SUSTAINABLE IMPROVEMENT OF ANIMAL PRODUCTION AND HEALTH

Edited by N.E. Odongo, M. García & G.J. Viljoen

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Contents

SESSION 5
ACHIEVING FOOD SAFETY AND SECURITY IN THE 21ST CENTURY

PREFACE

The world's poorest people, some one billion living mostly in Africa and Asia, depend on livestock for their day-to-day livelihood. To reduce poverty, fight hunger and ensure global food security, there is an urgent need to increase livestock production in sustainable ways. However, livestock production in developing countries is constrained by low genetic potential of the animals, poor nutrition and husbandry practices and infectious diseases. Nuclear techniques, when applied in conjunction with conventional methods, can identify constraints to livestock productivity as well as interventions that lead to their reduction or elimination in ways that are economically and socially acceptable. The challenge is how best to exploit these techniques for solving problems faced by livestock keepers within the many agricultural production systems that exist in developing countries and demonstrating their advantages to owners, local communities and government authorities.

This publication is a compilation of the contributions emanating from an international Symposium on 'Sustainable Improvement of Animal Production and Health' organised by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture in cooperation with the Animal Production and Health Division of FAO. It provides invaluable information not only on how nuclear and related techniques can be used to support sustainable livestock production systems, but also about the constraints and opportunities for using these techniques in developing countries; it also attempts to identify specific research needs and gaps and new options for using these techniques for solving established and emerging problems. As such, it is hoped that the information presented and suggestions made will provide valuable guidance to scientists in both the public and private sectors as well as to government and institutional policy and decision makers.

The Symposium comprised a plenary session and four thematic sessions, covering (i) interactions among nutrition, reproduction and genotype, (ii) livestock-environment interaction / productivity / climate (water / land / plants / heat / altitude), (iii) detection and control of transboundary animal diseases, including zoonoses, and (iv) animal product safety and food quality. The Symposium was attended by approximately 400 delegates from 100 Member States as well as representatives of international organizations including FAO, WHO, OIE and ILRI who presented and discussed strategies for the sustainable improvement of animal production and health, with particular emphasis on global food security, poverty alleviation and hunger reduction. The seriousness with which these topics were being tackled by Member States was shown in the results of their studies, presented in 53 oral presentations and 163 poster displays by an assorted group of researchers, veterinarians, policy makers, students and other animal scientists who attended the symposium.

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Director,
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in Food and Agriculture

Samuel Jutzi
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OPENING STATEMENTS

Ana María Cetto

Deputy Director General, Department of Technical Cooperation, IAEA

Dear Colleagues, Ladies and Gentlemen,

On behalf of the Director General of the International Atomic Energy Agency (IAEA) and on my own behalf, I have the great pleasure to welcome you to the Vienna International Center for this International Symposium on '*Sustainable Improvement of Animal Production and Health*'.

Let me start by briefly highlighting the mandate of the IAEA, and in particular, the Department of Technical Cooperation which I head. The IAEA, a specialised organization within the United Nations system, was set up as the world's 'Atoms for Peace' organization in 1957. The Agency works with its 146 (as of September 2008) Member States and multiple partners worldwide to promote the safe, secure and peaceful use of nuclear energy thus contributing to the United Nation's Millennium Development Goals for social, economic and environmental development.

The IAEA has three main areas of work or pillars underpinning its mission: Safety and Security, Science and Technology, and Safeguards and Verification. The IAEA is best known for its statutory roles in nuclear safety and security and the verification of international safeguards agreements relating to the non-proliferation of nuclear weapons. It is less known, however, for its role of helping countries mobilise peaceful and safe applications of nuclear science and technology for sustainable development. The Department of Nuclear Sciences and Applications is charged with the responsibility of implementing one of the Agency's Major Programmes, i.e. Nuclear Techniques for Development and Environmental Protection. The five key thematic areas of Water, Energy, Health, Agriculture and Biodiversity and ecosystem management that were identified in the Millennium Declaration of 2000 and reaffirmed in the Agenda 21 Action Plan and the World Summit on Sustainable Development in 2002 drive the Programme. The main programmes within Nuclear Techniques for Development and Environmental Protection are food and agriculture, human health, water resources, environment and radioisotope production and radiation.

These priorities are reflected in the structure of the Department of Nuclear Sciences and Applications, which utilises nuclear and isotope techniques, alone or integrated with other technologies, to assist countries in providing unique solutions to help solve the relevant Water, Energy, Health, Agriculture and Biodiversity topics. These techniques are employed in programmes addressing agricultural productivity and wider food security, improvement of human health, increased availability of water resources, assessment and management of the marine and terrestrial environments and industrial applications.

Of course, the Department of Nuclear Sciences and Applications focuses on those nuclear techniques and technologies that are indispensable to the Agency's mission or that have a comparative or competitive advantage over non-nuclear techniques in terms of cost-effectiveness, or are complementary to non-nuclear techniques. In developing and implementing this vision, there is a need for appropriate coordination within the Agency and with Member States, flexibility in adapting Agency programmes and activities to meet changing needs and to incorporate emerging technologies, the development of the necessary infrastructures, and the timely dissemination of information.

More than half of the Agency's activities in Nuclear Sciences and Applications are implemented through the Technical Cooperation (TC) Programme which helps to transfer nuclear and related technologies for peaceful uses to countries throughout the world. Through training courses, expert missions, fellowships, scientific visits, and equipment disbursement, the TC Programme provides the necessary skills and equipment to establish sustainable technology in the counterpart country or region i.e. Africa, Asia and Pacific, Europe and Latin America. The other half of the Agency's activities is implemented through Coordinated Research Projects (CRPs). The CRPs are research networks that stimulate and coordinate research, and foster the exchange of scientific and technical information by bringing together research institutes in both developing and developed Member States to collaborate on the research topic of interest. Currently, the Department of Nuclear Sciences and Applications is providing technical and scientific support to 901 TC projects and cooperates and collaborates in research and development activities in 77 CRPs across the globe. The research that is supported encourages the acquisition and dissemination of new knowledge and technology generated through the use of nuclear technologies and isotopic techniques in the various fields of work covered by the Agency's mandate. These programmes are supported by the FAO/IAEA Agriculture and Biotechnology Laboratory, situated at Seibersdorf, 35 km south of Vienna which provides scientific and analytical services to research projects, and training and quality assurance services in the area of technical cooperation.

To enhance cost effectiveness and efficiency, inputs from various stakeholders (Member States, donor agencies, UN organizations etc.) must be harnessed, for example, through cooperation, collaboration and information sharing. In order to take these factors into account in the development, review, and implementation of the strategy, the Agency needs a mixture of inputs from Member States and from technical experts. Of particular importance are international conferences, symposia and other meetings, which bring us to the topic of this Symposium: 'Sustainable Improvement of Animal Production and Health'. This Symposium will address aspects of:

- interactions among nutrition, reproduction and genotype;
- effects of environment on animal productivity;
- detection and control of transboundary, emerging and zoonotic diseases;
- achieving food safety and security in the 21 century

But why livestock?

Throughout history, science and technology have been powerful tools for human development and poverty reduction. For decades, the IAEA, in partnership with FAO, has assisted its Member States to produce more, better and safer food. The on-going 'Livestock Revolution', a demand-driven increase in livestock production, especially in developing countries, presents both opportunities and challenges. As countries experience economic growth, higher incomes and increasing urbanisation, consumers are able to diversify their diets to include more meat and dairy products. Livestock production is therefore one

of the fastest growing sub-sectors in developing countries, where it already accounts for a third of GDP and is predicted to become the most important agricultural sub-sector by 2020 in terms of added value. The increasing demand for livestock products is creating opportunities for improving the welfare of millions of poor people who depend on livestock for their livelihoods. However, changes in production, procurement, processing and retailing of food, along with environmental and food safety concerns, erosion of animal genetic resources and the threat of zoonotic and other emerging infectious diseases, threaten the potential of the poor to benefit from the on-going livestock revolution.

The food crisis — which began last year with soaring prices and food shortages — continues today in many countries. However, it has been overshadowed in recent months by the global economic crisis. Today, one in six people in the world is food insecure; almost one billion people. The choices we make now will determine how or whether we can feed ourselves in the future. If we get it right, we can have a thriving food economy. Speaking at the end of the two-day Madrid 'High Level Meeting on Food Security for All' — UN Secretary-General Ban Ki Moon said: 'We worked hard to bring food assistance to those that most needed it in 2008. This year it is going to be harder because of the financial crisis which impacts on food security, but we must remain focused on improving food production' which is the theme for this Symposium. Increasing agricultural productivity remains one of the most effective ways to combat hunger and poverty.

Ladies and gentlemen, I wish you fruitful discussions and a successful participation at the Symposium.

Modibo Traoré

Assistant Director-General, Agriculture and Consumer Protection Department, FAO

Dear Colleagues, Ladies and Gentlemen,

It is a great honour and pleasure for me to participate at this timely International Symposium on 'Sustainable Improvement of Animal Production and Health'. I wish to convey my sincerest thanks to Mr Burkart and the IAEA for the invitation and the opportunity to share the FAO, and other role-players', view on sustainable livestock production and health. I also wish to acknowledge and congratulate all those who have been involved in our successful and longstanding partnership of over 40 years since the inception of the Joint FAO/IAEA Division in 1964. As many of you are aware, the focus of this joint initiative is to support both FAO and IAEA Member States in the area of food and agriculture through the development, adaptation and transfer of nuclear and nuclear-related technologies in the areas of:

- sustainable intensification of crop production systems;
- sustainable intensification of livestock production systems;
- strengthening compliance with food and environmental safety standards through good agricultural practices.

However, Ladies and Gentlemen,

The challenges facing us in food and agriculture are enormous. The 2008 FAO report on 'The State of Food Insecurity in the World' shows that in 2007 — mainly because of soaring food prices — the number of hungry people in the world rose by approximately 75 million. With an expected increase of another 40 million people in 2008, it is currently estimated the world has 963 million malnourished people. This implies that almost one billion of the world's 6.5 billion people face hunger and are 'food insecure'. Global food security is becoming an issue for the first time since the Second World War. The UN Secretary-General Ban Ki-moon recently warned at the Madrid 'High-Level Meeting on Food Security for All' that the situation is likely get worse unless more is done to tackle the food security problem.

In the past, famine conditions usually affected only one country or region and the crisis was a result of a particular circumstance, such as drought, floods or civil unrest. The current food crisis, although overshadowed in recent months by the global economic and financial downturn, is a worldwide phenomenon and cannot be attributed to one single factor. It's the perfect storm of the increasing demand and variety (consumer preference) from emerging markets in Asia and Latin America, extreme weather cycles linked to climate change, and the diversion of food crops (maize in particular) from the human food chain to the production of biofuel. Our challenge is not only how to ensure adequate food for the current 963 million hungry people, but also how we are going to feed a world population of over 8.3 billion people by 2030. If food demand is to be met in future, increased outputs will have to come mainly from intensified and more efficient use of the land, water and plant and animal genetic potential, fisheries and forestry resources that smallholder farmers in developing and transition countries have at their disposal. Smallholder farms around the world are home to approximately two billion people, making up one third of global population thus representing a vital part of local and national consumption and international trade. A lack of water to meet daily needs is also a reality for many people around the world today. Water availability and access are key constraints to poverty reduction and food security. At the same time, action must be taken to arrest the destruction and degradation of the natural resource base.

Currently, seventy percent of the world's poor live in rural areas and are dependent on agriculture. Although global food prices have fallen in recent months, because of years of under-investment in agriculture, coupled with the increasing threat of climate change, future food security is not guaranteed, and in fact, the situation is likely to worsen. Urgent action is needed to prevent hundreds of millions more people from slipping into hunger because of the volatile food prices, increasing energy and water scarcity and the economic and financial crunch. Beside its direct benefits, agriculture also has important linkages with the rest of the economy and creates jobs in other sectors. Increased agricultural productivity would result in lower food costs, which in turn would help reduce poverty in both rural and urban households by lowering the high proportion of their household income currently spent on food.

But why livestock?

Approximately one billion people in the world today depend on animals for their livelihood. Animals provide protein, natural fertiliser and a cash income, and are also a source of draught power (ploughing, traction, irrigation). Because many developing and transition countries have realised high economic growth in recent years coupled with an expanding urban population, income growth is altering spending and consumer preferences. Global food demand is shifting from grains and other staple crops to processed food and high value agricultural products, such as vegetables, fruits, meat, and dairy. The increasing demand for livestock products e.g. increased demand for meat and dairy products from the growing middle classes of countries such as China and India as well as heavy demand for feedstock from the biofuel industry is creating opportunities for improving the welfare of millions of poor people who depend on livestock for their livelihoods. However, changes in production, procurement, processing and retailing of food, along with environmental and food safety concerns, erosion of animal genetic resources and the threat of emerging infectious diseases, threaten the potential of the poor to benefit from the on-going livestock revolution.

The world has about 5 billion hectares of agricultural land. Whilst 90 percent of the world's 1.4 billion hectares of arable land are increasingly being devoted to agro-export crops, biofuel and transgenic soybean to provide fuel for cars and feed for livestock, millions of smallholder farmers in the global South still produce the majority of staple crops needed to feed the world's rural and urban populations. In Latin America, about 20 million peasant production units occupying close to 60 million hectares, or 40 percent of the total arable land with average farm sizes ranging from 1–2 hectares, and producing 51 percent of the maize, 77 percent of the beans, and 61 percent of the potatoes for domestic consumption. In Africa, arable land comprises some 213 million hectares farmed by approximately 33 million smallholder farmers, representing 80 percent of all farm holdings in the region. Although Africa now imports large amounts of cereals, the majority of African farmers (many of whom are women farming less than two hectares of land), produce a significant amount of basic food crops with little or no usage of fertilisers and

improved seed. In Asia, arable land comprises slightly more than half a billion hectares is occupied by the majority of the more than 200 million rice farmers. Few farm more than two hectares of rice but they produce 91 percent of the world's production, with China and India growing more than half the total crop. Small increases in yields on these smallholder farms that produce most of the world's staple crops will have far more impact on food availability at the local and regional levels than the doubtful increases predicted for distant and corporate-controlled large monocultures on commercially managed farms incorporating such high tech solutions as genetically modified seeds.

The FAO/IAEA partnership, mandated through the Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture, has greatly contributed knowledge and assistance in capacity building activities for our Member States over the years. This longstanding collaboration, which began as a visionary initiative within the United Nations system, is being recognised increasingly by other international organizations as an excellent example of striving to work more closely together under 'ONE UN', to meet the Millennium Development Goals. The Joint FAO/IAEA Programme provides a synergy of resources and common aims in promoting the IAEA mandate on peaceful uses of nuclear technologies along with the FAO mandate in food and agriculture. Furthermore, the interdisciplinary approach of the Joint FAO/IAEA Programme plays a major capacity building role in these areas and supports the efforts of FAO directly. Sustainable livestock production systems require an integrated management approach to farming practices that take account of complex interactions between soil, water and crops, their linkages to livestock and plant pests, and their relationship to the efficient use of agrochemicals. Sustainable farming systems rely not only on the conservation and efficient use of resources that protect our environment, but also on other factors such as agricultural investment, government policies and meeting consumer's demands and perceptions related to rural development and farming operations.

However, current technologies for enhancing crop productivity, animal production and health, food quality and safety, and controlling plant and animal pests and land degradation may have to be modified in response to these new emerging global issues and our efforts re-directed. It is imperative that we rise up together as an all inclusive community to meet these challenges on food security and agricultural sustainability, and I look forward to the continued strengthening of our FAO and IAEA collaboration in providing solutions to these problems.

Thank you for your kind attention.

David Nabarro

UN System Coordinator for Influenza and Global Food Security

Dr Traoré, Excellencies, Colleagues,

I am most grateful for this opportunity to address you today. In January 2009 I was appointed by the United Nations Secretary General, Ban Ki-Moon, to serve as Coordinator of the United Nations System High Level Task Force for Global Food. This Task Force was established in May 2008 in response to the urgent need expressed by United Nations Member States for coherent efforts in response to both short and long term elements of food security. The Task Force brings together the work of UN system bodies, International Financial Institutions and the World Trade Organization.

The members of the Task Force are committed to supporting national and regional responses to food insecurity through a Comprehensive Framework for Action that covers a broad spectrum — first, on reducing the vulnerability of households and communities at risk of food insecurity (as manifested by hunger and high rates of malnutrition); second, encouraging investments in sustainable and productive agricultural systems that improve the resilience of smallholder producers; third, improving opportunities for marketing with the participation of producer organizations and engagement of private sector partners as appropriate; and fourth promoting trade in agricultural products that works fairly in the interests of all communities and countries.

Why the emphasis on smallholder farmers? In much of the world they produce the majority of the food that is consumed. They tend to have been left aside in agricultural development efforts — especially so in recent decades as investment in agriculture generally has fallen. Yet more than two and a half billion people depend for their income and nutrition on the efforts of smallholder farming households, particularly on the work of women farmers. They live in rural areas and also in the outskirts of cities, farming plots of less than two hectares. Some of them depend entirely on livestock, others farm with a mix of livestock and crop production. They make difficult choices in the face of uncertainty about climate, access to inputs, disease, crop losses and their opportunity to market their produce at a reasonable price. Most of them are women whose children are at risk of nutritional insecurity: frequently they have to choose between caring for a child that is unwell, and has special feeding or health care needs, and working on their land or tending the animals. Much animal rearing is done by children themselves.

The Comprehensive Framework reflects a three track approach. The starting point is a recognition of the absolute importance of people being able to enjoy their right to food. Then the emphasis is on actions to realise immediate outcomes — hunger reduction, immediate boosts to agriculture, sound policies on export restrictions and import tariffs and balance of payments support on the one hand, and the third emphasis is on longer term social protection, agricultural development, attention to regional and global trade, and action on complex policy questions on the other.

The framework, which reflects the combined food security aspirations of the whole of the UN system, highlights the importance of strong partnerships involving producer organizations and civil society, businesses, professional groups (including agricultural extension and veterinary services), and researchers at national, regional and global levels. It emphasises the need for those representing the interests of hungry people and smallholders in policy debates on short and long term aspects of food security.

The Task Force is chaired by the UN Secretary General with the Director General of FAO as Vice-Chair.

This meeting comes at a critical time. There is widespread concern among governments, farmers' organizations and civil society groups, reflected by members of our Task Force, that too many people are unable to enjoy the right to food and nutrition, to have the wherewithal to feed themselves and their families, and to be resilient in the face of economic shocks, climatic events or acts of violence. The UN Secretary General is deeply concerned that food insecurity and hunger are being experienced every day by at least one billion of the world's inhabitants. That is one person in six, or 14 percent of the global population, with a child dying of malnutrition every six seconds.

Much of your work is destined to improving the performance of the livestock sector. Unhealthy animal rearing practices in medium scale commercial operations can affect all who earn their living from animal rearing, especially those who keep a few animals in their back yards. They can also undermine the prosperity of the whole livestock sector, one which is growing at an extremely rapid rate. The prompt diagnosis of, and response to, diseases in animals is vital both for disease control and for assessing practices that are most likely to result in risks to animal health. This, in turn, is important not only for those who rear animals but also for the wider population given the importance of animal illness as a source for emerging disease in humans. At least two new pathogens capable of harming humans emerge each year, and 75 percent of these come from the animal kingdom. Frequently we do not know the potential pathogenicity of such organisms when they first emerge.

The work in which you are involved will have important repercussions for the short and long term health of people and their communities, and may also have implications for wider national prosperity and political stability.

Within our Task Force we work with nations as they contribute to national, regional and global partnerships for agriculture, food security and nutrition. We seek to help them mobilise and improve access to the resources that are necessary to initiate and sustain improved production, with Financial Coordination Mechanisms that gives them a better chance to access the investments they need in an integrated rather than piecemeal manner.

We will be guided in our work by the extent to which we are able to demonstrate reductions in hunger and poverty reduction as laid out in the Millennium Development Goals (especially MDG 1) through demonstrable improvements in production, agriculture-related income, and the contribution of agriculture systems to mitigation of and adaption to climate change.

I would like to focus now on the specific challenges to both animal and human health posed by influenza viruses, and the ways in which different national governments, regional bodies, political organizations and international institutions have worked together to address them.

During the last few years we have witnessed the agreement and application important standards for animal and human health to

the trans-boundary threats posed by disease. I refer specifically to the World Organisation for Animal Health (OIE) animal health standards and the Revised International Health Regulations (IHR 2005) developed by member states of the World Health Organisation. The IHR, for example, is an important intergovernmental framework and series of instruments for collective responses to infectious disease. The proper implementation of IHR 2005 depends on the full participation of national authorities and other stakeholders. Some of them question the extent to which systems for global governance on health reflect the interests of poor people and their nations: they question the value of globalised thinking and working.

A word on my own involvement in this field. I started out thirty years ago as a public health doctor working in rural communities in the Middle East and in South Asia, especially in Nepal. I focused on the determinants of resilience and nutrition in communities, and particularly on the problems experienced by women and children during the tough rainy months leading to harvest, when the demands of the fields and child care tended to compete, when money supply was tight, and when the health centres often lacked necessary medicines. For about five years I taught public health and nutrition, and for another ten years I worked as a civil servant with the British Government, in Africa and then in London. I joined WHO and served in various roles between 1999 and 2005. In September 2005 I was asked by the late J.W. Lee, the then WHO Director General, and Kofi Annan, the Secretary General of the United Nations, to move to New York. My remit was to help different parts of the UN system react to increasing political concern among Heads of State and Government, particularly from Southeast Asia, about the potential political, societal and economic impacts of a severe influenza pandemic.

I was asked to establish a temporary mechanism to ensure that the capacities of the whole UN system (technical human health and agriculture bodies, as well as our full range of social, political and economic bodies) are made available, in a coherent way, to the governments of our Member States.

During 2005 there was broad agreement on the scientific basis of work being undertaken on avian and pandemic influenza; outstanding research questions were also clear. These include a better understanding of risks associated with the movement of highly pathogenic avian influenza among poultry (particularly in ducks); the relative roles of wild birds, trade, and cross border movements in spreading H5N1 among birds; and the behaviour patterns that increase risks for human infection still needing some work.

WHO, FAO and OIE had established clear strategies for national actions to be undertaken: stamping out Highly Pathogenic Avian Influenza (HPAI) when identified — through quick and thorough action; reducing the threat to poultry through introducing biosecurity; monitoring wild birds and charting their movements so that where possible wild birds that might be infected with this virus could be separated from domestic birds; reducing the risk of human sporadic cases by limiting the degree to which humans would be in contact with infected birds, and then preparing to contain and then mitigate the next influenza pandemic when it happens.

The challenge for us was to ensure that governments gave these strategies the impetus necessary for their implementation, leading to the control of HPAI and preparedness for an influenza pandemic. The technical work had to be taken forward within the momentum of the emerging political environment. As well as ASEAN, the USA, the EU, Canada and Japan took political initiatives.

Within the UN Influenza Coordination Office we sought to align different international institutions — including the World Bank, the international organizations of the UN, the regional development banks, other international, regional and local research bodies and so on — and to encourage the collective pursuit of international normal and standards, with the specialized organizations (WHO, FAO and the OiE) charting a path for the rest of the UN system and the myriad of other organizations becoming engaged in work on avian and pandemic influenza.

From the start most of those who were involved in this work demonstrated unity of purpose and synergy of action. In general, coordination between the bilateral donors, the foundations, national governments, regional bodies and international non-governmental groups (including the Red Cross movement) was strong.

We have subsequently sought to identify the incentives that brought many disparate groups to work together. Finance was important, and the partnership mobilised over US$ 3 billion in assistance for avian and human influenza actions between 2005 and 2009. But this — on its own — cannot explain the extent to which national authorities have worked together on these issues. The funds that have been pledged are primarily made available to governments: they have moved comparatively slowly.

An International Partnership on Avian and Pandemic Influenza was established as a basis for this cooperation. Other partnerships were organised at regional level through the European Union, APEC, ASEAN and other regional groupings. Few of these partnerships were formal: most had real impact on the alignment and ways of working of their members.

We concluded that most of the groups working together on this issue recognised the value of working together, in synergy. They found it both operationally useful and reassuring in a situation where there was considerable political urgency and need for concerted action by institutions. Stakeholders from the public, private and voluntary sectors have valued the opportunity for coherence, joint working and participation. They have worked together on disease surveillance, reporting and response. They have joined together to support the evolution of an inclusive movement that enables hundreds of different stakeholders to feel at home within it.

Pandemic preparedness work has moved forward over the last four years thanks to the efforts of this broader movement, and the effort has been tracked through annual global progress reports using information from countries. The reports, which have involved the full range of UN system agencies and the World Bank, have served as the basis for collective accountability. The reports reveal that over the four-year period there has been more rapid reporting of HPAI and more effective, sustained responses to outbreaks of the disease in poultry. The OIE is now pursuing the elimination of H5N1 in the next few years. There has also been a massive effort to initiate pandemic preparedness work which we believe has stood us in good stead as the world faces up to the first outbreak — potentially pandemic — of a novel influenza virus of this century. Once again, our preparedness is being tested by the uncertainties around which way this particular threat will go.

Our Annual Reports identify seven factors for success. These are:

- consistent political commitment;
- resources and capacity to go to scale in response to a threat;
- interdisciplinary working (particularly animal health and human health) within countries and across borders;
- predictable, prompt, fair and sustained compensation schemes for those who lose property or animals as a result of control measures;
- strong engagement of public sector, private sector and voluntary agencies;
- clear and unambiguous communication of reliable information (and sharing of uncertainty as appropriate);
- the need for a viable and scientific response strategy.

Experiences with SARS and other diseases suggest that if information is kept from people they will not feel empowered to be part of the response.

What are the incentives for success? First is the availability of good quality and accessible information about HPAI outbreaks — based on good mapping of issues, tracking of progress and risk analysis. The information that is available has been synthesised and made available to those who need it through the efforts of international organizations in response to the needs of their primary clients. Without well functioning surveillance and reporting systems we are stuck: OIE and FAO have played major roles, working with the support of a number of Member States to establish better diagnostic surveillance and reporting capacity.

A second incentive is the ready availability of instruments, services and assets needed for effective action. These include the Global Outbreak Alert and Response Network (GOARN) in WHO and the FAO-OIE Crisis Management Center for Animal Health that provide a backbone for solidarity and international action. This encourages countries and other stakeholders to be engaged — they know that dependable systems exist that can help them.

A third incentive is the existence of the right legal codes (and means for enforcement) at country level — for controlling movements of animals, for ensuring compensation when animals have to be killed and for enabling the consistent nation-wide implementation of public health functions (especially in decentralised political systems).

A fourth incentive is the widespread appreciation among the public, of the pandemic threat and the need to be prepared. Unfortunately it has not proved easy to sustain the appreciation that animals, and ways in which they are cared for, can pose a risk not only for their own health but also for human health. The risk can be reduced by changed behaviour. The information and compensation needed to encourage behaviour changes are often not sufficient. It is vital that the potential for animals to serve as the source for diseases in humans, and *vice versa*, result in better attention to the animal-human health interface — what we tend to refer to as the 'One World, One Health' movement following the groundbreaking work of the wildlife conservation movement.

A fifth incentive is empowered and professional government servants — people in government who feel that they are in a position to take the initiative in the face of a disease threat. They sometimes do not believe that their own authorities, or international authorities, are working in their interests. This is a challenge. H5N1 — and other diseases — will not be controlled through compulsion and sanctions. It doesn't work. People start to hide, they do not explain, they do their best to avoid involvement. So it is absolutely essential to build the necessary trust for effective action.

There are a number of continuing challenges for our collective effort to control HPAI caused by the H5N1 virus and to prepare for pandemics.

The first is the continuing lack of adequate systems and capacities for data collection and surveillance, laboratory services and analysis, and for the management and use of information derived from the data. This applies to both animal and human health.

The second is the reality that some key groups (in some countries) are not fully engaged into the movement for pandemic preparedness. How to ensure that those who run the poultry industry in a HPAI-affected country see it as in their collective self interest to work together with the NGOs, the researchers, and governments on control and prevention of HPAI? This requires a continuous effort to build and sustain a movement. Movements wither away if they are not persistently supported and kept going.

The third challenge is to maintain trust. Committed professionals from countries in South East Asia worked with the Rockefeller Foundation to build the Mekong Basin Disease Surveillance Program over many years. This covers several different disease issues. It has generated trust between technicians across borders, has survived and continues to do well despite occasional difficulties at the ministerial or high political level. Similar systems are being established between Bangladesh, India and Nepal following their HPAI outbreaks in 2008 and 09.

We are all involved in this effort to build trust. We should ask ourselves, from time to time, whether we are contributing to trust as effectively as we could.

In conclusion, we who are involved in this work tend to want to implement the most appropriate (or 'right') actions. These norms must be well publicised, continuously reinforced in a very positive and embracing and open way and backed with good quality literature.

We need viable animal and human health services based on the best available technologies, and to be sure that the incentives for them to work well are the right ones. The OIE's PVS scheme offers us some valuable pointers.

It is worthwhile getting the incentives right so that pandemic preparations are successfully put in place. The reward may well be that when the next severe influenza pandemic strikes, millions of people survive who might otherwise be expected to die.

I acknowledge the contribution of my many colleagues in UN system agencies to the development of these ideas. The responsibility for the way in which I have presented them is mine alone.

Qu Liang

Director, Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture, IAEA

Dear Colleagues, Ladies and Gentlemen,

I am delighted to see that we have been able to attract such a high calibre of livestock scientists, practitioners and managers from all over the world. Welcome to Vienna and to this International Symposium on 'Sustainable Improvement of Animal Production and Health'. I would also like to record my appreciation to the private sector for the support given to this Symposium which has enabled us to channel more resources than would otherwise have been possible into ensuring that so many researchers and decision makers from developing countries are here this week. I believe we all share a common vision — the Millennium Declaration to 'spare no effort to free our fellow men, women and children from the abject and dehumanising conditions of extreme poverty."

Ladies and Gentlemen,

The current food crisis is unprecedented. A staggering one billion of the world's 6.5 billion people face hunger and are 'food insecure'. These are tough times for everyone, but they're especially desperate times for many poor communities and smallholder farmers in developing countries. The global food crisis has been blamed on a combination of the increasing demand and variety (consumer preference) from emerging markets in Asia and Latin America, extreme weather cycles linked to climate change, oil prices, and the diversion of food crops (maize in particular) from the human food chain to the production of biofuel. All this is true, however, in the past seven to ten years; the world has consumed more grain than its farmers have produced thus relying more and more on grain reserves. As a result, grain reserves are currently at their lowest level in the past 50 years. Furthermore, for decades there has been a decline in global agricultural scientific research and development funding in both developing and developed countries. Because it takes on average 15 to 20 years for a new piece of science and technology to be researched, developed and disseminated to end-users, the challenge facing today's farmers is how to overcome obstacles to sustainable food production and double the world's food production using less land, less water, fewer nutrients, and less technology.

The Joint FAO/IAEA Programme assists Member States of FAO and IAEA to develop improved strategies for sustainable food production through the use of nuclear and nuclear related techniques and is comprised of five sub-programmes covering: Soil and Water Management & Crop Nutrition, Plant Breeding and Genetics, Animal Production and Health, Insect Pest Control, and Food and Environmental Protection.

Briefly, the Soils and Water Management and Crop Nutrition sub-Programme assists national institutions in developing countries involved in agricultural production to develop integrated strategies and technologies using nuclear and nuclear related techniques to improve the efficiency of nutrient and water use within selected cropping systems, while conserving the natural resource base (soil, water, biodiversity, etc.). The Plant Breeding and Genetics sub-Programme assists Member States in the implementation of modern and competitive plant breeding programmes using radiation induced mutation and efficiency-enhancing biotechnologies to ensure food security through sustainable crop production. The Insect Pest Control sub-Programme assists Member States in implementing environmentally friendly and sustainable methods to control major insect pests of plants, animals and humans by focusing on area-wide integrated pest management approaches involving the sterile insect technique (SIT) to enhance food security. The Food and Environmental Protection sub-Pogramme assists Member States in their endeavours to ensure the quality and safety of food and agricultural commodities and facilitate international trade. The focus is on strengthening Member State capacities for applying international standards on irradiation and on using nuclear and nuclear related analytical techniques in the management of food and environmental hazards.

Last but not least, the Animal Production and Health sub-Programme contributes to the enhancement of global food security through the implementation of sustainable livestock production systems using nuclear and nuclear related techniques. Sustainable livestock production systems require an integrated management approach to farming practices that takes account of complex interactions between soil, water and crops, their linkages to livestock and plant pests, and their relationship to the efficient use of agrochemicals. We assist Member States to improve livestock productivity through the efficient use of locally available feed resources, adequate management practices and breeding programmes for indigenous and upgraded animals, and diagnostic tools and prophylactic measures for the control and prevention of animal and zoonotic diseases.

In all areas, support and guidance is provided in the formulation and implementation of activities that underpin Member States' national, regional and global development objectives through strategic, applied and adaptive research, technology transfer, capacity building, policy advice and information management. In all our activities, the overall objective is the development and use of novel nuclear and nuclear related technologies for a more profitable and sustainable agriculture, a secure food production system and a healthier environment.

How is this achieved?

This is accomplished by co-ordinating and supporting research, providing technical and advisory services, providing laboratory support and through scientific training and by collecting, analysing and disseminating balanced scientific, technical and policy-relevant information.

Co-ordinating and supporting research

Approximately 600 research institutions and experimental stations in Member States co-operate in 40 Co-ordinated Research Projects. Each project attempts to solve practical problems of economic significance for developing countries and involves collaboration among 10–20 institutions including those belonging to the Consultative Group on International Agricultural Research (CGIAR). Institutions in developing countries are normally awarded Research Contracts with nominal financial support, whereas those in the more developed countries participate through Research Agreements with financial support only for attendance at Research Co-ordination Meetings. These projects normally last for five years and the results are published either as an IAEA TECDOC or a special issue of a journal.

Providing technical and advisory services

The Joint FAO/IAEA Programme is also responsible for providing scientific and technical guidance and support to over 200 national and regional Technical Co-operation Projects, as well as for Inter-

regional and Regional Training Courses. These projects are financed by the Agency's Technical Co-operation Fund, FAO's Technical Co-operation Programme and through trust funds provided by donor countries and international funding agencies for the procurement of equipment and provision of expert advice and training (through fellowships and scientific visits).

Providing laboratory support and scientific training

The Joint FAO/IAEA Programme is supported in its activities by the FAO/IAEA Agriculture and Biotechnology Laboratory, situated at Seibersdorf, 35 km south of Vienna. The laboratory specialises in research, development and transfer of nuclear and nuclear related techniques including training of scientists through individual fellowships and inter-regional and group training courses in various disciplines. The laboratory also provides guidance on the introduction of analytical quality control and assurance into counterpart laboratories, and training in the maintenance of laboratory equipment and instruments.

Collecting, analysing and disseminating information

In addition to encouraging the direct transfer of skills and technology, the Joint FAO/IAEA Programme provides a variety of information services including conferences, symposia, seminars and advisory group panels, and the publication of technical and public information documents that arise from these meetings as well as from Co-ordinated Research Projects and Technical Co-operation Projects. The Programme also maintains contact and collaboration with Member States through joint scientific publications and other publications, such as newsletters, periodic reviews, and computer databases.

Going back to Animal Production and Health, let me re-emphasise the importance of the issues raised by Dr. Traoré in his address. Approximately one billion people in the world today depend on animals for their livelihood. We know that the global food demand is shifting from grain and other staple crops to more processed food and high-value agricultural products, such as fruits, vegetables, meat and dairy products in several transition economies such as Brazil, China and India which have enjoyed high economic growth in recent years coupled with an expanding, wealthier urban population. Although we tend to associate these changes with urban centres, the same change is happening in rural areas as well. Visit any small town or village in these transition economies and some developing countries and you will see a booming business in milk and other livestock products. This increased demand can only be met through the protection of animals from diseases, the selection of animals that give more meat and milk, and the optimal utilisation of local resources whilst protecting the environment to which the Animal Production and Health sub-Programme contributes through the use nuclear and nuclear related techniques.

Thank you for your kind attention.

SUMMARY AND CONCLUSIONS

I. PLENARY SESSION

Two keynote addresses were presented: Historic role of nuclear techniques in solving problems of animal health & production by Wyn Richards, and Decline in available world resources – implications for livestock production systems by Ron Leng. The relevant points that emerged from these talks were:

- The world demands more and healthier animals and animal products produced in an 'environmentally safe, clean, and ethical' way. This is imposing new challenges for animal scientists whose primary concern has been improving livestock productivity. Improving understanding and technologies in animal nutrition, animal reproduction and breeding, and animal health is critically important for food security, poverty alleviation and environment protection on a global scale. The Animal Production and Health Subprogramme of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture has developed, validated, and transferred to IAEA and FAO Members States sound, low cost and easy-to-use nuclear and nuclear related techniques coupled with convenient strategies to improve animal productivity and to efficiently manage diagnostic laboratories.
- These nuclear applications have undoubtedly spearheaded modern biotechnological research. For example, the most used disease monitoring technology is the enzyme linked immunosorbent assay (ELISA). ELISA assays were developed through research using radioactive isotopes (radioimmunoassay, Western blot), and many still use gamma irradiated pathogens as safe antigens (e.g. Rift Valley fever IgG and IgM ELISA's). Similarly, molecular diagnosis and characterisation techniques were founded using radioisotopic applications. In fact, the most sensitive and cost effective pathogen detection and characterisation applications (1-100 protein or nucleic acid molecules) still demand the use of isotopes. In modern biotechnology, nuclear applications will continue to play a major role.
- The IAEA was instrumental in the success of the global rinderpest eradication campaign through transfer of technologies, improvement in laboratory infrastructure and staff proficiency, and provision of methodologies and operational guidance. The laboratory groundwork laid then, now forms the basis of an increasingly successful animal health control programme in developing countries, demonstrating the sustainability of the interventions fostered by the IAEA in partnership with FAO.
- The world is faced with three simultaneous and interrelated crises that if not responded to appropriately will create chaos, i.e. climate change, increased oil demand and global resource depletion. There is an urgent need to respond to these challenges in order to produce and deliver food to maintain the present world population. The Food and Agriculture Organization of the United Nations (FAO). FAO has indicated that one billion of the world's 6.5 billion people face hunger and are 'food insecure'.
- The primary resource depletion is fossil fuel energy as the world has been using more fossil energy then is being discovered. Several countries have opted to cover part the demand by using bio-ethanol and bio-diesel, thereby impacting on land use and grain availability, and contributing to increasing world food prices. Cereal grain availability for industrial livestock production (pig, poultry and feedlot beef) will be highly restricted and the resulting shortfall in meat production will only be replaced by expanding ruminant animal production. Grain based animal production will become increasingly expensive. There is a greater need to intensify ruminant production on crop residues by applying better methods of treatment to improve digestibility and to prioritise protein supplementation that increases feed conversion efficiency.
- Water, the other major resource required for agriculture has also been depleted. Many of the world's large river systems are being drained for urban and industrial water supplies or for irrigating crops. Humans, animals, and plants compete for water and it is by far the most important limiting factor in livestock production. Although much research is being done, it needs to be focussed on overcoming the significant obstacles to sustainable food production in order to create the means to double global food production using less land, less water, fewer nutrients, and less technology to satisfy the expected demand.
- Global warming is causing serious deleterious effects to the environment. There is an evident increase in the incidences of extreme weather events such as storms, droughts and floods and, at the same time, changing temperatures may support the occurrence or recurrence of agricultural pests, diseases and their potential vectors.

II. ANIMAL PRODUCTION

- Much of the activities of the Animal Production and Health Subprogramme of the Joint FFAO/IAEA Division of Nuclear and Techniques in Food and Agriculture will have important repercussions on people and their communities, as it was clearly revealed through the various sessions in the symposium. To meet the demands and need of food of animal origin, to improve the efficiency of the large number of production systems in more than 20 livestock species reared under various environment and climatic conditions, and to provide sound surveillance disease programme, as well as national control and eradication programmes for transboundary and zoonotic diseases have to be the result of coordinated and joint efforts from several international organizations.
- The paramount of work ahead cannot be underestimated and therefore cannot be solved by few people or institutions. Livestock population is predicted to increase dramatically over next 40 years with an important impact on land use; but also, there is an annual loss of 150 million cattle and small ruminants from parasitic and infectious diseases and even greater losses in production from inefficient husbandry practices. These facts must be highlighted and that should be bear in mind when designing future activities. Besides this, papers and talks presented during the symposium showed that the coming world food crisis has three important factors to consider: the end of inexpensive energy era (and beginning of expensive inputs), global climate change, and global resources depletion including mineral fertilizers, irrigation water, soil fertility, and land use. Research work is highly needed to solve or alleviate much of these problems, and certainly the IAEA contribution with the development and application of nuclear and nuclear related techniques are utmost relevant; however, we must differentiate the target audience for application of nuclear techniques as the users in many cases are different from beneficiaries - but both of these are members of a much larger value chain. Research is highly needed but the results and its application in large scale has to be balanced, as proper evaluation and consideration to public needs, beliefs and cultural heritage are different within countries, regions and continents.
- Facts, data, results, and recommendations given by experts have resulted in a long list of valuable ideas, proposals, and procedures that can be used at various levels to boost livestock production, reduce hunger and improve health conditions in animals and

humans. Among them, manipulation of feeds and nutritional strategies to reduce methane production while improving body weight gain and milk yields, management practices to improve fertility and number of newborns, without high inputs, and therefore benefiting farmers with higher economical returns and the world with larger quantities of high quality food.

- Meat and dairy products are mainly produced by cattle, pigs, sheep, goat, and poultry, but there are several other species which are highly relevant for specific regions or countries, which need to be properly characterized and efficiently improved for the benefit of the local population. Among them, the buffalo is an important ruminant species in Asia that requires further research in both environmental effect on milk production and fertility using artificial insemination. Camels in the Arab world and camelids (alpaca, llama, and vicuna) in the Andean countries substantially contribute with meat, milk (camel) and fibre; yaks in China are essential animals for a vast number of people; and rodent species such as the cane rat in West and Central Africa and the Guinea pig in Peru and Bolivia are important sources of meat.
- Much of research data, methodologies, management practices, and health procedures produced and validated in the developed world cannot be easily transferred to developing countries, due to climatic, soil, genetic and cultural differences and therefore, the huge amount of valuable research has to be properly evaluated and in many situations adapted; however, in many other situations developing countries have to develop their own technology based on the existing species and breeds, and production systems. As an example, embryo transfer technology, widely available in the North, is not economically feasible under small farmer conditions despite efforts from Governments and interested parties. On this respect, the search and evaluation of locally available feedstuffs, the implementation of feeding trials, the emphasis on reduction of expensive concentrates, alternatives for oestrous synchronization, the use of artificial insemination on a fixed time without the need for heat observation, and the expansion and support of artificial insemination services were presented by many of the symposium participants.

Session 1: Interactions among Nutrition, Reproduction, and Genotype

Three keynote addresses were presented. These focussed on (1) nutrient requirement models for livestock to improve feed utilisation and animal performance under various environments, (2) the increase on milk yield in specialised dairy breeds and its relationship with fertility, body weight, and metabolic diseases, and (3) assisted reproduction techniques to obtain higher quantity of valuable embryos from high genetic quality animals for transferring to lower genetic value females. Additionally, 63 papers by scientists from 35 countries were presented orally or displayed as posters.

The main points emerging from this session were:

a) Multidisciplinary approaches for solving livestock constraints

- Integrated and multidisciplinary research approaches are needed to identify, test and solve or at least ameliorate productive constraints in developing countries, especially in rural communities with low education and without land ownership. In many cases, isolated interventions may result in adequate biological results but fail to significantly boost domestic dairy production because farmers do not see the economic gain associated with potential biological improvements. The integration of interventions covering nutrition, health, reproduction, and management can bring more economic benefits to small holder farmers and improve dairy production. The application of integrated interventions in dairying requires the synergistic action from the government, researchers, non-governmental organisations and farmers. It requires expertise from many inter-related fields, which calls for the creation of integrated action teams at different administrative levels.

b) Nutrition x reproduction x genotype interactions

- In tropical countries, there is usually a mismatch between the cattle genotype and the environment. The major problems associated with cattle production in these regions are diseases and biting flies, water shortage, heat stress, and scarcity of feeds. Cattle production is therefore characterised by high calf mortality, slow growth rates, low fertility and calving rates, low milk yield and carcass weight, and little attention is paid to disease resistance and heat tolerance. Farmers are demanding more adapted and productive animals. In this scenario, it is of great importance to consider indigenous breeds. Disease resistance and heat tolerance are particularly valuable traits in indigenous breeds compared with exotic ones reared in the same environment. However, little is known about the genetic composition controlling these characteristics. The rapid and growing knowledge of genes and genomes in livestock obtained by the assessment of a large number of DNA markers in phenotypic recorded populations could be used to localise and further characterise candidate genes of economic interest. The analysis of mutations is in essence the discovery and exploitation of the natural variation existing in the biological machinery with the aim of increasing the frequency of favourable alleles to the benefit of livestock breeding strategies.
- After calving, high milk yielder cows are unable to compensate for the increased energy demands by increasing feed intake, resulting in a negative energy balance. The consequent catabolic status is characterised by low insulin and glucose concentrations and reduced insulin sensitivity, leading to a massive mobilisation of body reserves. Animals can lose up to ten percent of their body weight during the first weeks post partum and the occurrence of first oestrus is delayed. This metabolic condition can directly hamper growth and function of the ovarian follicle as elevated free fatty acid and the low glucose concentrations are toxic for the granulosa cells and the oocyte. Some studies have shown showed that a higher proportion of non-viable embryos were flushed from lactating cows compared with non-lactating cows.

c) Feed and nutrients for improved livestock productivity

- Provision of nutrients should cover maintenance and production needs to a point that the investment generates an economic return for the farmer. Animals differ in their nutrient requirements according to their inherent genetic potential and the desired level of production. Nutrient requirements for maximum production in high genetic merit Holsteins in industrialised countries are fairly well known but still there is a need to improve the knowledge on the level of response for nutrients other than energy of specialised dairy breeds and indigenous crossbred animals under tropical and subtropical conditions where feeding, management, health, and climatic conditions are much less than optimal.
- There are multiple combinations of dietary ingredients that can meet an animal's nutrient requirements, which create variation in dietary costs when food resources are finite in supply. Seasonal

droughts and floods, and changes in cash crop production alter or limit feed availability from local sources and therefore impact animal productivity. Optimisation algorithms can be utilised to assess maximum production or economic returns given a set of constraints, especially those related to nutrient requirements and availability and accessibility of food supplies. Thus, it is important that animal performance models are capable of accurately predicting production responses when varying the quantity and quality of feeds.

- Rice straw, maize stover, wheat, and sorghum straw are major feed sources for ruminant in many tropical countries, especially during the dry season. The nutritional limitations of rice straw may be overcome by supplementation with concentrates, urea, or green forage. Among the latter, tanniferous tree foliages and shrubs are efficiently used together with agricultural by-products, mainly crop-residues, containing low levels of nitrogen to enhance rumen microbial fermentation and hence animal productivity. Rice straw is a highly fibrous feed and with low digestibility; therefore its retention in the gastrointestinal tract is longer, and within this period the fermentation process results in higher methane production which contributes to environmental; however, the combined use of rice straw as a cheap feed source with the supplementation of concentrates activates microbial fermentation in straw, resulting in a shorter retention time and increased feed intake, the final result being higher productivity (body weight and milk yield) without increasing methane production. Tree legume forages offer a cheap alternative to concentrate diets in ruminant nutrition
- Gamma irradiation of some animal feeds like cottonseed meal decreases crude protein disappearance in the rumen, allowing part of the protein to by-pass the rumen to the intestine which is beneficial for high producing ruminants.

d) Selection of livestock on genetic merit

- The resumption of ovarian activity in the cow includes the growth of healthy follicles, oestrus behaviour, and ovulation. The resulting oocyte will be fertilised and a viable embryo will be attached to the uterine wall. This process requires the release and inhibition of several reproductive hormones at various times. Any disturbance of the fine-tuned biological and mechanical events leads to failures in ovulation, fertilisation, or early embryonic death. Unfortunately, selection over the years for high milk yield has been at the expense of sustained reproductive efficiency.
- Overall improvements in the genetic merit for milk production in dairy cows in the last 20 years have led to a tremendous increase in milk yield; however, milk yield maximisation has been paralleled by a decrease in reproductive performance and higher incidence of metabolic and infectious diseases during the early post-partum period. Disappointing fertility results are a world-wide concern in the modern dairy industry as high milk yield is a major factor contributing to a reduced number of calvings per lifetime, days in milk and decreased longevity. Optimum reproductive parameters considered in the past as one calving per year, 1.8 services per conception, and 60 percent conception rate are no longer valid. Current figures, even in the USA and Western Europe show average calving intervals close to 400 days, more than two AI per conception and 40 percent conception rates in specialised dairy breeds.
- There is inadequate selection and handling of top quality alpacas. This is based on phenotype and leads to a further progressive deterioration of fibre quality. The scientific approach towards selecting animals (based on genotype) in combination with modern techniques for handling of gametes and embryos could ensure the preservation of highly valuable animals for high quality fibre. Making use of the sophisticated knowledge from assisted reproduction techniques to 'identify' genetically pure alpacas will allow obtaining a higher quantity of the most valuable embryos which could be transferred to lower genetic value females. This strategy could lead to a more rapid increase in the percentage of animals with good quality fibre.

f) Managing the ovarian cycle and the ova

- Optimisation of ovarian treatments in combination with cryopreservation of ovarian tissue, follicles, and oocyte are valuable strategies to improve the reproductive process, to create diversity, and also to save rare species from extinction.
- *In vitro* production of bovine embryos is currently a routine technique is several countries; however, there is a consensus among animal reproduction scientists that current techniques for the culture and maturation of oocytes are still inefficient compared with using *in vivo* ovulated oocytes. The hormonal treatments that are actually used to pre-treat animals before oocyte retrieval are suboptimal and do not rescue as many oocytes as expected, probably due to several external (seasonality, pasture, environment) and internal factors (disease, stress).
- A challenging new concept is that it might be possible to optimise oocyte quality by culturing (and 'treating') the oocyte-cumulus complex *in vitro* instead of the ovary *in vivo*. Several factors produced and secreted by the oocyte itself are essential to condition the cumulus-corona cells in producing essential metabolites that are transferred back into the oocyte by specialised communication (paracrine, gap junctional). It is possible today to produce these factors by recombinant technology and to use them in culture media.
- A technique that could greatly contribute to the genetic diversity is gamete banking. Ovarian tissue from valuable animals can be conserved (i.e. primordial follicles) by programmed freezing. Recent progress using vitrification in combination with high concentrations of cryoprotectants has been documented. The use of several small animal models may allow evaluating whether interference with the natural cycle by hormone treatment, collection of immature gametes and long-term culture or *in vitro* maturation might affect the imprinting of susceptible genes in oocytes.
- Current reproductive techniques and the complexity of the reproductive system in different species require a profound training in reproductive biology. The precise understanding of the changes in metabolic regulation of cells and tissues cultured *in vitro* is essential to know the precise boundaries within which the gametic culturist needs to operate in order to ensure normal healthy offspring.

Session 2: Effects of Nutrition, Reproduction, Genetics, and Environmental Factors on Animal Productivity

Four keynote addresses were presented. These focussed on (1) managing livestock in degrading environments, (2) foundations, fallacies, and assumptions of science for livestock development, (3) interactions among nutrition, heat stress, and reproduction in tropical cattle, and (4) early stirrings of landscape genomics. Additionally, 81 papers

by scientists from 35 countries were presented orally or displayed as posters.

The main points emerging from this session were:

a) Dealing with enteric methane production

- Highly fibrous diets not only decrease feed-use efficiency, but also increase methane production in ruminants. Reducing methane emissions from ruminants therefore has implications not only for efficient animal production but also for global environmental protection. Computer applications for diet formulation must consider the need to reduce greenhouse gas emissions from ruminants.
- Mitigation of methane emission is essential to protect the environment from the greenhouse effect and at the same time improve feed conversion efficiency. Some strategies to mitigate methanogenesis are: (1) non-productive or low productive animals should be replaced with either high producing animal be they indigenous cattle/buffalo or crossbred cattle; (2) any effort made to enhance degradability of poor quality feeds (e.g. urea ammoniation of straw) results in an improvement in nutrient availability accompanied by a decrease in methanogenesis, (3) use of plants containing secondary metabolites (saponins, tannins, lignins, essential oils, etc.) that are effective against methanogenesis and ciliate protozoa.

b) Grazing and feeding behaviour

- Well managed livestock on either a grassland or mixed crop/livestock system offer a highly efficient method of increasing the production of high quality food with minimal environmental impact. There is clear evidence that livestock are not inherently damaging to rangelands or farming landscapes, and, in fact, may be required for their sustained health and profitability. Moderate to heavy grazing has, in some cases created highly resilient and ecologically sound systems. On the other hand, both over- and under-grazing are likely to result in a loss of species diversity. As livestock have the ability to select plants with higher digestibility, adequate nitrogen (crude protein) and low or manageable anti-nutritional compounds, in under-grazing situations loss of diversity will be accompanied by a decline in nutritive value and palatability, and reduced ability to deal with toxins. In the case of over-grazing the decrease in diversity and nutritive value will be accompanied by a decrease in feed availability. Management of livestock, vegetation, and the interaction between the two is critical for productive of grazing of such fragile environments.
- Where loss of diversity is a consequence of previous over-grazing, complementary feeding can improve feed intake, feed conversion efficiency, and therefore productivity. Examples include the provision of energy supplements to improve the utilisation of plants that contain high levels of non-protein nitrogen or to facilitate the breakdown of anti-nutritional compounds in the forage. Similar strategies can be applied to revegetation with selective planting to complement the composition and availability of other feed resources.
- Grazing and feeding behaviour can be further assessed with the use of stable isotope techniques applied to feed and water intake. These techniques have the potential to optimise the combination of livestock production and ecological stability that will be required for the long-term productive use and revegetation of degraded landscapes.

c) Towards intensive management systems in developing countries

- Consistent management systems based upon intensive inputs plus a compliant consumer market exist in industrialised countries; however, the social, physical, and economic environments in developing countries where most livestock are located are very variable, often unreliable, and distant from large markets. In 2007, half the world population lived in rural locations and 3 billion of these are dependent upon or associated with livestock. While there is a move in a few locations to set up intensive livestock units to serve the growing mega-cities in urban parts of the developing world, a high proportion of livestock and their keepers seek a sustainable life for themselves and their extended families in rural areas.

d) Environment, heat stress and animal productivity

- Efficient reproductive performance of lactating dairy cattle in tropical/subtropical environments throughout the world is impacted by a multiplicity of factors such as: the physical environment, social-economic status of producers, available nutrients, adaptability and genetic composition of cattle, intensive or extensive management systems, and available reproductive technologies. Seasonal periods of reduced fertility are associated with concurrent increases in temperature and humidity, availability of nutrients, and elevations in body temperature detrimental to ovarian function, oocyte competence, and embryo development.
- Development of low impact sustainable agriculture and a growing use of adapted breeds are of priority to most countries in the world, and are particularly important to developing countries/emerging regions. The level of adaptation of livestock breeds to their environment has to be measured in order to reach better understanding of the relationship between environment and the adaptive fitness of livestock populations, as well as to favour production systems based on adapted breeds.
- Bulls that transmit a high tolerance to heat stress have daughters with higher pregnancy rates, a longer productive life, but lower milk yields. Continued selection for milk yield without consideration of heat tolerance will likely result in greater susceptibility to heat stress. Various genes regulate heat tolerance such as the slick hair gene that contributes to a greater tolerance of lactating dairy cows to heat stress that likely improves fertility. By knowing the gene sequences of the bovine genome, identification of heat tolerance genes of *Bos indicus* breeds offers the potential of introducing these genes into less heat tolerant breeds.
- An array of refined reproductive technologies is available to better manage the reproductive performance of dairy cows. Since high temperature causes embryonic death during the first three cleavage divisions, embryo transfer of more advanced healthy embryos at day 7 will bypass the early heat sensitive period to partially restore pregnancy rates. Development of vitrification procedures for storage of *in vitro* produced embryos that develop normally post-transfer of the embryo will increase the impact of this reproductive strategy. Dairy heifers can now undergo timed artificial inseminations (TAI) with high fertility during summer to avoid seasonal breeding and parturitions. Optimised TAI programmes coupled with heat abatement systems reduce the impact of poor oestrus expression during summer.

e) Breeding management: crossbreeding and embryo transfer

- An efficient way to produce milk in the tropics is the direct cross between *Bos taurus* and *Bos indicus* under well-structured crossbreeding programmes; however, the problem arises when the farmer faces the challenge to breed the crossbred animal because crossing back with *Bos taurus* the resulting cow is quite vulnerable to the harsh environmental conditions in the tropics and crossing with *Bos indicus* the offspring will be deficient in milk production. An alternative is to transfer F1 embryos to F1 dams, hence avoiding the hazards of crossbreeding. Although the technique of embryo transfer has been available for many years, there are several pitfalls at least under tropical conditions, which need to be considered. Among them, embryo transfer programmes in small community farms is difficult because the selection of recipients is restricted to a few animals in the herd and even worse if the distance between farms is large; the super-ovulatory response can be directly related to the follicular dynamics at the moment of treatment and experience showed that the number of healthy embryos evaluated by their resistance to freezing and their degree of apoptosis was affected if the embryos were produced in the spring or the autumn.
- Timed artificial insemination are often advantageous to cattle producers because they reduce the time and labour required for the detection of oestrus and allow all animals to be managed in groups rather than individually. A wide variety of effective TAI programmes have been successfully developed but their use on a particular region must be validated. The selection of suitable females is of vital importance as the practitioner must ensure that cows are non-pregnant and with adequate body condition; otherwise, the treatment will fail.
- The monitoring of reproductive hormones, through radioisotopic techniques such as radioimmunoassay, in conjunction with field protocols for sampling, collection of behavioural and biological data, and the use of computer software applications, developed by the IAEA have proved highly advantageous for obtaining a better understanding of the reproductive physiology of livestock species, in identifying and ameliorating limiting factors affecting reproductive efficiency (in high-input-high output dairy production units, medium scale farms using modern technology, small-scale livestock farms and pastoral farms), in providing diagnostic tools for ensuring proper AI timing, for monitoring ovarian cyclicity, identifying anoestrus and non-pregnant females, and in assisting AI centres and services. Individual or corporate farmers, by using simple but well established and validated field and laboratory protocols, can monitor and evaluate the performance of their animals and farms. Animals can thereby reach sexual maturity and first parturition at an earlier age, offspring obtained at a higher frequency and in return, farmers can achieve higher and sustainable economic returns.
- The efficiency and sustainability of embryo transfer programmes should be critically evaluated. Data from a number of these programmes in Mexico was evaluated to determine the cost involved in the preparation of the donor and embryo recovery, the average number of embryos recovered, the percentage of animals pregnant, the production of the embryo itself. It was found that the cost for a replacement heifer was US$ 2 640 dollars which surpassed by far the commercial cost of a crossbred heifer (approximately US$ 900).

f) Camelids and milk production

- Old world camelids (Dromedary and Bactrian camels) are important sources of milk in rural areas of many arid countries; however, extensive production systems cannot guarantee constant quality and quantity raw milk for the market. Some attempts to develop large scale camel milking farms in Dubai draw attention to camels as a potential source for high quality milk and meat in developing countries. Average milk production per lactation was 2 467 ± 79.4 kg (340 ± 7.9 d) with some animals able to produce up to 8 000 kg per lactation.

g) Molecular genetics and livestock productivity

- Molecular genetics opens new horizons for changing the life processes of animals in ways that have great potential to improve their health and production. The scientific imagination sees the universe of DNA as an opportunity to reshape biodiversity to yield ever more efficient economic performance. Caution is needed. Emerging genome research shows that control of gene expression is far more complex than conveyed in current genetic models. For developing countries use of radical molecular genetics raises issues of risk, accountability, authority, power, ownership, morality, the nature of animals, and the sustainability of life for rural people in remote areas. Perhaps of greatest importance is the growing evidence for epigenesis. The mammalian genome is a complex of DNA, RNA of many types and proteins which seem to be engaged routinely in passing information around that modifies gene expression. Since this raises the question of information being fed into the genome and the genes from the environment, the whole issue of adaptation of livestock to differing environments is open to review.
- There are indigenous breeds with some degree of enhanced resistance compared with exotic ones reared in the same environment, especially for gastrointestinal nematode infections, diseases due to mycotoxins, bacterial diseases including foot rot and mastitis, ectoparasites such as flies and lice, and scrapie, and small ruminant transmissible spongiform encephalopathy. Therefore through genomic studies using radiolabeled nucleotides in DNA hybridisation, DNA characterisation, and hybrid mapping procedures, activities are focusing on the identification of molecular markers of economic interest. This will open possibilities to select and breed animals for enhanced resistance to disease.
- The number of available environmental data bases (NASA SRTM, NASA MODIS, Norwich CRU, etc.) is gradually increasing and their quality improving (better resolution). Most of these global data sets are freely available through the Internet and can be used for a comparable description of production environments worldwide. On the other hand, the amount of molecular data to be analysed will more than likely expand rapidly, and the next generation of sequencers will allow researchers to obtain genomic data faster and at a lower cost. Several alternative low cost sequencing technologies are under way, and the $100 complete sequencing of individuals is about to become real.
- Researchers in molecular biology have begun to consider new approaches for analysing the large data sets produced by the 'next next' generation sequencing technologies. On the GIS and statistical side, it is necessary to stress the importance of recording geographic coordinates in any new project involving animal sampling campaigns, and to improve related software. As the association analysis process is rather straightforward, the challenge will mainly be to improve the efficiency and robustness of algorithms.

- The cultivation of genetically modified plants has increased worldwide from 1.7 (1996) to about 114 million ha. Currently, soybeans, corn, cotton, and canola are the most important genetically modified plants. They are modified mainly for agronomic traits but the second generation should contain more valuable nutrients (e.g. amino acids, fatty acids, vitamins, enzymes etc.) or less anti-nutritive substances (e.g. mycotoxins, inhibitors, allergens etc.). Both chemical analyses and animal studies have revealed no significant differences between feeds from genetically modified plants and their isogenic counterparts and hence strongly support their substantial equivalence; also no significant differences were found in digestibility and animal health.

III. ANIMAL HEALTH

- The need to intensify and increase food production to meet the needs of the ever growing human population for nutritious and safe products demands more efficient means to diagnose and control animal diseases and to measure food and feed contaminants. The most prominent challenges that arose from the Symposium focussed on the increase in human population, new farming systems, increased movement of animals in world trade and the alterations in ecosystems brought about by climate change and the geographical distribution of pathogens or their vectors. Against this background, resource poor developing countries will increasingly be faced with an increasing prevalence of infectious diseases due to both known, as well as hitherto unknown emerging diseases. A significant proportion of the latter, over 60 percent, are likely to be zoonotic diseases, the greatest proportion of which will probably be associated with wildlife and therefore the domestic and wildlife interface is very important (e.g. HIV, avian influenza, rabies, Ebola, Rift Valley fever). This will challenge our perception of surveillance, requiring capacities at national and international levels to diagnose infections in the animals, rather than identify a problem zoonosis after it has spread to humans.

  Faced with this situation, it will be important for countries and the international community to prioritise activities in terms of resource allocation and focus on:
  - the most important endemic diseases;
  - the most important zoonotic diseases;
  - the most important exotic diseases;

- In spite of the many advances in our understanding of diseases and their epidemiology there are still gaps in knowledge, for instance in the global economic burden of zoonoses. Better insights are needed into the transmission pathways for opportunistic zoonotic infections, and at the same time to understand disease epidemiology in each host species, particularly wildlife reservoirs, since persistence of infection will slow down attempts to effectively control diseases. The global effort for surveillance and research on emerging infectious diseases is poorly allocated with much of the scientific endeavour being located in regions where new pathogens are least likely to appear. There is an urgent requirement for allocation of resources for surveillance of emerging diseases in Africa, Asia and South America. Such studies should include targeting at-risk people to identify emerging diseases before they become large scale problems. Zoonoses of wildlife origin are particular threats and it would therefore be useful to identify new potentially zoonotic pathogens in wildlife to forecast risk from emerging diseases. Zoonotic diseases are frequently under-reported and there is a pressing need for cross disciplinary approaches to fill knowledge gaps in research and diagnostic needs, and define responsibilities between veterinary and public health authorities to ensure integrated surveillance and control of diseases.

- There is a comprehensive range of new generation (nuclear and nuclear associated and nuclear related serological and molecular) diagnostic techniques to detect animal diseases. The list is long and includes both direct and indirect methods. On-site diagnostics for foot and mouth disease (FMD) and avian influenza using disposable automated sample preparation units have been developed with communications systems that enable instant reporting of results from even remote locations. Moreover, handling is optimised and indeed simplified to reduce human (field and health worker) and laboratory technician exposure. Rapid, specific penside diagnosis has also been made possible for peste des petits ruminants (PPR) by the development of a loop-mediated isothermal amplification (LAMP) PCR.

- Global trade in livestock and livestock products will continue to increase as it is driven by demand towards an ever increasing protein-based consumption. However, biosecurity and in particular, infectious diseases, along with other factors will limit this trade. Since the greatest biosecurity risk is posed by the live animal, investment in processing capacity at or near livestock production sites and prior to export has the potential to significantly reduce biosecurity risks. Trade in the processed product and not the animal could be a way for livestock producers to trade their way out of the poverty trap in developing countries. This would be of greatest benefit in those areas currently excluded from exporting due to biosecurity concerns and disease risks in national herds.

Session 3: Transboundary, emerging and zoonotic diseases

Five keynote papers were presented focusing on (1) emerging and re-emerging zoonotic diseases, (2) early, complex and rapid diagnostic technologies, (3) climatic changes, seasonality and the dynamics of infectious diseases, (4) an overview of FAO's Emergency Prevention System for Transboundary Animal and Plant Pests (EMPRES) and the FAO. OIE and WHO Global Early Warning System for Animal Diseases (GLEWS), and (5) World Organisation for Animal Health (OIE) activities for the global improvement of animal disease detection and control. In the open section, 39 papers and posters were presented by scientists from 27 countries.

The main points arising form the session were:

a) Emerging and re-emerging zoonotic diseases

- Recent outbreaks of infections with influenza H5N1 and H1N1 and the occurrence of HIV have drawn the attention of the general public to the problems of communicable diseases; nevertheless, communicable diseases have always impacted on mankind and although they might not nowadays present such a problem in the industrialised world this is not the case in developing countries where they have been, and continue to be, a major cause of morbidity and mortality. However, the more frequent movement of people between countries together with the movement of animals have increased the likelihood that diseases can spread more rapidly to areas where they are not normally found.

- Another factor that might in the future contribute to the problems of diseases is that climate change could lead to certain diseases increasing their range and affecting countries where they have not previously been a problem. There is a wide diversity of

protozoa, helminths, viruses and bacteria that infect animals, but have the potential to infect man; these zoonotic diseases represent the majority of newly emerging diseases now occurring, and they present a considerable challenge both for diagnosis and control. Where domestic animals provide a reservoir of infection, control and eradication of the disease is often viewed as being difficult or impossible and there is a tendency to neglect such diseases. Amongst those considered in this respect are intestinal protozoa, food-borne trematode and cestode infections and vector-borne protozoan infections like human African trypanosomiasis, leishmaniasis and Chagas' disease. Molecular biology has played a pivotal role in helping define these diseases in terms of their epidemiology, pathology and diagnosis as well as contributing to monitoring and surveillance. This is exemplified by the studies on Chagas' disease that have considerably altered our understanding of both the parasite and the insect vector, allowing their genetic subdivision and relating these parameters to their geographic distribution and the sylvatic cycle of development in reservoir hosts. Molecular characterisation of the liver fluke and the snails that harbour the infective stages have enabled a more clearly defined understanding of the epidemiology of human and animal infections.

- In spite of examples such as these, there are still major problems in dealing with zoonotic diseases. Although there is usually a general understanding of their epidemiology, at the specific, local level they may not be as well defined. More attention needs to be placed on establishing appropriate multi-disciplinary networks of health professionals to cooperate in managing the various strategic and political implications of controlling zoonotic diseases. Critically, much more basic field work needs to be done in endemic areas, applying not only the modern molecular methods, but also the increasingly neglected disciplines of medical entomology and malacology.

b) Diagnostic technologies

- Transboundary animal diseases disrupt trade and cause enormous economic damage; for instance, the last outbreak of FMD in the UK caused losses of Euros 12 billion. Long-standing problematic TADs like FMD and classical swine fever and various food-borne diseases have been joined by newly emerging diseases like highly pathogenic avian influenza (HPAI), swine 'flu and bocaviruses. For all of them however, there is a need to provide early and rapid diagnosis using the most up-to-date technologies. This aim is being met through the OIE Collaborating Centres for Biotechnology-Based Diagnosis of Infectious Diseases. These Centres, together with their collaborating partners have achieved considerable success in developing cost effective strategies for disease diagnosis and control. Among their achievements is the development of multiplex PCR and ELISA assays for classical swine fever that can be used to assist control of the disease worldwide, including in wildlife. For vesicular diseases, multiplex diagnosis using padlock probes can identify FMD, swine vesicular disease virus and vesicular stomatitis virus. These diseases present a similar clinical picture and conventional diagnosis is complicated and time-consuming, increasing the likelihood that they could spread further. The microarray system detects and identifies all three viruses in just a few hours.

- HPAI presents a major problem, both as a serious economic threat and a potential zoonotic infection in humans and there is a need for improved laboratory techniques for both virus detection and typing. Again, padlock probes allow identification of avian influenza viruses and subtyping within one platform, instead of needing to carry out 25 separate tests.

- Bluetongue has recently become an emerging disease in Europe and there was an urgent requirement for developing new virus detection techniques to replace the complicated cumbersome ones generally in use as well as providing a means for identifying the many serotypes. Primer-probe energy transfer (PriProET) with high diagnostic sensitivity and specificity now allows detection of all 24 serotypes. The necessity for rapid, on-site diagnosis in emergency situations, or in situations where laboratories have only minimal facilities is being addressed by the development of techniques such as the INVADER assay for classical swine fever and LAMP tests where results can be read by the naked eye using sophisticated portable PCR machines with built-in communications links.

c) Climate change and disease

- Climate change is widely acknowledged as being a significant factor likely to affect the occurrence and pattern of disease globally, and predicting what might happen in the future, or indeed, what has already happened to the epidemiology of infectious diseases is a matter of debate. The impact of animal disease in sub-Saharan Africa is considerable, overall leading to a 24 percent reduction in productivity, especially in livestock species important to the rural poor. For example, an outbreak of Rift Valley fever in eastern Africa caused losses of US$ 130 million and resulted in a decline of 20 percent% in household income. In trying to predict what might happen we must indentify those diseases that are more likely to be sensitive to climate change and what are the main drivers, i.e. temperature, rainfall, drought etc. How will these changes be manifest — by increased incidence and prevalence of infection or by increased virulence? Some pointers to climatic effects are already known. Outbreaks of anthrax are associated with heavy rainfall and drought and high temperatures; PPR is linked with the onset of the rainy season or dry, cold periods; African horse sickness (a vector-borne disease) is associated with a combination of drought and heavy rainfall; West Nile virus (another vector-borne disease) has been linked with severe droughts in Europe, the Middle East and the USA. A warmer, wetter world is likely to favour vector transmitted diseases and pathogens with intermediates hosts like helminths and gastrointestinal parasites. Among the effects of higher temperature could be an increase in vectorial capacity and range expansion e.g. bluetongue virus and its vectors in Europe. Nevertheless, predicting what is likely to happen in the future is not an easy process; the changes will be complex e.g. range shift, range expansion, population amplification. Socio-economic factors are also likely to impinge upon the process as there may be alterations in the environment that change farming practice and livestock production. Also, predictive models might not be sufficiently accurate when targeted at the local level.

d) Wildlife and emerging transboundary diseases

- Over half of the occurrences of emerging infectious diseases during the past ten years have been caused by zoonotic infections and nearly three quarters of them originated from wildlife, a trend that has been observed for the past 50 years. Their emergence is often driven by environmental changes that affect ecological systems, sometimes brought about by man-made agricultural changes. Diseases and pathogens that are exchanged between humans, wildlife and livestock should receive more attention in

the form of targeted, active surveillance, in order to improve timely detection, identification, molecular typing, and monitoring. Understanding the human-wildlife-livestock interface is being achieved by the establishment of collaborative networks that ensure capacity building, wildlife disease surveillance and studies of migration pathways to understand disease ecology and pathogen maintenance. For instance in the case of avian influenza the FAO plays a leading role in a scientific taskforce comprising over 100 individuals, governmental and non-governmental organisations that provide science based guidance for decision and policy makers

e) Improving animal disease detection and control: role of the OIE

- Ensuring food security is a goal that will not be met unless there are coordinated efforts to ensure that livestock productivity is maintained by the improvement of disease detection and control. Central to this tenet is the OIE that has the mandate of improving health all around the world. The OIE stands as the arbiter of the legal framework for controlling regulations on international trade and is one of three reference organisations responsible for international standards. Its objectives are to ensure that there is international collaboration in promulgating proper livestock health standards for international trade and animal disease surveillance, thereby contributing to food safety and security. It also provides the expertise for ensuring that there is international collaborative effort to control animal diseases and improve the resources for national veterinary services.
- These aims are achieved through the publication of international standard monographs on animal health, diagnostic tests and vaccines that describe in detail the various needs for ensuring livestock health and welfare. To ensure competency in the delivery of veterinary services OIE operates a Performance of Veterinary Services (PVS) tool that seeks to ensure components such as human resources, technical capability, interaction with stakeholders and access to markets are fulfilled. Over 170 Reference Laboratories in 32 countries facilitate disease diagnosis, supply reference reagents, provide training and organise proficiency testing. A further 30 Collaborating Centres assist in coordinating collaborative studies and harmonising procedures for animal disease regulations. The OIE network is enhanced by Twinning Projects that link an OIE Reference Laboratory or Collaborating Centre with a national laboratory, thereby improving expertise and providing sustainable diagnostic capacity to OIE standards.

f) Coordinating responses to the problems of transboundary diseases

- Transboundary diseases not only inflict direct economic losses but also affect trade since sanitary measures are often put in place to restrict the movement of animals and sale of livestock products in affected countries. In order to enable more effective response to TADs that directly impact on hunger, malnutrition and poverty, FAO created EMPRES, and in the area of animal health provides the information, training and emergency assistance to help control livestock diseases, including newly emerging diseases. Early warning is a vital component of the strategy and FAO. OIE and WHO have developed the GLEWS that will coordinate the response of the three organisations. In this way a more effective response to outbreaks of disease, especially those with zoonotic potential, can be realised.
- Peste des petits ruminants (PPR) is just one of the diseases that is a priority for EMPRES; since its discovery in Africa in the 1940s the disease has spread throughout Africa, the Middle East and Asia, so that over one billion small ruminants are at risk from this disease. FAO aims to progressively control PPR by stressing the importance and impact of the disease to national governments and other stakeholders and conducting epidemiological and socio-economic impact studies. The introduction of appropriate diagnostic tools will lead to a strengthening of surveillance and more effective emergency responses.
- For FMD a sophisticated Progressive Control Pathway, implemented in 2009, has been developed to assist the progress of individual countries in managing disease on the way to establishing freedom from infection. It is planned to have in place an active programme covering all seven major endemic regions from 2010, hoping to achieve in most endemic countries reduced circulation of the FMD virus by 2020, thereby offering safer trade from an increased number of countries.

g) Making the right choices for disease control

- By improving animal and human health, food safety and quality and facilitating access to trade for developing countries the Millennium Development Goals will become a possibility. One way of assuring this is to provide the means to accelerate the development and distribution of the means to control animal diseases in both Europe and the developing world. This is the aim of the European Technology Platform for Global Animal Health (ETPGAH) whose agenda sets out to address the views of stakeholders on the research and development needs in the short, medium and long-term.
- A number of thematic initiatives have been identified that will progress these aims. In the first place, diseases will be targeted based on appropriate prioritisation models that have been peer-reviewed and accepted by funding agencies. In Europe, the emergence of new disease conditions, either natural or by bioterrorism is an issue that requires immediate consideration. Since wildlife diseases rank first as a source of new pathogens, the introduction of sophisticated technologies as screening assays is a priority especially where zoonoses are involved to ensure that such pathogens are rapidly identified.
- Research funding will be targeted towards the priority diseases, but efforts would be made to identify existing gaps in our knowledge and understanding to enable more effective implementation of the strategy. New technologies will need to be assessed to ensure that they are used to their full potential and provide maximum benefits. There is very little information on the current research effort in priority diseases, nor on the availability of products from manufacturers of animal health products, including vaccines, pharmaceutical and diagnostic kits so it is necessary to identify those that are available. Finally, although the major aim of the ETPGAH is to provide adaptive technologies, it is vital that fundamental research in areas such as host-pathogen interaction, immunology, epidemiology genomics and bioinformatics is supported to ensure the development of new tools can be sustained

Session 4: One Health

Three keynote speakers presented papers dealing with One World One Health and there were a further 27 papers and posters in the general section presented by participants from 19 countries.

The main points arising in this session were:

a) One world One Health: what can be learned from the farm to the fork?

- The dynamic interaction and convergence of animals, humans the environments they occupy has created a situation in which the health of each group is interconnected. This is exemplified by the fact of the 1 500 or so diseases known to affect man nearly 60 percent are brought about by pathogens that have crossed the species barrier. The need to increase animal productivity together with the increased trade in animals and animal products may well be one of the most important risk factors with regard to infectious diseases. It is clear therefore that current attitudes of the animal health, public health and food industries to the management of disease will have to undergo a paradigm shift if the challenges of providing healthy animals together with food security, food safety and optimal health for people are to be met. The 'One Health' initiative seeks to improve collaborative interaction between the veterinary and medical professions to meet these challenges.
- In testing food, the emphasis should be on carrying out strategic testing based on the likely risk and there should be more effort in integrating data for agriculture, food and animal health. In the case of wildlife this is a particularly under researched area and it will be necessary to look further into the impact wild animals might have. There should be improvements to food control systems to allocate funds to sensible data gathering, including disease burden estimations. There also needs to be a combination of active surveillance and new typing systems to enable source attribution. Overall, risk can be reduced through efficient interventions.
- International regulations governing zoonotic diseases are already in place through the WHO International Health regulations and the OIE Terrestrial Animal Health Code. For major animal diseases the GLEWS enables the sharing of information between FAO, OIE and WHO. GLEWS is triggered by a number of events such as high mortality or morbidity in animals and/or humans, an unusual first occurrence or events associated with an unknown pathogen. It also considers the likely risk of international, transboundary spread that might lead to restrictions on international trade or travel.
- The International Food Safety Authorities Network (INFOSAN), a global WHO/FAO undertaking in collaboration with OIE promotes the exchange of food safety information, assists countries strengthen their capacity to manage food safety risks and responds to international food safety problems. Some 174 countries belong to INFOSAN.

b) One World One Health: is there a need for a global research agenda?

- Although there are efficacious tools for control of zoonoses, few have proved sustainable in resource poor countries. Since they impact on public and veterinary health, livestock production, and international trade and community development their management and control must be cross-sectorial in their inception. However, cooperation between these different sectors is often not encouraged for various reasons including institutional barriers and perceived 'mandates' of responsibility. Of considerable importance is identifying the sector that is most likely to benefit from control of zoonoses, and indeed, who will pay for the activities?
- Zoonoses tend to be neglected diseases; there is no common indicator of the effects, and they are often under-reported due to the poor capacity of developing countries to recognise and diagnose them. There is no measure of the effect, measured in disability adjusted life years (DALYS) for even the most common zoonoses such as anthrax or cryptosporidiosis and almost no global assessment. Wildlife reservoirs are of critical importance in control of zoonoses but there is insufficient knowledge of their ecology and epidemiology in the various host species affected. However, it is clear that the persistence of disease in wildlife reservoirs will hamper efforts to eliminate disease.
- Various One Health initiatives have been adopted including MED-VET-NET the European Network of Excellence for Zoonoses research. Med-Vet-Net's aim is to develop a network of excellence for the integration of veterinary, medical and food scientists, in the field of food safety, at the European Level, in order to improve research on the prevention and control of zoonoses, including food-borne diseases. The Network will also take into account the public health concerns of consumers and other stakeholders throughout the food chain. ProMed-mail is the global electronic reporting system for outbreaks of emerging infectious diseases and toxins. A central purpose of ProMED-mail is to promote communication amongst the international infectious disease community, including scientists, physicians, epidemiologists, public health professionals, and others interested in infectious diseases on a global scale. It encourages subscribers to participate in discussions on infectious disease concerns, to respond to requests for information, and to collaborate in outbreak investigations and prevention efforts.

c) Integrating laboratory results from animal food and human investigation

- Several sources allow for the introduction and transmission of food contamination, from feed –farm – slaughterhouse – processing — retail — consumption and at each point in the chain, the stakeholder is responsible for safety of the product. Therefore coordination of control actions is required with good communication between the many stakeholders. Information is needed on the source of contamination, the route of transmission, the most effective means of controlling or containing the problem and what is the likely public health burden. Various tools will be applied including suitable diagnostics, epidemiological modelling and decision support tools.
- There are many stakeholders in ensuring food safety, including ministries of agriculture and heath, veterinary authorities, public health authorities as well as reference laboratories and the food industry itself. A central coordinating body can ensure that there is appropriate collaboration and communication between these diverse organisations, providing information on all aspects of identifying, tracking, controlling and disseminating data from different sources. Integrated surveillance should encompass food, human and animal data and there should be a network among the various laboratories involved, including data sharing and standardisation of methods and definitions.
- The food trade is increasingly globalised; products seemingly originating form one country can be found to have ingredients that have been sourced in several other countries, possibly form a different continent. Hence there can be a rapid spread worldwide by the movement of food. This requires international oversight and this is ensured by FAO/OIE/WHO through GLEWS and INFOSAN.

Session 5: Achieving Food Safety and Security in the 21st Century

Three keynote speakers presented papers and 13 scientists from 10 countries presented their findings orally or with poster demonstrations.

The main points arising in this session were:

a) The global market for livestock and livestock products and biosecurity

- The enormous increase in demand for livestock and livestock products had been driven by increasing urbanisation and the shift away from cereal based diets. This has been accompanied by a movement in production from temperate and drier areas to warmer, more humid and disease-prone environments. Large scale industrial production near urban centres is increasingly common, often with emphasis on pigs and poultry. There is expected to be a significant shift of production to developing countries, with these contributing over half the milk, and two thirds of the global meat production by 2030.
- In relation to food security and the global market in livestock, the greatest concern is for the likely risks of disease being introduced to the national herd in the receiving country by the importation of live animals. This compromises the ability of developing countries to trade internationally, especially where veterinary services are inadequate. An alternative approach is to trade in processed products produced close to production areas. By investing in managing biosecurity at the processing stage the risks form disease would be mitigated. This would provide considerable benefit to those areas that are presently excluded from trading due to biosecurity concerns. This approach has had some success in Kenya and Ethiopia and deserves wider application.

b) The future of aquaculture and its role in contributing to food security

- Aquaculture started as a fresh water food production system in Asia, but has since spread to all continents and other aquatic ecosystems so that now a wide variety of both fresh water and marine species are produced. The range of enterprise covers both small scale, family based operations up to commercial, international industrial scale production. Aquaculture is still greatest in China and the Asia Pacific region, but in other regions, the scale of production, although much lower, is often of greater value.
- The importance of aquaculture is seen in relation to the decline in capture fisheries over the last 30 years. Many species are disappearing due to overfishing and perhaps a third of species are no longer available. Hence aquaculture is the fastest growing food sector in the world as the growing global population demands more aquatic food products. The annual growth rate over the last 50 years averages about 9 percent and is now valued at US$ 70 billion. The present trend is for continuing intensification of farming activities and increased diversification. There will need to be new production systems in which there will be more influence by the markets, trade and the consumer. Regulatory procedures will require better management practices.
- Aquaculture is limited in sub-Saharan Africa, its contribution being only <1 percent and the per capita consumption of fish has dropped. There is plenty of potential for development and growth of aquaculture and it is a high priority for this region. There will need to international cooperation and a focus on regulation, sustainable development and diversification while at the same time diminishing the impact on wildlife habitats.
- Aquaculture will be faced with various environmental constrains as a result of global climate change caused by increased temperatures, variable weather patterns, and the availability and quality of freshwater. Pollution and diseases will always be threats.
- Disease results in lower productivity but it also incurs costs to the producer in the form of the preliminary diagnosis, then control will require additional use of microbial compounds with the possibility of residues in the final product that compromises product marketing. Environmental degradation of the rearing area might also occur. The most important aspect of disease is the possibility of spread through trade, both national and international. Although local pathogens are the most prevalent form of disease outbreaks, introduction of exotic pathogens through trade is a reason for epidemics e.g. the spread of white spot disease of shrimps. However, endemic pathogens, internal transfers of live aquatic animals and factors such as poor biosecurity, water quality and bad husbandry are more common causes of outbreaks than international trade. The OIE, through the Aquatic Animal Health Standards Commission provides guidelines on health measures to ensure the safety of international trade and prevent the transfer pathogens for aquatic animals.

c) Production of biopharmaceutical compounds in plants

- Plants have a number of advantages as producers and delivery vehicles for antigens and other compounds. Antigens are more stable and protected against degradation and, in addition, no cold chain is required. To date nearly 200 heterologous proteins have been expressed in plants, including hormones, antigens, enzymes, growth factors and antibodies. Approximately a quarter of them are for veterinary use.
- Producing proteins in plants is cost-effective; it is cheap compared with systems based on tissue culture or milk from transgenic animals and the yields are higher than in these systems. Cereals have been used quite frequently, with yields in rice and corn in the order of 1–2 g/kg. The maintenance costs required for large scale production are low, but the cost advantages of oral delivery are the main advantages. Edible vaccines remove the need for trained personnel to administer injections, the cost of materials is lower and animals can be vaccinated via their normal feed; there is no need to gather them specifically for injection.
- Since large quantities of antigen can be produced, oral doses can be greater than those considered for injection. Oral delivery will stimulate a serum antibody response and also stimulate substantial mucosal immune responses. Hence, plant-based vaccines are particularly suitable for combating intestinal pathogens and those targeting other mucosal surfaces. Proteins expressed in grains can be stored in a dehydrated and stable condition for several years at ambient temperatures. Since plant based vaccines are sub unit vaccines, the safely concerns of live vaccines are removed.
- The market in veterinary pharmaceutical products is valued at US$ 15 billion and market conditions are favourable for developing new products for the livestock market, particularly for respiratory and gastrointestinal diseases. Amongst areas of opportunity are the development of multivalent vaccines for poultry diseases to replace current vaccines that require injection and to seek new avenues of development such as in fish farming where diseases are not currently controllable by vaccination.

d) Support for vaccine development

- The Global Alliance for Livestock Veterinary Medicine (GALVmed) established with funding from the UK Department of International Development seeks to identify gaps in the availability of vaccines or drugs in developing countries. It has a target list of two avian diseases, three diseases of swine, four of small ruminants and four of cattle. These include major transboundary diseases like PPR, contagious bovine pleuropneumonia (CBPP), African swine fever (ASF), Newcastle disease and avian influenza. *Theileria parva*, the cause of East Coast Fever is the first disease that GALVmed is providing the means for a sustainable vaccine. Supplies of the original vaccine are now depleted and GALVmed is supporting the preparation of new material at the International Livestock Research Institute (ILRI) to ensure continuity of control of the disease. The aim is to register the vaccine in Kenya, Uganda, Tanzania and Malawi and transfer the manufacture and distribution to the private sector to enable long term sustainability. It is anticipated that improvements in the production process will lead to price reductions.

PLENARY SESSION

Nuclear and Related Techniques for Solving Problems of Animal Production and Health: Development Context and Lessons Learned

J.I. Richards[1]

ABSTRACT

In reflecting on the role nuclear and related techniques have played in resolving problems of livestock kept by the poor in developing countries, the author acknowledges previous reviews in this area which dealt with the use of these techniques in generating new information on the elaboration of metabolic pathways, in unraveling the complexity of disease transmission, in understanding feedbacks in the endocrine system in relation to reproductive and metabolic hormones, and in attenuating parasites for vaccine production. Clearly the early benefits, perhaps with the exception of vaccine irradiation work, had more intellectual/scientific than economic/practical value. This short review, however, considers developments to date from the perspective of both the users and beneficiaries of such knowledge. It is only in the last 30 years or so that new knowledge and innovative technologies based on nuclear and related techniques have been used in the field to address challenges facing livestock keepers. Interestingly, these developments occurred in parallel with new knowledge in the physical and biological sciences — particularly in the use of plastics in bio-medicine, in fluid transfer and analytical throughput systems, in the computerisation of analytical technologies, in the measurement/detection of ultra-low levels of radiation in immunology, and in molecular biology. Also, there has been a palpable increase in interest of the business sector in developing robust and/or field-oriented technologies for use in the South e.g. nuclear and other marker-linked assay systems for the measurement of a wide range of key antigens and antibodies. In reviewing these developments, the author has attempted to place them within a development context and to draw out lessons learned particularly from the perspective of their contribution to solving problems of animal health and production and contributing to achievement of the Millennium Development Goals. Ten lessons are identified for consideration by FAO/IAEA, donors, policy makers, research managers and researchers to make the use of nuclear and related techniques more applicable and available for users, and to ensure the benefits of such techniques address the needs of poor livestock keepers. They include: a less reductionist approach to research; a more holistic approach to research on livestock systems; more diverse public and private research partnerships; longer, more sustained research trajectories; more support for extension/technology transfer; better communications (and resources) to facilitate wide-scale adoption of research products; more emphasis on monitoring and evaluating the outputs of research on livestock production and livelihoods; and greater advocacy of evidence for the key contribution of livestock and livestock research for development.

Key words: *livestock, research, poverty, nuclear, innovations, lessons.*

INTRODUCTION

This paper does not detail the history of using nuclear and related techniques in livestock development since this has been done previously by acknowledged experts in the field (Mulligan 1976; Thatcher et al., 1986; Robertshaw, 1986; Wright et al., 1986; Poppi, 1986; Annison and Leng, 1991). Rather, an attempt is made to locate the use of nuclear techniques within an agricultural developmental context and to highlight some lessons learned from previous experiences which may enable future investments in the development and application of such techniques to be put to better use in resolving problems of animal production and health, particularly as they impact on the needs of the poor. This paper also attempts to put the work on livestock of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture into a global context.

The publications emanating from the work of this Division are not included in the list of references provided as they are too numerous to cite and are widely accessible through the Division's web site at http://www-naweb.iaea.org/nafa/aph/index.html. They were, however, reviewed in the process of preparing this paper which benefitted also from an internet search to capture global contributions in this field. However, it should be emphasised that unlike most other papers in these Proceedings, this is not a conventional research paper — but a review which attempts to identify lessons learned from past experience in developing and applying the techniques concerned.

The development context under which innovative nuclear and related techniques are employed is described including the challenges faced by livestock keepers in developing countries: poverty, hunger and food security, climate change etc; the special case of Africa; the emerging role of the new economic powers (BRIC countries — Brazil, Russia, India and China); and the increasing demand for livestock products at the global level. The paper also describes the processes used in identifying appropriate research; the sustainability of R&D support; and the dissemination pathways traditionally employed to relay innovative research products to target beneficiary groups.

The lessons learned from the foregoing are grouped into 10 discreet but related issues. These are directed at a range of stakeholders involved in nuclear applications for livestock development

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— policy makers, donors, research managers, researchers, academic institutions, public/private extension agencies, civil society, farmers associations, providers of goods and services, knowledge managers, the media, consumers etc.

CONTEXTS

World Hunger, Health and Poverty

With grain yields currently increasing at 1.1%/annum and the world's population growing at 1.2%/annum the ability to satisfy food needs are gravely threatened. Concurrently, there is increased demand for feed grain to satisfy the ever-increasing demand for animal protein especially by the new burgeoning economies, where grain is increasingly being used as raw material for fuel energy, and reserves of grain and other food staples are at an unprecedented low. Consequently, Malthusian predictions are not unthinkable, particularly in Africa. The latest reports from FAO and other global contemporary information sources indicate that the number of hungry people in the world increased in the past two years by 75 million to 963 million. Among the causes cited are: decreasing areas of productive farm land for food, higher input prices, greater demand for and increased price of food and climate change (FAO, 2002 and 2008a; World Bank, 2008a; Holmes and Nabarro, 2009). Consequently, every day, some 16 000 children die from hunger-related causes — one child every five seconds (FAO, 2002; Black and Bryce, 2003; UNICEF 2008; Bread for the World, 2009). In essence, hunger is the most extreme form of poverty, where individuals and families are unable to grow, do not have access to, or simply cannot afford to satisfy their most basic need for food. This is particularly disastrous for families suffering from HIV-AIDS, malaria, dysentery etc (WHO/UNICEF, 2004). The food security issue has become so severe that it is impacting on world stability with food riots in over 40 countries over the last two years (Population Reference Bureau, 2006; Brown, 2009).

World food prices remain high despite small recent decreases (FAO, 2008b). One major cause for soaring food prices has been the rapid growth in demand for biofuels, which has diverted land from food production. Rapid economic growth and urbanisation too, particularly in Asia, have resulted in a high demand for meat and consequently for maize and soybeans for livestock feed; and in some parts of Africa, improved economic growth has increased the demand for staples such as maize. Meanwhile, growth in agricultural productivity especially in developing countries continues to remain static or is declining. Growing water scarcity and negative climate change effects are also increasingly affecting food production and prices. Poor and food insecure households are among the hardest hit by rising food prices and subsequently by the current global economic recession.

The Green Revolution in Asia in 1960–1980, and increased investments in agricultural research and development (R&D) in the North up to the early 1980s contributed to a doubling in global grain and livestock production which exceeded population growth needs — and prompted the slogan 'end of famine forever'. The consequent 'mountains' of stored grain, meat and dairy commodities caused much public criticism and political concerns in the North. In response, overzealous policies were put in place to limit production e.g. restrictive milk quotas in the European Union (EU) in 1983 to lower the butter 'mountains'. Concurrently, much of the excess was 'dumped' in the South; this inhibited local production and compromised trade and investment in agriculture there. A quarter of a century later, just as demand for food is exceeding supply, the stores are empty and many former farmers have found alternative employment.

Investments in global agricultural R&D also fell dramatically beginning in the early 1980s from two percent growth year-on-year to 0.6%/annum from 1990 onwards; the relative investment in livestock R&D was skewed even worse. Experience shows that research products in the agricultural sector take a generation or so to filter through to farms at best, so the consequence of this underfunding in agricultural R&D means there are fewer potential technologies available for farmers to use than would otherwise be the case.

Developments in the BRIC Countries

The economies of the four BRIC countries have one thing in common — fast growth. They are undergoing rapid industrial and agricultural expansion, have strong consumer and export growth, enjoy substantial Foreign Direct Investment, and are in the process of becoming much larger forces in the global economy (Mityayev, 2009; Kumar and Fodea, 2009; Beijing Review 2009).

Brazil's wealth derives from an abundance of mineral resources, agricultural products such coffee, soybeans, sugar, oranges, cocoa and tobacco, livestock products including beef, poultry and leather footwear; and wood products such as pulp, paper and plywood. Equally rich in natural resources, the vast territory of Russia, the world's largest country in terms of area, is endowed with great reserves of oil, natural gas, metals and timber, which together account for more than 80% of exports. On the other hand, the economy of India is booming as a result of its huge population, which provides a variety of service skills such as a thriving information technology (IT) sector. It is also the world's largest milk producer — the majority of this being generated by 1–3 cow/buffalo herds. And China's dominance in global manufacturing is due to a vast low-cost labour market and a dynamic entrepreneurial approach.

The Special Case of Africa

Africa is the poorest continent on earth, and the only one, despite all the technological advances that are filling stomachs and pockets elsewhere, that has actually grown poorer over the last 35 years. Approximately two-thirds of sub-Saharan Africa's population is dependent on agriculture and the sector is responsible for generating one-third of the continent's GDP. With its vast arable land, Africa has the potential to feed its citizens and to be a significant global exporter of food. After independence, investments in R&D continued to improve production and productivity of crops, livestock, forest products and fish. There was also a significant increase in monoculture, and greater specialisation and intensification; and there were attempts to sustain productivity and overcome trade barriers. Yet today, Africa must import much of its food and rely on food aid to feed many of its people. Most countries in the continent are now poorer than they were 50 years ago; half of sub-Saharan Africa's over 600 million people live on an average of US$65 cents/d — and the majority of poor subsistence farmers lack the money to buy seeds, fertilisers, tools and basic resources; and for numerous reasons, mostly associated with risk, financial institutions are reluctant to provide credit to small farmers.

The issues farmers face are enormous — access to water and shelter; prices of and access to basic foodstuffs and agricultural inputs; huge levels of mortality and morbidity in the human and animal population, some but not all due to avoidable conditions such as contaminated water; lack of protection from infectious pests/diseases; more frequent and severe droughts and floods exacerbated by climate change; the continuing debilitating effects of malaria, yellow fever and AIDS, and a host of energy-sapping parasites all exacerbated by poverty and hunger; problems of governance, land

ownership, security, political/social unrest; institutional corruption; national and individual debt; national and regional coordination difficulties; coping with climate change including more frequent and severe droughts; and now suffering the worst of the global food and financial crises (Delgado, 1995; Cleveland, 2007; World Bank, 2008b, ONE International, 2009). Many of the richer and better educated Africans see a dismal future too — about 40% of Africa's privately held wealth is held offshore.

Efforts by the world's rich countries to solve Africa's problems over the past few decades have largely been of a reductionist nature, and have been spectacularly unsuccessful; those countries that have prospered have tended to do so by their own efforts and addressed issues in a more holistic way.

Development assistance in the form of appropriate technologies in agriculture can help provide the technical expertise to move smallholder farmers out of poverty, but this has to be accompanied by an effective extension/advisory service, better access to inputs and markets, and a revitalisation of national agricultural research systems. Unfortunately, international and national development assistance for agriculture has declined dramatically over the last two decades. This despite the fact that the World Bank estimates that growth in the agricultural sector in Africa is four times as effective at reducing poverty than growth in other sectors (World Bank, 2008). Yet over the past 2–3 years, Official Development Assistance (ODA) to agricultural development in Africa has begun to increase; and as part of the African Union's Comprehensive Africa Agricultural Development Programme (CAADP http://www.nepad-caadp.net/) African governments have pledged to commit ten percent of national budgets to agriculture and reach the goal of a year-on-year increase of six percent in agricultural productivity by 2015. In addition, the 2008 G8 Summit in Hokkaido and the January 2009 UN Summit on Food Security signaled the urgency of both short-term and long-term needs in food security and agricultural productivity. Also, The UN's Comprehensive Framework for Action (CFA) lays out a set of actions for donors and international financial institutions to act upon (Holmes and Nabarro, 2009).

Global Livestock Issues — Past, Present and Future

The livestock sector is the fastest growing agricultural sector and has been predicted to continue growing at high rates for the foreseeable future. Global demand for meat and milk has more than doubled in the developing world over the last 20 years — driven largely by the new affluence in BRIC countries. This 'livestock revolution' is largely satisfied by semi-industrial production systems involving cereals as the primary energy source — in direct competition with more direct human demands.

Livestock production has been shown to make an important contribution to national economies as well as to increasing incomes and security at the community and individual levels. Mechanisms to escape from poverty are inextricably linked to livestock. Consumption of livestock products has important health benefits and a modest amount of animal protein in the diets of African children appears to improve their mental, physical and behavioural development (Neumann and Bwibo, 2003). As a proverb in the Horn of Africa goes*: if the animals die, then the people will die too*. Approximately one billion poor people in the developing world rely on livestock for their livelihoods: for their nutritional wellbeing, for social standing, for financial security, for draft power, for dung for fuel and fertiliser, for their hides and skins etc.

However, despite the many new developments in animal nutrition, breeding, husbandry practices, reproduction and animal health over the last 50 years — some of which have filtered down to the developing world, there are still annual losses of 50 million cattle and water buffalo, over 100 million sheep and goats and countless poultry from parasitic and infectious diseases (FAO, 2009). Many more succumb from inadequate feed and water supplies, poor husbandry practices, inappropriate policies and ignorance.

A major new challenge to livestock today is the conflicting evidence and consequent media frenzy regarding their contribution to climate change (Cline, 2007; Pachuari and Reisinger, 2007; Stern, 2006, IPCC, 2007; Nyong, 2007; Noble, 2007; Human Development Report, 2007/2008)). According to a recent report from the UN's Food and Agriculture Organization, livestock production, dominated in the West and increasingly in BRIC countries by large-scale factory farming, is responsible for 18% of the world's greenhouse gas emissions (FAO, 2006), a bigger share than that contributed by all of the world's transport. Yet the global livestock population is predicted to increase dramatically over the next 40 years: cattle from 1.5 to 2.6 billion; and sheep and goats from 1.7 to 2.7 billion. Simultaneously, grazing intensity is projected to increase by 50% globally by 2030 — with substantial land degradation potential. As a consequence, livestock are receiving a very bad press in the North and there is pressure to reduce consumption of livestock products and/or increase their price through some form of carbon taxation. Yet as stated above, livestock production provides an essential pathway out of poverty. Rich and poor worlds are thus colliding when it comes to the value of livestock production and consumption of livestock products. But who is advocating for the key contribution of livestock for bettering the lives of the poor?

Science therefore, and the generation of objective evidence through the use of nuclear and other techniques, can serve as an honest broker in the complex and often controversial debate over livestock and environmental issues. The 'truth' may be inconvenient to some, but clear empirical evidence is needed in this discussion. The global agricultural research community needs to develop a more comprehensive, integrated agenda to provide crucial, objective evidence on the trade-offs between food security, livelihoods and the environment. It is surely not beyond man's wit to protect the livelihoods of poor livestock keepers while also conserving environmental resources. The foregoing begs the question why livestock research findings have not had a greater impact on livestock development and poverty reduction.

LESSONS LEARNED FROM RECENT USES OF NUCLEAR TECHNIQUES

Effective Development Occurs when the Poor are Empowered to Articulate their Needs and Concerns

Whereas many people are familiar with the eight Millennium Development Goals, the one which most recall is the one which addresses a monetary target — "to reduce by one-half those who subsist on less than US$1/d". But what are the true indicators of poverty? Most subsistence farmers do not have the opportunity to earn money from their small land holdings or to earn a wage from labouring; and subsistence farmers represent the majority of poor farmers in most African countries, e.g. in Rwanda, over 90% are subsistence farmers. Their basic human needs include access to food, water, shelter and clothing. Another measure of poverty, the UN's Human Development Index, looks at quality of life factors including access to education, health systems and credit. Others consider human security indicators — whether people have the assets or skills to survive shocks such as poor rainfall, while others stress the importance of empowerment and participation in decision making, including the right to information and knowledge. Therefore, when (or if) scientists undertake

market research in relation to the issues affecting the poor, they need to specify which poverty indicator is being addressed by the research and not restrict the vision statement to improvements in agricultural commodity or productivity indicators which may not automatically be beneficial to the poor.

In the North, indicators of successful agriculture and livestock research at the project level are normally increased production on a per ha or per investment basis. These indices are in common use and many of the research benefits accrued are marketed through public/private extension systems to commercial farmers, and the extra income earned goes to improving the livelihoods of the farming community. Unfortunately, this model has been transferred wholesale to the South — and is largely inappropriate for two main reasons. Firstly, the vast majority of small-scale crop-livestock farmers and pastoralists seldom if ever receive advice from government extension agents. There are many reasons for this — but in general, the number of farmers far outweighs the capacity of the extension services and so they are generally not fit for purpose. With very few exceptions, the extension systems are generally grossly underfunded and ineffective; the criteria of success are not appropriate and incentives to adopt new technologies or to innovate seldom exist. And secondly, animals are often kept for reasons not understood by Western influenced researchers, e.g. for good reason, the Maasai herders in East Africa believe that a cow/bull should not be bred to be heavier than the ability of two men to lift it to its feet during a drought. How this contrasts with the breeding philosophy from the North.

The Development and Transfer of Nuclear Techniques have to be Appropriate for Need and Local Circumstances

Up until the 1970s nuclear techniques contributed largely to learning — biochemical pathways, and physiological mechanisms in animals and man, and the use of radiation to produce attenuated vaccines. Thereafter, there was greater emphasis on the application of these techniques to problem solving in animal feeding, reproduction, breeding, genetics, animal health etc. Examples include:

- *nutrition and feeding:* the use of isotopes (deuterium) in the investigation of metabolism in the rat in the 1930s; the use of stable (^{15}N) and radioisotopes (^{14}C, ^{32}P and ^{35}S) in characterising biochemical and metabolic pathways in the 1950s–1980s; the development of feeding standards for most livestock species in 1960s; and in some applications such as in evaluating new diet formulations from mixtures of feeds for livestock in marginal environments, e.g. various radioisotope markers in *in vitro* systems such as the Rusitec in the 1980s and 1990s.
- *reproduction:* the use of competitive protein-binding assays (based on ^{3}H) for various steroid and protein hormones in the 1960s and 1970s to the use of rapid (^{125}I-based) radioimmunoassays for quantifying levels of progesterone, luteinising hormone, follicle stimulating hormone etc. in biological fluids in the 1970s–1990s to assist in detecting oestrus and diagnosing pregnancy in farm animals and improving the efficiency of artificial insemination (AI), natural service and animal husbandry practices. Whereas these were widely adopted over this period in many developing countries and found to be extremely useful tools by researchers in support of farmer needs, their subsequent transformation into enzyme marked immunoassay technologies resulted in much wider use as pen-side tests.
- *animal health/disease diagnosis:* the use of (^{32}P-based) DNA probes and the development of enzyme linked immunosorbent assays (ELISA) for a range of key livestock diseases including rinderpest, foot and mouth disease, brucellosis, peste des petits ruminants, Newcastle disease etc in the 1990s – early 2000s have been enormously successful in contributing to national and global eradication and control of these diseases (viz. rinderpest). Recent collaborative work with international diagnostic centres has also produced kits which distinguish vaccinated from naturally infected animals — a key development in animal health practice in the developing world; these procedures are being proposed as candidates for commercial kit application. Also, a range of exciting new related and complementary technologies, including genetic modification (GM) and RNA interference are being assessed for their potential.
- *insect and other pests:* the eradication of disease vectors which affect livestock — such as tsetse flies and New World screwworm- where gamma radiation or X rays are used to sterilise male flies prior to mass release through use of the sterile insect technique (SIT) has also been used successfully in North Africa and on the island of Zanzibar to eradicate these pests. And the use of stable isotopes to follow the migration of wild birds is an important component in the control of the avian influenza virus.
- *structural and functional genomics as associated biotechnologies* will become really important to agriculture in the future as they begin to unlock the mysteries of disease and the characterisation of breed traits. One goal will be to accelerate disease research by enhancing the genomic tools used to explore how bacteria cause disease. Also, sequencing of the cow genome (Elsick et al., 2009) provides new information about mammalian evolution as well as bovine-specific biology and points the way to research that could result in more sustainable food production. Whereas these are molecular diagnostic developments, they are often characterised as nuclear-related because of the pioneering work in DNA technology using ^{32}P and ^{35}S markers and their continued use in labeling genetic markers e.g. in Southern blots.

Whereas the successful use of these techniques in increasing basic knowledge of biological systems cannot be denied, their successful application in addressing the needs of the poor cannot be argued so strongly, at least as yet. Critics question whether these specific developments have addressed user needs or simply the interests of the scientific community — and whether adequate market research was undertaken up-front to identify and prioritise problems facing poor livestock keepers and in 'marketing' the products adequately as is the norm in private sector research.

The Most Useful Applications of Nuclear and Related Techniques Occurred as a Consequence of Parallel Innovations in Other Sciences

Particularly crucial were developments in immunology (Wu, 2006), in plastics chemistry, in radiation detection, in liquid delivery systems (Martin, 2001), in miniaturisation, in throughput speed technologies, in complementary enzyme marked systems (Engvall and Perlman, 1971), in DNA chemistry and in genomic sciences (Elsick et al., 2009) etc. These developments would not have become marketable products without the key involvement of the private sector, particularly the equipment manufacturers and the diagnostic companies who saw unique opportunities in the application of much of the above particularly in the field of human medicine both in the North and South. There is still a key role for blue-sky research and research into potential 'marriages' between nuclear techniques and other innovations in science and technology. Some of the developments in genomics illustrated in these Proceedings exemplify these exciting innovations.

Nuclear Techniques are Likely to Further Significantly Influence Development only when:

- animal science researchers collaborate more closely with those in other disciplines within agricultural systems — crops, pests, residues etc, and with other sectors which affect the systems in which poor livestock keepers operate — human health, education, infrastructure, policy development;
- the agricultural systems within which livestock and their keepers co-exist are better understood;
- researchers address both long term and contemporary issues which affect livestock keepers e.g. access to food, adaptation to climate change, disease control, sustainable feed resources, simple breeding practices, pen-side diagnostics etc.

The Different Needs of Target Users and Beneficiaries are Better Understood

Research needs to be balanced and considered — between the demands for better livestock trade by commercial farmers, to the increased livestock production/productivity and associated livelihoods benefits of small farmers, to the improved production of livestock under challenging environmental conditions faced by subsistence farmers and pastoralists. The target groups for the information generated through the application of nuclear techniques also need to be defined and addressed. Researchers need to be clear whether they are developing technologies for *users* or/and for *beneficiaries* — accepting that both are members of much larger value chains of institutions that span the farm-to-fork continuum.

The Benefits of Wide-scale Use of Nuclear Techniques in Development are Effectively Communicated

Based on the outputs of the FAO/IAEA's Coordinated Research Programmes over the last 30 years, the Joint FAO/IAEA Division has generated an excellent series of publications. There is also an unique series of laboratory training manuals. The professional quality of these publications is extremely high and their benefit to third world livestock systems 'potentially' enormous. However, there is little objective evidence of the impact of this information on livestock production or practices.

One major reason for this may be poor communications practices. Public sector research has a great deal to learn from private sector approaches to the research process, including better communication. The private sector traditionally devotes as many resources to communication (product marketing, publicity, branding, promotion, sales etc.) as it does to the research and development of a product. And prior to the R&D activity, significant investments are made in communications (market research) to ascertain exactly what clients need. Compare this with the tiny investments made by the public sector in communications and it becomes more understandable why public sector research findings are not more widely adopted. The situation has been exacerbated in many developing countries by collapse of national extension services during the debt crisis of the 1990s; and most of these have still not been rebuilt.

To improve the situation, there is need for root and branch change in the mind sets of research institutions and donors involved in development. Despite the fact that researchers are now encouraged to add value to their research products through working on-farm, by writing extension products and even advising farmers directly, they have not been trained in this area, there are no financial or career development incentives to go the 'last mile', and there are too few to address the multitude of clients. Thus, there appears to be an urgent need for public sector research to employ and train professional communicators and marketing specialists, and allocate appropriate budgets to enable them to undertake this work within and outside current extension systems. As an indicator of the importance assigned to this sector, some donor research programmes now allocate up to one-third of their research budgets to communications to facilitate greater out-scaling of relevant research findings to value chains.

Communication strategies also need to be part of research programme planning and implementation. Indeed, in a recent report by Perera (2006) who reviewed the successes and missed opportunities in the work of the Joint FAO/IAEA Programme, there was an acceptance by many decision makers interviewed that better access to appropriate information by animal scientists and field practitioners was essential if the impact of innovations on poverty reduction are to be realised.

The internet search made for this review also highlights a communication issue in need of attention. Approximately 90% of the references resourced originated from, or referred to, FAO/IAEA Research and IAEA Technical Cooperation projects. The remaining 10% were from the USA, India, Israel and Brazil — and most of these came from their respective Atomic Energy establishments. Whereas it may be comforting for the Joint FAO/IAEA Programme to know that its work is at the centre of activities in this field, it does illustrate a problem that is consuming the attention of many donors — lack of evidence of widespread application of research findings and innovation to non-nuclear research laboratories and field operations. This is not uncommon at all. For instance, in managing the UK Department for International Development's (DFID's) numerous livestock research projects in Africa, Asia and Latin America (www.lpp.co.uk) and more recently its Research Into Use (RIU) Programme (www.researchintouse.com) the author became increasingly aware of the strong disconnect between generators and users of new technologies, practices or policies, and also between the users and beneficiaries, irrespective of whether these research products had nuclear labels or not.

Need for Champions to Advocate a More Significant Role for Livestock in Development

There is general agreement that the livestock R&D community needs to become more vocal in advocating evidence-based support for the contribution of livestock and livestock research to development. Regarding 'advocate to whom?' the answer must include the media, parliamentarians and other decision makers, donors, the general public, the private sector — institutions not normally targeted by researchers. All animal scientists have a role to play in lobbying and in potentially challenging government on policies with evidence based on objective scientific data. They also have a responsibility to inform the press appropriately and prevent the catastrophic media blunders such as the recent misuse of the term 'swine flu' and its extreme consequences on the pig industry world-wide (El-Awady, 2009) However, while accepting that the generation of such evidence is a keystone to effective advocacy, it must be acknowledged that the publication records of many southern scientists in peer-reviewed journals leaves much to be desired — and some question the value of further donor investments in research in the South until this record is improved.

Issues in need of advocacy include: i) increase ODA for agriculture and livestock; although 75% of the world's poor live in rural areas in developing countries and depend on agriculture for their survival, a mere four percent of ODA goes to agriculture and less than one

percent to livestock, ii) in sub-Saharan Africa, GDP growth originating in agriculture is about four times more effective in raising incomes of extremely poor people than GDP growth originating outside the sector, iii) a dynamic 'agriculture for development' agenda can benefit the estimated 950 million rural people in the developing world who live on less than US$1/d, most of whom are engaged in agriculture. Agriculture needs more prominence across the board. At the global level, countries must deliver on vital reforms such as cutting distorting subsidies and opening markets, while civil society groups, especially farmer organisations, need more say in setting the agricultural agenda.

Partnerships Strengthen the Development Effort

These include in research, in development, with industry and with marketing. The work of the animal health group within the Joint FAO/IAEA Programme has been exemplary in this regard. For instance, by engaging with global organisations and initiatives such as FAO's Global Rinderpest Eradication Programme (GREP), with the World Organisation for Animal Health (OIE), and the Emergency Prevention System for Transboundary Animal and Plant Pests and Diseases (EMPRES), the diagnostic and training elements provided by the Programme contributed to international coordination mechanisms to verify freedom from rinderpest and promote its global eradication while providing technical guidance to achieve these goals. Such partnerships have also enabled the Programme to be influential at the global scale in the control of foot and mouth disease, trypanosomosis, brucellosis and peste des petits ruminants.

Even more innovative is the development of the 'OneHealth' programme between FAO and WHO which addresses zoonotic diseases from both human and animal perspectives. Whereas such partnerships have generated positive outcomes, the world's agricultural R&D institutions remain largely autonomous at heart and there is need for agreement both on their 'territorial' mandates and strategies — and for greater collaboration especially at the programme/project level. This includes the UN specialised agencies, the CGIAR and the global and regional fora for Agricultural Research for Development. Other global initiatives such as the Livestock Community of Practice www.cop-ppld.net/testsite for Pro-poor Livestock Development being facilitated by the International Fund for Agricultural Development (IFAD), is an inclusive partnership of livestock practitioners, managers, researchers and other actors involved in livestock development (Community of Practice) through which there is exchange of experiences, management of relevant knowledge and support for learning as an instrument to achieve better uptake of knowledge. And as for research priorities, the Inter-Agency Donor Group for Livestock Production and Animal Health Research (IADG — see www.cop-ppld.net/) website) meets annually to address how it might coordinate funding activities to significantly increase the level of adoption of new livestock technologies, practices and policies and justify the expenditure of public funds in generating such information. This Donor Group identified the top priorities for future livestock research for development as process issues in support of previous technical investments in research: i) communications, ii) advocacy, iii) research into use, and iv) vaccine delivery systems.

R&D Processes are Significantly Improved

The fixation by donors and research implementers to employ disciplinary biased and short duration (three-year) projects to address development issues is inappropriate. History reveals that successful projects are more holistic in nature, have an agricultural systems perspective and a longer time trajectory (minimum five years). Equally frustrating to development is the inability to disseminate and transfer new technologies, practices and policies to users as evidenced by the work of the RIU programme. However, the FAO/IAEA Programme has an unique partnership model between the research-oriented activities of its Coordinated Research Projects and the technology transfer and scaling-up services offered by the IAEA and FAO Technical Cooperation Departments. Whereas this partnership could be greatly strengthened, it provides a research and transfer model for other agencies and donors to follow. There is also increasing evidence to show that focusing research on the needs of the several institutions within the farm-to-fork value chain results in greater adoption and impact than when the research findings are directed solely at poor farmers. Finally, it is evident that greater involvement of the private sector in research activities and/or as value chain recipients of new knowledge improves the likelihood of research being adopted and generating value at the community level or wider.

Monitoring and Evaluation Processes are Employed routinely to Assess Progress and Performance

Despite the rhetoric surrounding the use of M&E practices in development, there is a general reluctance by the scientific community involved in development to willingly engage in this area — both in monitoring the progress of a project or process and in evaluating achievements to date or at end of project. Direct evidence is normally available on the *outputs* generated as a consequence of projects involving the use of nuclear and related techniques in support of livestock research. These might include statistics on the technologies transferred such as individuals trained and data on the successful harmonisation of in-country laboratories (practices and performance) which conform to international standards. There is normally less evidence of the benefits to livestock keepers at the project level such as might be generated through the wide-scale use of technologies such as ELISA in support of livestock disease eradication or control; or at the programme level, particularly when such technologies are employed through partnership agreements with specialist global institutions. Some more tangible evidence sometimes exists of increases in livestock trade as a consequence of the use of such techniques in the control of trade-related diseases such as foot and mouth disease and rinderpest. However, despite these successes, objective evidence on the benefits of such programmes to the livelihoods of poor farmers is seldom available and there appears to be satisfaction in capturing information 'solely' on the benefits to livestock themselves. Whereas such achievements are laudable, there is an unfortunate reluctance to go the 'last mile' and assess the benefits to livestock farmers.

Regarding nuclear technologies in support of reproduction or animal nutrition, again there is much subjective evidence about improvements in livestock productivity at the local (project) level as a consequence of investments in the development and use of nuclear and related techniques but the benefits to poor livestock keepers remain largely speculative. Thus in response to the query: "has research on nuclear techniques contributed to achieving any of the MDGs or else contributed to wide-scale increases in livestock production/productivity", there is no hard evidence for the former and little for the latter. Consequently, efforts to assess the value of investments in nuclear techniques for the benefit of livestock farmers are few and far between (Vose, 1994; Dargie, 1989). With such a dearth of information, donors are understandably investing larger proportions of their project 'spends' on M&E to ensure the effectiveness of project management and to justify the expenditure to taxpayers particularly in these days of fiscal constraints.

CONCLUSION

Nuclear and related techniques remain as cutting edge tools in scientific discovery — and need to be developed further and be applied more to address contemporary issues in agricultural development. However, appreciating the drivers which impact on poor livestock keepers and understanding the farming systems context under which they work is central to any effective agricultural R&D effort — with or without the use of nuclear techniques.

This review has identified 10 lessons to enable greater effectiveness of nuclear techniques in resolving problems experienced by poor livestock keepers in the field. There is a need for more diverse partnerships; a less reductionist approach to research, and a more holistic approach to research on livestock systems; a longer perspective — more sustained support to technology transfer; better communications (and resources) on the application of nuclear techniques on agricultural practices to discreet groups is at the heart of wide scale adoption of research products; the need for greater advocacy on the key contribution of livestock and livestock research for development — a responsibility that all animal scientists need to assume since there are many doubters; more emphasis on monitoring and evaluating the outputs of research on livestock production and livelihoods. Sadly, whereas there may well have been benefits to both livestock and their keepers at a local level there appears to be very little objective evidence available to confirm this on a broader scale. Notwithstanding, the products of research on and with nuclear and related technologies are vital parts of the armoury required to generate technologies and knowledge to satisfy the world's basic needs for food; they need and deserve a better shop window.

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Decline in Available World Resources: Implications for Livestock Production Systems

R.A. Leng[1]

ABSTRACT

The world is faced with a triple crisis: climate change, peak oil (the end of inexpensive energy) and global resource depletion. These are interrelated and interactive which makes the subject to be discussed in this paper extremely complex. The certainties are that there will be great changes to contend with in the future in order to produce and deliver food to maintain the present world population, let alone a balanced diet for everyone. The global financial meltdown is seen as having a critical role in determining future animal production strategies. The age of scarce and therefore high energy costs now dawns, and will be marked by the decline of oil and all that depends on it, including financial capital. It heralds large changes in the financial and related political structures. Without rationalization of the use of oil there seems to be little chance of continuing growth in industrialised or developing countries. Inexpensive oil allowed food to be produced cheaply but this will change greatly as oil prices rises create the potential for major disruptions in food availability. Peak oil will affect other resource availabilities. Agriculture has received inexpensive chemicals and fertilisers on which high crop yields have been predicated, including: nitrogenous fertilizers manufactured from natural gas and phosphates reserves which have peaked. The world is now dependent on extracting phosphate fertilizers from low grade rock phosphate at high energy costs. Irrigation waters from aquifers have also been depleted and rivers overused. The advent of peak oil with the ultimate high cost of fuel and therefore power for irrigation will clearly cause a return of vast areas of highly productive crop land back to rain-fed cropping, pasture or desert with major loss of food productivity. Soil erosion and fertiliser run off from cropping systems are also major concerns as tillage and crop management have eroded the top soil of large areas of land that will inevitably lead to decreased crop yields. The dependency of the industrialised countries on imported oil has seen a headlong development of biofuel from sugar cane and maize mainly in Brazil and the USA respectively and bio diesel from plant oils, creating major cereal food/feed grain shortages. The expectation is that world cereal grain availability for livestock production will be highly restricted, with a major decline in industrialised or commonly termed factory farming of livestock. Herbivores are likely to be used more extensively with time, particularly the ruminant and the rabbit. Global warming cannot be ignored in any discussion on future agriculture. Increasing sea levels will undoubtedly remove considerable areas of fertile delta and weather patterns will change, leading to at times more intense drought and or flooding rains. In some areas lack of synchrony of river flows (from glacial melt) with irrigation requirements may reduce multiple cropping areas. Warming also carries with it the risk of decreased crop production as rice yields decrease by 10% for every °C rise in night time temperatures. Resource depletion threatens any attempt by countries to grow their economies and has the potential to lower world crop production by direct or various flow-on effects. It is suggested that the world is now entering a time where intensive animal production will become increasingly expensive as competition for food, feed and fuel develops. The animal production industries based on herbivores will need extensive development, exploiting a wide range of waste by-products of agriculture or biomass from land not dedicated to food or biofuel production. The high cost of fuel will also see a gradual return to animal power in agriculture. The downside of this will be the increase in ruminant livestock with potential increase in enteric methane production. A new prospect for limiting methane production in fermentative digestion indicates that this could be reduced substantially in the future.

Key words: *climate change, peak oil, resource depletion, biofuels, agricultural by-products, ruminants, methane emissions.*

INTRODUCTION

Campbell (2005) speaking in Edinburgh at the Association for the Study of Peak Oil stated: "The first half of the age of oil now closes. It lasted 150 years and saw the rapid expansion of industry, transport, trade, agriculture and financial capital, allowing the population to expand six-fold. The financial capital was created by banks with confidence that tomorrow's expansion, fuelled by oil-based energy, was adequate collateral for to-day's debt. The second half of the age of oil now dawns, and will be marked by the decline of oil and all that depends on it, including financial capital. It heralds the collapse of the present financial system, and related political structures".

These predictions have been ignored by politicians, at great cost, particularly to developed countries and the implications of peak oil on future strategies for agriculture and animal production have been little discussed (Leng, 2002). To delve into changes that are likely to the world's livestock production systems without considering the complex and interacting factors that affect the outcomes makes this task seemingly impossible at the time of writing. However, the present food and financial crises have brought out some highly informative recent publications (Brown, 2009; Nellemann et al., 2009: Scherr and Sthapit, 2009).

Deepening economic turmoil is prioritising resources in the industrialized world; the emphasis is on decreasing the 'politically unacceptable', loss of jobs by increasing consumption and therefore resource utilisation, in order to return countries to growth (or business as normal). But 'kick starting' economies and increasing

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development in emerging economies, without decreasing demand for fossil energy, may rapidly reverse economic recovery by increasing fuel prices (Campbell, 2005). Historically, global economic growth has never occurred without a simultaneous increase in the use of energy (Hirsch, 2009). All nations of the world use oil, but for a few nations, coal is an important component of the fuel mix. Unless economic policies ensure a decreased demand for fossil fuels relative to production, there seems little prospect of a permanent recovery from depression in industrialised countries and little chance of substantial growth in the underdeveloped economies. The second half of the age of oil has major implications for world agriculture in both industrialised nations and in the emerging economies, particularly of India and China (Brown, 2009).

Livestock production systems will be differentially affected depending on the level of industrialisation and therefore dependency on fossil energy resources. The changes that occur in industrialised nations will inevitably impact on the less well developed countries. For instance political decisions such as subsidising first and second generation biofuels will have significant effects on food availability on world markets and therefore the availability of food for emergencies created, for example by adverse weather conditions or militant actions, particularly in heavily populated countries (Runge and Sanauer, 2007).

In the following discussion it is argued that herbivorous animals, particularly ruminants, but also small herbivores, fed on agro-industrial or under utilised feeds will need to dominate the meat and milk production requirements of future generations. However, animal production is not simply a matter of producing food; it is also relieving the ill health of resource poor people. Poor health often results from essential nutrient deficiencies in people mainly on cereal based diets which animal products can rectify. In 2000, one billion people were chronically undernourished and the number is growing. Animal products, even in tiny quantities, support physical and intellectual development of young people and pregnant mothers (Waterlow, 1998).

LIVESTOCK FARMING

Livestock farming began with early hunters and gatherers as they settled into crop production and harnessed livestock for transportation and eventually the heavy tasks such as tilling the land. Pastoralists as they became farmers used ruminants as a source of power, and milk and meat production were secondary issues. Similarly birds and pigs were initially scavenging animals, feeding on left over or spilt food, by-products and natural fauna. Instinctively people recognised the nutritional role of animal products which provided essential amino acids and micronutrients, deficient in cereal based diets.

It was only with the advent of inexpensive energy and therefore feed (energy and protein) that livestock production diverged from its integrated roles in food production to industrialised production systems. Inexpensive grain and/or energy allowed the economic development of a number of intensive meat and milk production systems including grain-based dairy and beef production, intensive production of carnivorous (highly energy dependent) and herbivorous fish (highly grain dependent) and other smaller and specialised systems such as intensive rabbit and guinea-pig meat production.

In the non-industrialised nations, animals mostly remained an integrated component of farming, but with the availability of inexpensive grain, peri-urban pig and poultry production developed. The shift in the relationship between fossil fuel energy and food and meat or milk production began around 1970 with major changes in the cost of oil. For 20 years prior to 1970 a bushel of wheat cost about the same as that of a barrel of oil but the equivalent cost increased four-fold in recent times (Brown, 2009). Before there was abundant inexpensive fuel, world population was constrained below one billion people: it increased dramatically to 6.7 billion over a few years as food production was ramped up by mechanisation and increasing farm size, irrigation, improved seed varieties, improved agrochemicals such as herbicides and increased fertilizer availability and inexpensive distribution systems. At the same time cheap energy also aided improved healthcare and modern medicines decreased infant mortality and increased longevity. Together these allowed populations to grow at a fast rate and the world population is predicted to be eight billion within two to three decades.

There has, however, been a price to pay for the green revolution; farmers and businessmen were inevitably exploiting finite or non renewable resources. A number of these resources, just as with oil, appear to have peaked and their reduced availability in the future will have detrimental effects on the world's ability to feed its population and provide balanced diets, particularly for the resource poor of any country.

FOOD AND FEED RESOURCES TO MEET A WORLD POPULATION OF 8–10 BILLION PEOPLE

The food requirements and the resources to meet a projected human population of 8 billion people in the decade 2030–2040 appear to be unattainable. The required resources have been estimated by Vance (2001) and are shown in **Table 1**. As oil becomes scarce it appears that food production costs will escalate and increasingly divert a higher proportion of income in the developed countries to maintenance energy of people including their food and transport costs. In the less rich societies, the cost of food may develop into

Table 1. Agricultural production and resource use (Vance, 2001).

Item	1960	2000	2030–2040
Food Production (M Tonnes $x10^{-9}$)	1.8	3.5	5.5
Population (10^9)	3	6	8
Irrigated Land (% arable)	10	18	20
Cultivated land (ha $x10^{-9}$)	1.3	1.5	1.8
Water stressed countries	20	28	52
N fertiliser use (Tg)	10	88	120
P fertiliser use (Tg)	9	40	55-60

major famine risks and all countries will need to develop their own food security measures

Climate Change is Likely to Reduce Overall World Crop Yields

The most significant effects of climate change on agriculture arise through changes in weather patterns and instances of droughts and inundating rains (see Stern 2007). Increasing temperatures in tropical countries can have a slowing effect on photosynthesis and hence plant growth. For example, rice yields in Asia are declining by ten percent for every degree Centigrade rise in night-time temperatures (Peng et al., 2004). A recent report (CGIAR, 2007), on the effects of increased environmental temperatures on wheat production in the Indo-Gangetic Plain, indicates a massive decrease in yields. This area produces 15% of total world wheat annually, about 90 million tonnes. Under the climatic conditions expected to prevail in 2050, this wheat mega-environment will shrink by just over half, mainly through shortening of the growth period as a result of heat stress early and late in the wheat growing season. This threatens the food security of about 200 million people (CGIAR, 2007).

The predictions on climate change are for increasing rain in some areas but less rain in others and rainfall patterns will become more variable and storms more intense (Stern, 2007), leading to increasing crop failures. Already in 2009, catastrophic falls (20–40%) in food production appear to be probable because of drought in countries that are major food producers and or exporters (de Carbonal, 2009). Some countries that normally have food surpluses are already imposing export restrictions on grains because of high international prices and if de Carbonal's observations are correct, food prices will soar. Whether the widespread incidence of drought this year is a result of climate change is debatable but it is difficult to dismiss the possibility. Recent models suggest that global warming is likely to reduce world agricultural output by between 16% and 3%. However, the effects will not be spread evenly with productivity in the tropical, developing countries likely to be reduced disproportionately by 21–9% (Cline, 2007) The spread of estimates are associated with the uncertainty of the benefits of increased atmospheric carbon dioxide on plant growth (carbon fertilisation). Global warming will also open up land for grain production in the Northern Hemisphere, but far away from the main centres of population. However, the warming of the Artic region appears to have potential to disrupt the monsoon that would have enormous social and economic impacts for the two billion people of South Asia (Pearce, 2009). Melting of the permafrost, may also result in more methane produced from decomposing organic matter and dissociation of methane hydrates, which together may be the cause of the recent upturn in atmospheric methane after a decade of stabilised concentrations (Pearce, 2009).

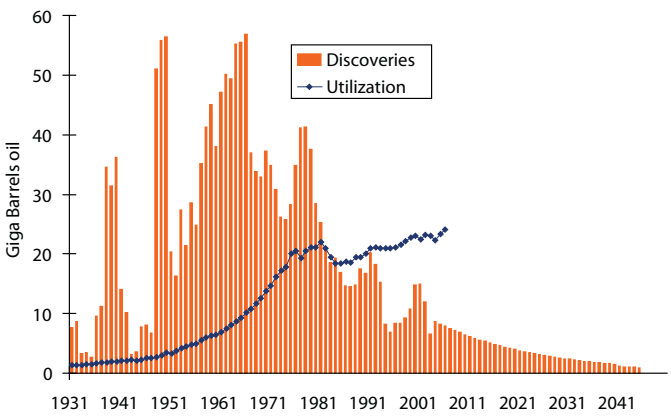

Figure 1. World oil discoveries and the trend in world utilisation of oil (from Campbell 2005).

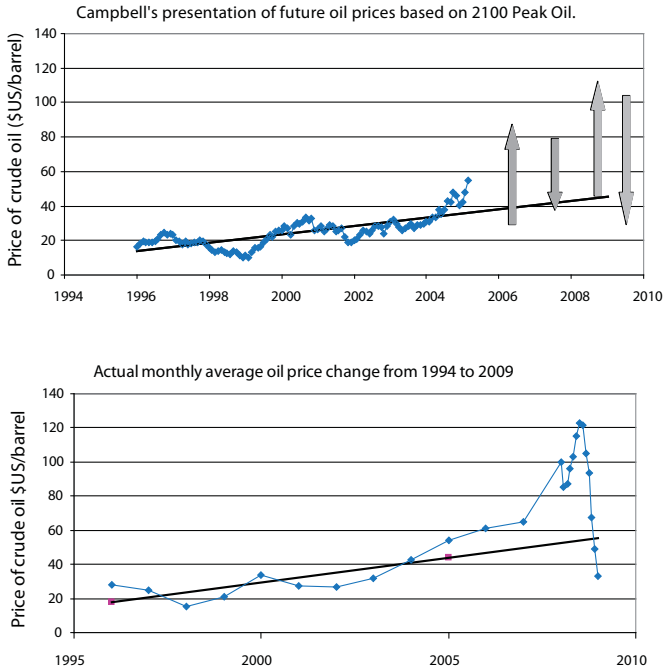

Figure 2. The change in the price of oil from 1994 and the predictions of recession and recovery according to Campbell (2005).

MAJOR INFLUENCES ON FUTURE LIVESTOCK PRODUCTION SYSTEMS

Peak Oil

It must be emphasised that fossil fuel availability is likely to be the main driving force for change in present animal production systems. The most important aspect at this time is that oil production and oil demand are finely balanced and production has out-paced new discoveries since 1981 (**Figure 1**) and in all probability oil production has peaked (for discussion see Brown, 2009).The upward trend in oil price has been building for several years and Campbell in 2005 predicted vicious cycles of price shock–recession–demand fall–price collapse–recovery–price shock. This potential pattern is being borne out by the global financial downturn being experienced at the time of writing. Campbell's predictions are shown in **Figure 2** with the actual price of oil to this year. The 'easy to extract' fossil fuels have been depleted and increasing world demand for fuel will inevitably force world fuel prices to rise (Campbell and Leherrere, 1998), but with a saw-tooth pattern over time, as periodic recessions lower demand and price, allowing both to rise again thereafter (Campbell, 2005). Interacting factors also indicate that food availability and food prices will be compromised directly or indirectly by flow-on effects of oil depletion (Leng, 2002, 2005), possibly the most important being

the diversion of land from food crops to production of biofuel and the variable effect on food costs (Runge and Senauer, 2007).

Industrial Biofuel Production and Food Grain Availability

The twin threats of peak oil and global warming have resulted in politically driven development of large-scale production of biofuel from sugar cane in Brazil, and maize in the USA. Both countries have huge importation costs for oil. The USA uses 25% of the world's oil production, but imports 70% of its needs, making it extremely vulnerable to world oil supply (Beddor et al., 2009). Biofuel production from cereal grains with competition for food and feed has major implications for human welfare and livestock production worldwide (Runge and Senauer, 2007). It is not the intention here to focus on the net efficiency gains in transport fuel, however the amount of energy returned in ethanol from growing maize and processing the starches through to alcohol is hotly debated but barely positive (Patzek and Pimentel, 2006; Patzek, 2007; Pimentel et al., 2009). Even the advocates for this industry fail to estimate a net energy gain in the manufacture of ethanol that is much greater then a gain of energy represented by the by-products of the industry (distiller's by-products). These are, however, important resources for the animal feed industry, as the protein is in a form that increases the efficiency of ruminant production based on agro-industrial by-products. The latter are likely to be a major resource for future meat and milk production from ruminants (see later) provided a problem associated with antibiotic residues can be over come (Philpott, 2009).

The industrial production of biofuel is creating major conflicts over food for humans, feed for animals and feedstock for liquid fuels. The Earth Policy Institute predicts that ethanol production claimed 50% (or 140 million tonnes) of US grain in 2008 (Brown, 2009). The balance between maize exports and maize used for ethanol in the US indicates the extent of the potential effects on world food supplies (see **Figure 3**, from Brown, 2007). However, combinations of the financial meltdown and recent lower fuel prices have emphasised the irrational and unfolding disaster of agrofuels; one-fifth of the ethanol industry's production capacity in the USA — most of it less than five years old — has shut down (Staff, 2009).

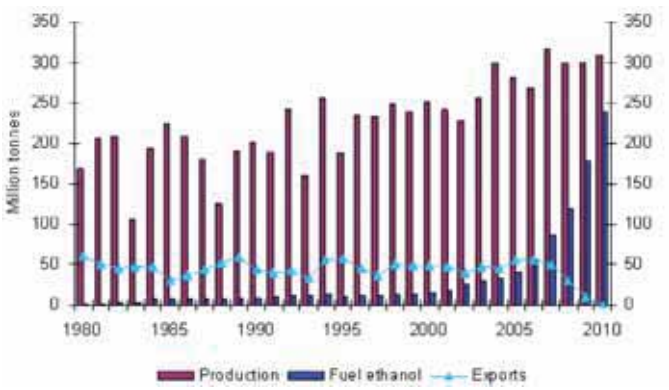

Figure 3. Production of maize in the USA and the calculated availability of grain for export and ethanol production.

Decline in Mechanised Agriculture and Food Production

Increasingly expensive fuel will have a major influence on the use of traction power, with the developing countries embracing more reliance on this with considerable effects on crop yields. Mechanisation in agriculture was a critical component in increasing crop yields (see Verma, 2002). The ability of farms to move from one or two crops per year to two -three crops a year is dependent on precision timing of farming practices such as land preparation, seeding, irrigation, herbicide application and harvesting times. Such farming practices also require high fertiliser inputs and are best suited to conservation practices such as no-till. To time these inputs effectively will be much more difficult using animal traction. This will reduce, in places, the number of crops that can be taken annually and also will make impossible some conservation practices, such as direct drilling which are not easily done with draught animals. Draught animals may also require some of the food grown but as mature animals, their feed requirements can usually be met from crop residues. In addition, the effects of vagaries of weather that may result from global climate change will not easily be rectified when animal power is the main source of cultivation energy. For instance 'catch up' in cropping procedures may not be possible for flood or drought related delays in planting or harvesting of a crop.

Other Resource Depletions with Implications for Agriculture

Water is the most potent resource for plant growth. Agriculture accounts for 70% of human water use and two-thirds of the available fresh water is used for irrigation (Revenga et al., 1998). There is a growing body of opinion that water may ultimately limit world food production (Postel, 1999). However, this area has been discussed earlier and is therefore not continued here. Major factors that will interact with the peak oil and global warming in reducing world food production have been discussed previously (Leng, 2005) and more recently by Brown (2009) and Nellemann et al. (2009). They include:

1. Availability of water as affected by:
   - increasing demand with increasing population;
   - changing weather patterns having adverse effects on crop production through global warming and the incidence of drought and inundating rains;
   - over-use of river water;
   - reduction of water run-off or reduced synchronisation of run-off water as mountain ice decreases, particularly where it supplies major water sheds such as the Gangetic plains or for that matter the Murray Darling basin in Australia and Californian croplands watered by the Sierra Nevada;
   - aquifer water draw-down for industrial and irrigation purposes (e.g. the aquifers under the Texas high plains)
2. Availability and cost of nitrogenous fertilisers. These are produced directly from fossil fuels which will rise in cost and inevitably result in reduced fertiliser application, particularly by resource poor farmers and farmers in marginal areas who may not apply fertiliser in areas where crop failure is a risk.
3. Availability of other fertilisers whose cost will be determined by extraction and transport costs. Mineral fertilisers such as phosphates are mined and high quality ore deposits are now approaching peak supply (Dery and Anderson, 2007).
4. vailability of crop land that is increasingly being lost to agriculture through erosion, sea level rise, construction, salination and pollution.

5. Changing and increasingly unfavourable environment for plant growth in vast areas of the present food bowls of the world through climate change (Stern, 2007).

The future impacts of peak oil, global warming, resource depletion and an on-going financial crisis are difficult to predict and cannot be realistically incorporated into any quantitative model, as none exist that assesses agriculture using an holistic approach and that takes into account future constraints (foreseen and unforeseen). Possible future reductions in cropping areas and crop yields have been tentatively predicted by Nellemann et al. (2009). These data are presented in **Figure 4**. The tripling in the world grain harvest since 1950 was due to the ability of farmers, with modern seed varieties and access to fertilisers, pesticides and mechanised conservation technology, to increase the number of harvests produced per year in Asia, together with increased yields generally. Examples of this are the double or triple cropping of rice in southern China, southern India, and South-east Asia (Brown, 2009). A gradual return to draught power can be expected as fuel becomes expensive with world depletion. This will also signal the need for more labour in agriculture.

In addition it appears that by 2050 significant sea level rise will inundate some coastal lands and river deltas (UNEP, 1989), often the most productive cropping areas. The amount of land consumed by the sea can only be guessed at with the present uncertainties of global warming scenarios. However, these latter factors appear to represent greater threats to world food production than those considered by other authors and some estimates are included in **Figure 4**. If drought, such as is being experienced in a number of countries at the present time (de Carbonnel, 2009), returns in the next 20 years and the reduction in food production is superimposed on the other potential contractions of crop yields (**Figure 4**), famine (see also Cribb, 2007) is likely to effect more people in more countries and the developing countries will not be the only countries affected.

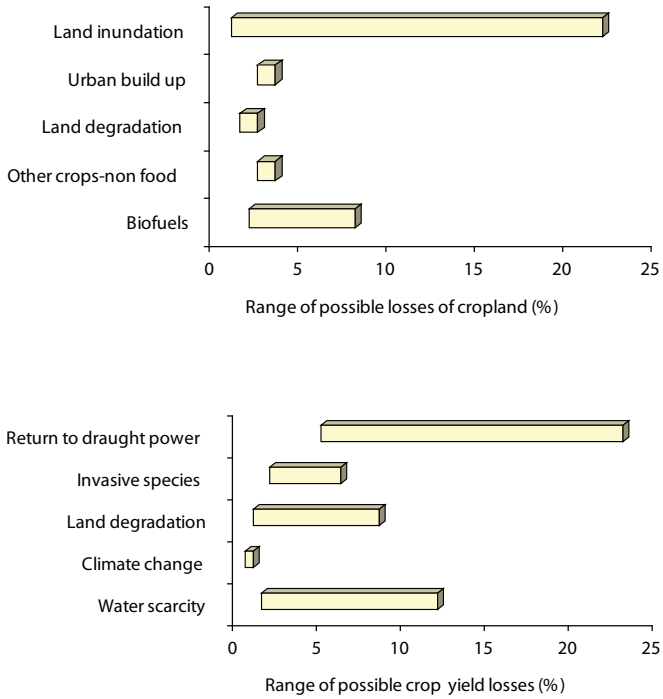

Figure 4. Possible ranges of cropping land losses and reduction in crop yield by 2050 (adapted from Nelleman et al., 2009). Note: the values for land inundation and return to draught power are estimated by this author.

Feed Grain Availability and Price are the Major Factors in Future Animal Production

Eighty percent of the world's food supply is derived from cereal grains consumed directly or as meat and milk from animals fed mainly grain, including fish (Pimentel et al., 2009). Worldwide food production per capita has actually declined over the last two decades and world production has lagged behind world demand in most recent years (Pimentel et al., 2009). The Livestock Revolution (Delgado et al., 1999) was predicated on surplus world grain supplies and that the relative price of grain would not rise significantly in 50 years. The quantities of feed grain needed (calculated to be over 900 million tonnes) to meet the projected meat and milk production demands in industrialised and developing countries will clearly not become available.

WHERE TO WITH FUTURE ANIMAL PRODUCTION SYSTEMS?

Industrialised Countries

The confluence of changes discussed above, together with financial constraints that are impacting the world, indicates that in the industrialised countries, the first and rather rapid change will be a changed pattern of use of discretionary funds by people to reduce expensive food in their diet. Expensive animal products may see people moving down the food chain, reducing their excessive consumption of meat, but retaining funds for transport. A major reduction in intensive farming of animals fed grain appears to be inevitable. This will initially be at the expense of feedlot beef which, because of the low efficiency of conversion of grain to meat, will be more costly relative to pig and poultry production. Poultry and inland herbivorous fish farming may also be initially favoured because of the highly efficient utilisation of concentrates which is greater then that associated with pig production.

Reduction in meat consumption, particularly in the developed countries, should free up large amounts of grain and supply may keep pace with food grain production, but the chances are that grain for the biofuel industry will increase. However, the by-products of maize ethanol will make up some of the shortfall of feed for all classes of livestock (Babcock et al., 2008). As meat production is scaled back, ruminant production may be re emphasised but with a greater dependence on grazing and the use of crop residues in a system that uses polyculture to reduce the needs for fossil fuel embedded inputs and minimise their 'long shadow' (Steinfeld et al., 2006).

In parts of Europe where rabbit meat is popular the potential to use non concentrate feeds may see this industry expand based on cellulosic biomass. Future agriculture in industrialised countries will be forced to return to ecologically sound farming practices that produce food, meat and milk locally and with a minimum of artificial inputs using modern (yet to be designed) and energy-efficient strategies and machinery that uses the minimum of fuel.

Milk consumption will also contract with major effects on the systems that use high yielding cows that must be fed concentrates to achieve their potential milk yield. The need to produce milk from cellulose biomass will de-emphasise industrial milking units for more sophisticated systems employing cattle with lower yields but the potential to use lower quality feeds and provide meat in addition to milk. In the longer term the changes will be towards those predicted for the developing countries (see below).

The huge amounts of by-product feed from industrial alcohol production from grain (distillers dried grains and solubles referred to as DDGS) provides a source of nutrients for all animal industries. It appears that these can be included in the typical beef feedlot diets, up to 30% and some will find their way into pig and poultry diets (Babcock et al., 2008). It is estimated that annual DDGS production in the USA will surpass 40 million metric tonnes as early as 2011 (Tokgoz et al., 2007). However the high costs of drying and or transport of wet material are serious limitations to their use, except in the vicinity of distilleries. Residues of antibiotics in the by-product may also be a future constraint.

Developing Countries

Developing countries have embraced some of the systems of intensive livestock production. These have usually been based on the models of the West but generally situated in peri-urban areas where imported grains were cheaply and easily accessed. As the price of oil and grains increase there will be a substantial reduction in these industries. These will be replaced in countries such as Vietnam by development of rabbit production based largely on forage resources from land that is not cultivatable (Preston and Van Thu, 2008). The development of rabbit feed resources from water spinach grown on waterlogged or inundated land is a very good example of likely development (see Pok Samkol et al., 2006). Farming of herbivorous fish such as carp in poly-culture is likely to be increased but high water demands may limit its growth. The high cost of fuel in developing countries is likely to enforce a return to animal power mainly from large ruminants but horses and donkey numbers will surely also increase. This scenario occurred in Cuba following the loss of cheap fuel from the former Soviet Union in 1990 (Henriksson and Lindholm, 2000). Milk and meat production will continue, mainly using agro-industrial by-products but the high cost of imported feed emphasises the need for efficient use of locally available resources to increase production per animal, increase feed conversion efficiency, and replace the decrease in white meat production.

Ruminants Fed Forages Offer the Most Reliable Source of Protein in the Future

Forty per cent of the Earth's land surface supports the majority of the world's 3.3 billion cattle, sheep, and goats. Most of these pastoral areas cannot be economically cropped; half are moderately degraded; and five percent are severely degraded, particularly the communal lands. Forage requirements of the large livestock population in nearly all developing countries appear to exceed the sustainable yield of rangelands and other forage resources such as agro-industrial by-products (Brown, 2009). On the contrary, Savory (2009) suggests that grazing livestock have been vilified as a major cause of climate change when they are a vital part of the solution. With an holistic approach, a major reversal of desertification can be achieved, that can draw down greenhouse gas by sequestration in organic soil carbon (see Savory, 2009 for discussion).

The nutritional value of forage is the prime limitation to ruminant production in most developing countries. Poor nutrition leads to low levels of production and increases the time for animals to grow to maturity or slaughter weight, increasing the quantity of feed needed. This often suggests that feed quantity is a primary limitation to livestock production. Simply supplying nutrients that are deficient in these roughages (generally a source of fermentable nitrogen, various minerals and a source of by-pass protein) to ruminants under poor management can increase productivity many fold, and since young animals grow more quickly and reach the age of slaughter sooner, they use considerably less feed and more animals can be fattened on the same land area. The scientific basis of feeding supplements to ruminants fed on poor quality forages has been discussed in a number of papers (Preston and Leng, 1987; Leng, 1990; Leng, 2004), and the efficient use of such feeds is a major way to increase animal protein for human consumption in the future.

Crop Residues Must Become a Major Resource for Livestock Production in the Future

The world produces just less than two billion tonnes of cereal grains which is accompanied by about the same yield of straw. Straw has a number of uses; it is fed to ruminants, mostly without appreciation of production responses that could be achieved with treatment and supplementation; it may be burned to facilitate multiple cropping practices and it is, in places, harvested for other commercial purposes or ploughed back into the land. Much speculation has centred on the prospects for producing second generation cellulosic ethanol fuel from straw. However the logistic constraints of moving the huge amounts of straw to centralised processing plants appears to be a formidable barrier. This is fortunate because it appears to be one of the few feed resources available to increase animal protein production in the future. There is a major case to be made to retain straw organic matter as soil carbon (Lal, 2007). Nevertheless the major long-term action of straw on soil carbon is through its least digestible components i.e. lignin. This is 100% excreted by the animal and therefore if manure is returned to the land there is little reason why straw cannot be fed to livestock provided the land is not exposed to erosion by maintaining a cover of biomass.

Crop Residues can Support Surprisingly High Levels of Ruminant Production

Crop residues particularly straw can support moderate to high levels of production in ruminants provided efficient means of treating the straw to enhance digestibility and any deficiencies of nutrients in a diet are corrected. If additional by-pass protein is then provided, levels of production and efficiency of use of biomass for growth and milk production are greatly improved (Leng, 1991 and 2004). The improvement in utilisation of straw by ruminants by adhering to these simple principles has been well demonstrated (Preston and Leng, 1987). In India, milk production (largely from cows fed straw), has escalated by the application of good nutritional principles among other applications (Banarjee, 1994). In the northern wheat belt of China cattle growth rates on straw with enhanced digestibility approached 0.9 kg/d or 75% of the rate that could be achieved with similar animals fed grain-based feedlot diets (Cungen et al., 1999). At these growth rates the numbers of animals that can be fattened on the same quantity of untreated straw increases 10–13 fold (**Table 2**). In multiple cropping areas, the wet season rice crop of straw is mostly wasted but with preservation methods that also improve digestibility, this rice straw is now being harnessed to efficiently feed ruminants (personal observation).

At the present time there is enormous need to implement known treatment and nutritional strategies to improve straw use by ruminants. All countries, must quickly begin to put in place known technologies to use crop by-products efficiently for ruminant production and at the same time relieve the pressure on over-grazed pastoral lands. Mechanisation of straw treatment/preservation is the first step for development of supplementation strategies. Cheap grain removed the stimulus for massive application of roughage fed ruminants, but with good support farmers can fill the coming vacuum of animal protein supply by directing the available resources into

Table 2. The potential of balanced supplementation to increase meat production from young cattle fed low quality crop residues treated to increase digestibility. The calculations are based on the data from research in Hebei, China as reported by Dolberg & Finlayson (1995).

Cottonseed supplement fed [kg/day]	0	0.25	0.5	1.5	2.0	2.5
Lwt gain [g/day]	63	370	529	781	829	892
Straw consumed to produce 100 kg Lwt [tonnes]	6	1.1	0.92	0.56	0.48	0.46
Cottonseed cake consumed [tonnes] to produce 100 kg Lwt	0	0.1	0.1	0.14	0.22	0.24
Number of animals that can achieve an extra 100 kg of Lwt on 6 tonnes of straw	1	5+	6+	10+	12+	13+
Protein meal requirements [tonnes] to allow 100 g Lwt gain per group of animals fattened	0	0.5	0.6	1.4	2.6	3.1
Conversion of protein meal to live weight [g Lwt gain/g feed concentrate]	–	1.2:1	0.93:1	0.48:1	0.26:1	0.31:1

the efficient feeding of ruminants. The vast majority of ruminants, which number some 1 800 million large ruminant equivalents, are low producing and can be upgraded to moderate to high levels of production with modern technology. There are two billion tonnes of straw that could be converted into animal products with a feed conversion efficiency of about 10:1 This could produce 200 million tonnes of live animals annually which could support four billion people at 25 kg/year. Thus with information transfer and political will, ruminant production systems could be the major source of animal protein in the future.

The Downside of Ruminant Production from Poor Quality Roughages

The majority of the world's ruminants are in developing countries. Globally, ruminant livestock produce about 80 million tonnes of methane annually, accounting for about 28% of global methane emissions from human-related activities (Johnson and Ward, 1996). In developing countries, the majority of ruminants are supported on forages of intermittent or poor nutritional value. In general, growth rates, milk production and reproductive rates in these systems are extremely low compared with the genetic potential of these animals (mostly about 10% and rarely exceeding 30%). Mostly (with exceptions in particular areas), cattle grow to maturity or slaughter weight between four and five years, cows produce their first calf at four to five years and then, on average, every two years. Milk production is often below 1 000 litres/lactation (Preston and Leng, 1987). Cows may be kept largely to produce draught oxen and in some specialised systems they are kept for the production of dung (which is valued as a fuel) and a number of other minor purposes (e.g. as an investment, for recreation and for religious purposes). Slow growth, low milk yield and poor reproductive performance result in poor feed conversion and a large methane output relative to product output (Leng, 1991). The benefits of high growth rates as a means of reducing methane production per unit of meat production have been confirmed from direct measures of methane output (**Figure 5**). Provided growth rates (in cattle) are between 0.7 and 1 kg/d, methane production will be minimised and these upper levels of growth are being achieved with cattle fed crop residues (see for example Dolberg and Finlayson, 1995). In addition at these growth rates it is possible to produce quality meat for all the major markets of the world. Thus an answer

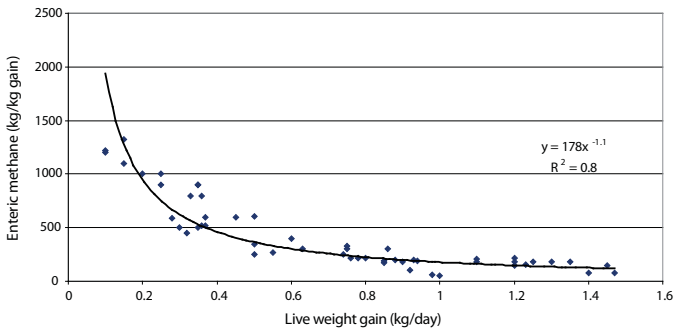

Figure 5. The relationship between Lwt gain of cattle and enteric methane production/ kg of gain (see Klieve.and Ouwerkerk (2007) for sources).

to world meat shortages, when industrialised production systems become too expensive, is to develop ruminant production systems from crop by-products in industrialised countries and ensure a large input into research and extension in the developing countries to achieve the levels of production at minimal cost to the environment and without increasing livestock numbers.

Potential of Nitrate as Fermentable N Source for Ruminants Fed Poor Quality Forage

Recent studies suggest that the fermentable nitrogen requirements of ruminants on diets based on low protein cellulosic materials can be met from nitrate salts (Trinh et al., 2009), and this potentially reduces methane production to minimal levels (Leng, 2008). Trinh et al. (2009) demonstrated that with adaptation, young goats given a diet of straw, tree foliage and molasses grew faster with nitrate as the fermentable N source as compared with urea. This is a major step forward in ruminant nutrition which should create a paradigm shift in animal protein production. If methane production is lowered significantly when nitrate is fed in low protein diets consumed by ruminants, it will remove a major barrier to replacing much of the monogastric production lost because of the unavailability of feed grain in the future.

In the same way that arguments are developed to support future ruminant industries the supply of animal protein can be enhanced using rabbit fed forages (Lukefahr, 2007). Their major attributes include ability to utilise cellulosic biomass efficiently, coupled to a high fertility with ability to breed every six weeks producing multiple offspring.

CONCLUSIONS

Ecological, bio-diverse, local agriculture combined with holistic land management that can reverse desertification and increase carbon stored in land is part of the solution to global warming and food scarcity. The world's farmers will steadily adopt these procedures as the required resources become scarce and prices move upwards. The alternative is that their land will be rendered infertile. Under these conditions even the developed countries will recognise that priority must be given to ruminants and other herbivores that transform biomass into food resources with minimum jeopardy to the environment.

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SESSION 1

INTERACTIONS AMONG NUTRITION, REPRODUCTION AND GENOTYPE

Interactions Between Nutrition, Heat Stress, and Reproduction In Cattle Within Tropical/ Subtropical Environments

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ABSTRACT

A series of approaches are presented to partially alleviate harsh environmental stresses on productivity and fertility of lactating dairy cows. The first area involves the use of various environmental facilities and management to provide heat abatement in order to enhance performance. A second complementary approach is utilisation of classical genetics that also includes introduction of heat tolerant genes into less heat tolerant breeds. A variety of reproductive technologies and their application to manage seasonal periods of infertility associated with heat stress are documented. Lastly, integration of nutritional and reproductive management strategies to improve postpartum immune competence, health and reproductive performance are presented.

Key words: *Heat tolerance, timed artificial insemination, seasonal infertitlity, selenium supplementation.*

INTRODUCTION

Efficient reproductive performance of lactating dairy cattle in tropical/ subtropical environments throughout the world is impacted by a multiplicity of factors such as: the physical environment, socio-economic status of producers, available nutrients, adaptability and genetic composition of cattle, intensive or extensive management systems, and available reproductive technology. Seasonal periods of reduced fertility are associated with concurrent increases in temperature and humidity, availability of nutrients, and elevations in body temperature detrimental to ovarian function, oocyte competence, embryo development, and placental-foetal growth.

Modification of the Environment

Implementation of heat abatement facilities can enhance both pregnancy rates and milk production. Heat abatement is dependent upon optimising heat exchange via convection, conduction, radiation and evaporation. Collier et al. (2006) reviewed extensively the dynamics of environmental management and subsequent impacts on the lactating dairy cow. The system to be used depends upon the local environment (e.g. arid to tropical) and includes the use of shades (reduction in solar radiation), sprinklers and fans under shade structures (enhances evaporative cooling from the skin surface), fans and sprinklers in the holding areas and/or exit lanes from the milking parlour, fans and sprinklers in free-stall facilities (e.g., cooling cows along the feed lines with sprinklers and fans) and evaporative cooling systems (i.e. cool the air that ultimately surrounds the cow). Although a shade structure partially alleviates heat exposure from solar radiation, there is no alteration in air temperature or relative humidity; consequently, additional cooling strategies are required for lactating cows in a tropical/subtropical environment. A benchmark reference point for lactating cow status is a surface skin temperature of 35 °C. Below this temperature all four routes of heat exchange are possible, and the micro environment to sustain a skin temperature at or below 35 °C avoids reductions in milk yield.

These types of environmental management systems need to be optimised for the region of application and integrated with the production potential of the area. For example, in many tropical areas, the period of stress most often extends for an extended period of the year and is coupled with diseases, parasites, and low nutritional inputs. Obviously, a system under this environment needs to incorporate a management plan that not only protects animals from periods of thermal stress but provides more stringent health care, well-being and nutritional inputs to reach the production potential of the animal unit in the system. Such systems involve increased capital investment to allow maximal performance of high-producing animals. A system of environmental management comprised of intermittent cooling with sprinkling and forced ventilation throughout the heat stressful period in Israel, improved conception rates (Wolfenson et al., 2000). However, fertility levels were not restored to levels typical of what is found during the winter months. Complete elimination of heat stress by intensive and frequent use of the sprinkling and ventilation cooling system was able to achieve summer conception rates comparable to that recorded in winter. Thus short periods of hyperthermia appear to have compromising effects on reproductive processes.

Genetic Strategies to Increase Milk Production and Reproductive Efficiency

Conventional crossbreeding between *Bos taurus* and local *Bos indicus* cattle (F_1) has been a strategy to improve resistance to thermal stress but always lowers milk yields in the F_1 generation compared with the *Bos taurus* purebred dairy cow. An alternative breeding programme to improve local cattle (e.g. B*os indicus* cattle) is upgrading to B*os taurus* dairy cattle (e.g. Holstein). As the percent *Bos taurus* breeding increases, the need for environmental management

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becomes greater. Nevertheless, lower percentage upgrades often out-produce local native cattle with minimal management inputs. Fluctuations in environmental conditions (climate, feeding, management, etc.) from year to year at the same tropical location can be important in determining the preferred genotype of dairy cows. McGlothlen et al. (1995) showed during a 27-year period that in 14 good years, upgrading of Butana cattle (*Bos indicus*) to over 70% Holstein or Ayrshire in Sudan resulted in greater annual milk yield/cow. However, in 13 poor years highest yields were obtained from three-breed crossbreds, 3/8 Holstein, 3/8 Ayrshire, and 2/8 Butana.

Within the Holstein breed in the USA, the Predicted Transmitting Abilities (PTAs) for milk yield and heat tolerance were determined from 172 411 sires and 10.5 million cows (Bohmanova et al., 2005). Heat tolerance PTAs of sires ranged from -0.48 to 0.38 kg milk/temperature humidity index (THI) unit above 72/d; milk yield PTAs for sires were between -8.9 and 7.9 kg/d. Based on estimated heat tolerance PTAs, the 100 most and 100 least heat tolerant sires were examined. Bulls that transmit a high tolerance to heat stress have daughters with higher pregnancy rates, a longer productive life, but lower milk yields (Bohmanova et al., 2005). Continued selection for milk yield without consideration of heat tolerance likely will result in greater susceptibility to heat stress. Conversely, selection of bulls for heat tolerance will likely result in a decrease in milk yield. This is to be expected because as average production per cow increases the metabolic heat output increases making cows more susceptible to heat stress.

Since genetic variance for heat tolerance exists in dairy cattle, there is the likelihood that specific genes controlling heat tolerance can be introduced into the gene pool of the population. One such gene is the slick hair gene (*slick hair*) originally described in Senepol cattle, subsequently identified in Carora cattle, and introduced into Holsteins by crossbreeding (Olson et al., 2003). The gene has been mapped to chromosome 20 (Mariasegaram et al., 2007). Animals with the dominant allele have a very short and sleek coat. Holstein (75%) x Carora (25%) crossbred dairy cows in Venezuela with slick hair coats had lower body temperatures in heat stress conditions than those with the wild-type hair coat (38.58 °C < 39.09 °C) and produced more milk (6 389 > 5 579 kg, 305-d milk yield; Olson et al., 2003). The superior thermoregulatory ability associated with the slick phenotype is apparently the result of increased convective and conductive heat loss and decreased absorption of solar radiation. During an experimentally imposed heat stress, slick haired lactating Holstein cows (i.e. out of Holstein cows sired by 75% Holstein and 25% Senepol bulls heterozygous for the slick hair gene) had lower vaginal temperatures and respiration rates than wild-type lactating cows (Dikmen et al., 2008). In either the indoor environment of a free-stall barn with sprinklers and fans or the outside environment during the heat stress period, sweating rates were greater for the slick-haired cows (indoor: 57 vs. 43 g/hm^2; outdoor 82 vs. 61 g/hm^2). Lactating cows with the slick-hair gene had a greater sweating rate which is deemed very important during hot stressful periods; since at an air temperature above 30 °C, 85% of heat loss from the skin is through evaporation. Consequently, the slick hair gene is a candidate gene for incorporation into dairy cows that would improve regulation of body temperature and production potential in heat stress environments.

Differences between thermal adapted breeds and non-thermal adapted breeds extend to early developmental stages of the embryo (Hansen, 2007). *Bos indicus* embryos are less adversely affected by elevated temperature in culture than Holstein or Angus embryos. Furthermore, B*os taurus x Bos indicus* embryos, in response to an *in vitro* heat shock, have a higher rate of blastocyst development acquired through *Bos indicus* genes that contribute to the presence of thermotolerance factors from the oocyte or imprinting of certain embryonic paternal genes. For example, embryos produced by insemination of Brahman oocytes with Angus semen were more thermotolerant than embryos produced by insemination of Holstein oocytes with Angus semen. However, there was no difference in thermotolerance between d four embryos produced by insemination of Holstein oocytes with Brahman semen versus embryos produced by insemination of Holstein oocytes with Angus semen. With the known gene sequences of the bovine genome, future identification of heat tolerance genes of *Bos indicus* breeds offers the potential of introducing these genes into less heat tolerant breeds.

Hansen (2007) reviewed various strategies to improve embryonic survival in dairy cattle during heat stress, and utilisation of timed embryo transfer was effective in studies at both Florida and Brazil. Embryos transferred into recipients at 7–8 d after either an oestrus or injection of GnRH, to induce a programmed ovulation, will by-pass the thermosensitive periods of the oocyte or early embryo. Pregnancy rates are enhanced with embryo transfer during periods of heat stress because transfers are made with embryos considered transferable that were not exposed to heat stress or were developmentally competent to be transferable. Natural embryos that were cryopreserved from superovulated donors or fresh embryos produced *in vitro* improved pregnancy rates but cryopreserved embryos produced *in vitro* did not enhance pregnancy rates.

Several studies have documented the beneficial effects of adding insulin-like growth factor 1 (IGF-1) to bovine embryos produced *in vitro* on both enhancing the rate of blastocyst development and reducing the magnitude of elevated temperature effects on inhibition of blastocyst development and apoptosis (Moreira et al., 2002; Hansen, 2007). Furthermore, *in vivo* embryo transfer of *in vitro* produced embryos that were cultured with IGF-1 increased pregnancy and calving rates during heat stress but not during the non-heat stress seasons.

Reproductive Management to Improve Seasonal Herd Fertility

An array of refined reproductive technologies is available to better manage the reproductive performance of dairy cows (Moore and Thatcher, 2006). Synchronised timed artificial inseminations (TAI) prior to the heat stress period will improve herd pregnancy rate. The strategy is to obtain as many pregnancies in the entire breeding herd before the infertile heat stress period begins. Since high temperature causes embryonic death during the first three cleavage divisions, embryo transfer of more advanced healthy embryos at d seven will bypass the early heat sensitive period to partially restore pregnancy rates, as described above. Development of vitrification procedures for storage of *in vitro* produced embryos, which develop normally following post-transfer of the embryo, will increase the impact of embryo transfer during the heat stress season. The following three reproductive management systems can contribute to seasonal breeding programmes.

Timed Artificial Insemination in Dairy Heifers

A major limitation for using AI in dairy replacement heifers is the time and effort connected with daily oestrous detection. Systems for timed artificial insemination in dairy heifers have resulted in poor responses in pregnancy per timed artificial insemination (P/TAI). An efficient system for TAI in heifers will allow producers to synchronise a large number of fertile heifers either just before the hot season, or during the hot season to sustain calving patterns throughout the year, or to synchronise a breeding programme so that subsequent

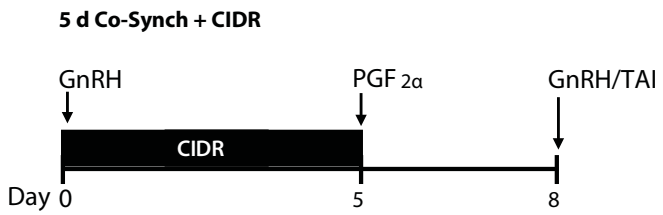

Figure 1. Diagram of the 5 day Co–Synch + CIDR protocol for the timed insemination of dairy heifers. Heifers received an intravaginal CIDR® insert and injection of GnRH on day 0; 5 days later CIDR insert was removed and $PGF_{2\alpha}$ administered; on day 8, GnRH was administrede and heifers received timed AI.

parturitions are occurring at a time when seasonal availability of nutrients is optimal.

Two replicates of heifers (n = 203 and n = 214 for replicates 1 and 2, respectively) from a subtropical environment in North Central Florida were synchronised for a first TAI with the 5-d Co-Synch + CIDR (controlled internal drug release insert containing progesterone) protocol (**Figure 1**) with one injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$); (Rabaglino et al., 2010). Non-pregnant heifers at 30 d after first TAI were resynchronised for a second TAI following the same protocol initiated on the same day when the diagnosis of non pregnancy was performed. A single sire was used for the first TAI in both replicates and the second TAI of the first replicate, whereas three additional sires were used for the second TAI of the second replicate. Three and two technicians performed the AI for the first or second services, respectively.

Pregnancy per TAI for the first TAI was 60.3% at 32 d (251/416) and 58.2% (242/416) at 60 d. Pregnancy loss between 32–60 d of gestation was 3.6% (9/251). There was no significant effect on P/TAI of replicate number, technicians, or the interaction replicate number by technician.

For the second service, P/TAI was 52.1% (86/165) at 32 d and 47.3% (78/165) at 60 d. Pregnancy loss was 4.8% (8/165). Pregnancy per TAI to the second TAI at 60 d was 58.4% (45/77) for the first replicate and 37.9% (33/87) for the second replicate. Difference in P/TAI for second service was significant (P = 0.01; OR: 0.30; 95% CI: 0.12–0.76) between replicates. The effect of service sires tended to affect (P = 0.10) P/TAI to the second TAI, and they were 11.1% (1/9), 50.0% (14/28), and 36% (18/50) at 60 d after AI for sires 1, 2, and 3 of the second replicate, respectively. There were no differences in P/TAI between technicians and the interaction of technician by replicate number was not significant.

From a reproductive management perspective, the overall percent of heifers pregnant after two synchronised inseminations encompassing a 64 d period was 77%. The programme requires no heat detection and involves two eight-day periods with heifers being handled three times in each period. Our practical experience is that pregnancy rates are acceptable when done in the summer months probably because the heifers are not lactating. The lower P/TAI to the second TAI compared with the first TAI tended to be due to the sires. Herd- or animal-level factors affect fertility in dairy heifers (Donovan et al., 2003), and most herd level variation is caused by variation among inseminators and service sires (Ron et al., 1984). The five-d Co-Synch + CIDR protocol (**Figure 1**) is an efficient reproductive management programme to achieve acceptable P/TAI in dairy heifers. Cost per heifer to implement the programme is $US 16 for purchase of GnRH (2), CIDR and $PGF_{2\alpha}$.

Timed Artificial Insemination versus Natural Service with Bulls

Both expression and detection of oestrus are often suboptimal in dairy herds due to high milk production and seasonal periods of heat stress, which impair reproductive efficiency. Despite considerable advantages for AI, a significant number of dairy producers use natural service (NS) for their breeding programme or in part of their breeding following several initial artificial inseminations. A common perception among dairy producers is that NS is comparable to AI because human errors in oestrous detection are avoided with bulls. However, a comparison of reproductive performance between NS and TAI, two breeding systems where efficiency of oestrous detection is not a factor, is lacking. A study was undertaken to compare reproductive performance of lactating dairy cows bred by natural service (NS) or timed AI (TAI; Lima et al., 2009).

The study was conducted between November 2006 and March 2008 in a commercial dairy farm of 2200 Holstein cows located in north central Florida (subtropical). Cows were housed in free-stall barns with fans and sprinklers for forced evaporative cooling during the warm season. Cows (n = 1055) were blocked by parity and enrolled to receive either NS or TAI. Cows in both groups were presynchronised with two injections of $PGF_{2\alpha}$ given at 42 and 56 d postpartum. At 14 d after the last $PGF_{2\alpha}$ injection, cows in the TAI group were enrolled in an Ovsynch protocol (d 0 GnRH, 7d later $PGF_{2\alpha}$, 56 h after $PGF_{2\alpha}$ injection a second dose of GnRH was given, and 16 h after the second GnRH cows were TAI). Cows in the TAI group were resynchronised with an intravaginal insert containing progesterone inserted 18 d after TAI and removed seven d later, when GnRH was given. Cows were examined by ultrasonography on d 32 after TAI; non- pregnant cows received $PGF_{2\alpha}$ and GnRH 56 h later followed by TAI 16 h after the GnRH injection. Non pregnant cows in TAI group were re-inseminated up to five times using the same scheme.

Cows in the NS group were exposed to bulls 14 d after the second $PGF_{2\alpha}$ injection, and ultrasonography was performed 42 d after exposure to bulls to determine pregnancy status. Nonpregnant cows in the NS group were re-examined by transrectal palpation combined with ultrasound every 28 d until diagnosed pregnant or 223 d postpartum, whichever occurred first. Cows diagnosed pregnant in TAI or NS were re-confirmed 28 d later to determine pregnancy loss. All bulls underwent a breeding soundness evaluation and were rested for 14 d after 14 d of cow exposure. The bull:cow ratio in the NS group was one bull per 20 non-pregnant cows

The proportion of pregnant cows in the first 21 d of breeding did not differ between groups (NS, 34.2%, 175/512; TAI, 37.4, 203/543)**.** The overall 21-d cycle pregnancy rate, which included a total of eight and five service opportunities for NS and TAI, respectively, was not different between groups (25.7 and 25.0% for NS and TAI, respectively). However, the daily rate of pregnancy differed (P = 0.05) between groups and was 15% greater (Adjusted Hazard Ratio = 1.15; 95% Confidence Interval = 1.00 to 1.31) for NS than TAI (**Figure 2**), which resulted in fewer median days open (111 vs. 116 d). The survival curves did not differ until 150 d postpartum, when they began to separate. Nevertheless, at 223 d postpartum, which was the end point of the study, the proportion of pregnant was greater (P = 0.001) for NS than for TAI (NS = 84.8% and TAI = 76.4%). Cow d at risk for pregnancy were not different between NS and TAI, being 30978 and 29424 d, respectively. The greater proportion of pregnant cows observed in the NS group at the end of the study is attributed to differences in breeding dynamics between groups (**Figure 2**). In the NS group, bulls had the potential for daily detection of oestrus and breeding of non-pregnant cows. On the other hand, due to the TAI re-synchronisation scheme, non-pregnant cows in thisgroup

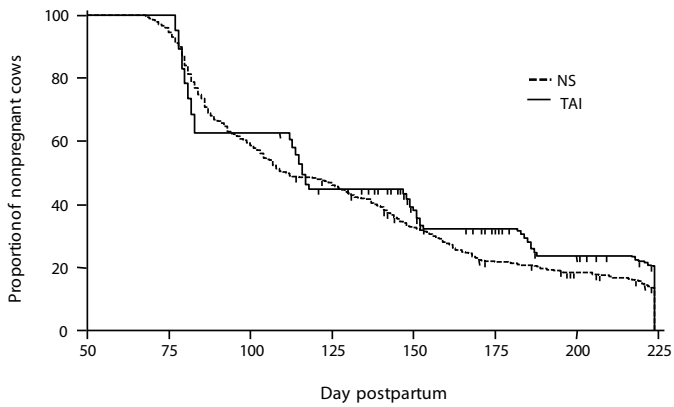

Figure 2. Survival curves for proportion of non pregnant cows by day post partum for cows bred by natural service (NS) or timed artificial insemination (TAI) in the first 223 days postpartum

required 35 d to be re-inseminated and thus the number of d to become pregnant increased. However, within this scenario, up to 223 d postpartum cows in the TAI group had only five opportunities to be bred compared with a potential eight times for cows in the NS group. A greater number of non-pregnant cows in the NS group had earlier opportunities to be bred than TAI cows under the same 21-d cycle pregnancy rate; consequently the final outcome for median time to pregnancy favoured the NS group. Primiparous cows had a greater proportion of pregnant cows and daily pregnancy rate than multiparous cows at 223 d postpartum. The THI was the criterion used to determine effect of season (warm or cold) on reproductive performance. The maximum daily THI was categorised as cool, when THI < 72 or warm when THI ≥ 72. The THI on the day of the first TAI or the first day of exposure to bulls was used in the statistical analysis. Cows receiving their first breeding during the cool season had increased (P < 0.01) pregnancy in the first 21 d of breeding (41.2% vs. 27.7%), 21-d cycle pregnancy rate (27.5% vs. 22.5%), and daily pregnancy rate (AHR = 1.22; 95% CI = 1.06 to 1.41).

In spite of applying a heat abatement system during the warm season, pregnancy responses were decreased. However, it is important to indicate that no interaction between treatment (i.e., NS and TAI) and heat stress was observed for the proportion of cows pregnant in the first 21 d of breeding or pregnancy rates.

In conclusion, NS and TAI are two breeding systems that can be used strategically to minimise problems related to detection of oestrus, but the extended inter-insemination interval in TAI reduces daily pregnancy rate because these cows have fewer opportunities for breeding. Of course, use of TAI with semen from bulls with high daughter pregnancy rates and transmitting ability for milk yield is an advantage compared with unproven NS bulls.

Timed Artificial Insemination of Grazing Lactating Dairy Cows

A TAI programme is a reproductive management platform for grazing lactating dairy cows to establish a seasonal breeding period such that cows can be inseminated prior to the heat stress season with subsequent parturitions occurring at a time of abundant pasture for grazing. Sufficient advancements have been made in controlled breeding to obtain optimal pregnancy rates and such a programme was conducted in two Florida commercial dairies (Ribeiro et al., 2009). For the period of March and April, average and range THI were: 65 and 60 to 70, respectively.

Objectives were to evaluate reproductive performance of grazing dairy cows subjected to different presynchronisation and resynchronisation protocols. Lactating cows (n = 1264) in two dairies were blocked by parity, breed (Holstein, H = 458; Jersey, J = 185; and Holstein/Jersey Cross, C = 621), and d postpartum, and then randomly assigned to one of four treatments in a 2 x 2 factorial experiment: 1) Presynch: two injections of $PGF_{2\alpha}$ given 14 d apart and starting the TAI protocol 11 d later; 2) G6G: injection of $PGF_{2\alpha}$ followed three d later by gonadotrophin releasing hormone (GnRH) and starting the TAI protocol 6 d later. The TAI protocol consisted of GnRH on day 0, $PGF_{2\alpha}$ on d 5 and d 6, and GnRH+TAI on d 8. On d 12 after the TAI, half of the cows in each presynchronisation received one of the two resynchronisations: 1) resynchronisation control (RCON) cows were observed daily for oestrus and inseminated starting on d 19 after TAI; 2) resynchronisation CIDR (RCIDR) cows received a CIDR from d 12–19 after the TAI and were observed for oestrus and inseminated between d 19 and 35. At d 35, cows were exposed to bulls for a 65-d period so that the entire breeding period for the programme was 100 d. Pregnancy diagnoses by ultrasound were done at 30 and 60 d after first TAI, 30 and 65 d after the resynchronised AI, and at 30 d intervals following introduction of bulls.

Pregnancies per AI (P/AI) for the treatment groups are presented in **Table 1**. The P/AI following the first TAI (P/TAI; 44.3%), to resynchronised detected oestruses (P/RAI; 16.8%), and to the bulls (P to Bulls; 21.4%) did not differ between the four treatment groups. The overall pregnancy rate for the 100-d breeding season was 82.5%. The only statistical difference detected between treatments was that pregnancy loss following first service TAI between d 30 and 64 of pregnancy was lower for the Presynch protocol (8.1%, 25/310) than the G6G protocol (12.9%, 40/309; P<0.05). Holstein lactating dairy cows had lower P/TAI at each of the three breeding periods (i.e.

Table 1. Effect of presynchronisation (PRE) and resynchronisation (RES) treatments on pregnancy (P) rates of grazing lactating dairy cows in a 100-d breeding season.

PRE	RES	N	P/TAI		P/RAI		P to Bulls		P at 100 days	
			n	%	n	%	n	%	n	%
Presy[1]	RCont[2]	318	142	44.7	62	19.5	61	19.2	265	83.3
Presy[1]	RCIDR[3]	314	143	45.5	48	15.3	61	19.4	252	80.3
G6G	RCont[2]	316	132	41.8	56	17.7	82	25.9	270	85.4
G6G	RCIDR[3]	309	140	45.3	45	14.6	65	21.0	250	80.9
Total		1257	557	44.3	211	16.8	269	21.4	1037	82.5

[1]Presy = presynchronisation; [2]RCont = resynchronisation control; [3]RCIDR = resynchronisation CIDR; See text for specific protocols

Timed AI, Resychronised AI, and Bull) such that overall pregnancy at the end of 100 d was lower for Holstein (71.4%, 324/455) than Jersey (89.7%, 165/184) and Crossbred (88.5%, 547/618). Mean milk production for Holstein, Jersey and Crossbred cows milked twice daily was 28 ± 0.7, 18.7 ± 1.5 and 22.2 ± 0.8 kg/d, respectively.

Nutritional Effects on Reproductive Efficiency

Clearly reproductive performance of today's high producing dairy cow is sub-optimal, and reproductive management systems have been developed for both intensive and extensive management systems as demonstrated above. Coupled with increases in milk production are major advancements in housing, nutritional management and health programmes that need to be collated with reproductive management programmes to sustain or enhance reproductive performance. This is considered essential because the next generation improvements in herd pregnancy rates are to increase reproductive competence of the lactating dairy cow during the periparturient and postpartum periods prior to the time of designated breeding. Nutritional strategies have been developed to improve health and reproductive performance with the feeding of specific nutraceuticals. A nutraceutical is defined as a product isolated or purified from feeds that is demonstrated to have a physiological benefit or provide protection against chronic disease. There are several nutraceuticals such as fatty acids, certain minerals, and vitamins that appear to have beneficial effects on production (i.e. reproduction and milk production) and health responses of lactating dairy cows.

Feeding of Supplemental Fatty Acids to Improve Reproductive Performance

The postpartum uterus undergoes dynamic changes associated with uterine regression, reabsorption of uterine tissues, and providing a localised immune response via the action of neutrophils to manage intrauterine populations of bacteria. Santos et al. (2008) reviewed the dynamics of dietary long chain fatty acids of the diet as factors influencing reproduction in cattle. Several of the eicosonaoids exert pro-inflammatory actions (e.g. PGE_2), and PGF_{2a} appears to be involved as well with neutrophil function as related to phagocytosis of bacteria. Feeding fatty acids (e.g. linoleic acid) during the periparturient period could act as a precursor for the biosynthesis of prostaglandins of the 2 series that may benefit postpartum health of the cow. Later on during the postpartum period (i.e. beginning at 30 d postpartum), it may be reasonable to feed fatty acids (e.g. eicosapentaenoic acid, EPA) that lead to the biosynthesis of fatty acids that are anti-inflammatory in nature (e.g. suppress neutrophil function and cytokine secretion). This would reduce possible residual inflammatory responses in the uterus associated with carryover effects of subclinical endometritis or as reported in repeat breeder cows, further enhance the immune-suppressive environmental state of pregnancy, and also reduce the potential luteolytic peaks at the time that the conceptus is suppressing PGF_{2a} secretion in order to maintain the corpus luteum for pregnancy maintenance.

In a recent Florida study, Silvestre et al. (2008a and b) randomly allocated cows (n = 1 582) into two experimental transition diets beginning at approximately 30 d before the expected date of parturition and continued until approximately 30 d postpartum. After 30 d all cows within each transition diet were allocated randomly into the experimental breeding diets that were fed until approximately 160 d postpartum. Experimental transition and breeding diets differed only in the source of supplemental fatty acids. Transition diets consisted of calcium salts (CS) of palm oil (PO, 47% C16:0; EnerGII) or CS of safflower oil (SO, 64% C18:2n-6; Prequel 21) and breeding diets consisted of CS of PO (EnerGII) or CS of fish oil (FO 11% of C20:5n-3 + C22:6n-3, StrataG). All CS of FAs were manufactured by Virtus Nutrition (Corcoran, CA, USA) and supplemented at 1.5% of dry matter. Diets were formulated to meet or exceed NRC (2001) nutrient requirements for net energy of lactation (NE_L), crude protein (CP), fibre, mineral and vitamins and fed to obtain intakes of 200 and 400 g/d of CS of FAs, for pre- and postpartum cows, respectively. Diets were fed as a total mixed ration twice daily targeting 5% refusals. The experimental design encompassed four treatment groups to test the effects of feeding SO during the transition period and FO during the breeding period. Feeding PO as a saturated fatty acid control in both periods would not account for possible carry-over effects of the transition diets into the breeding period.

Blood samples were collected from sub-samples of cows at enrollment (n = 18) and in the postpartum period (n = 47) at parturition (i.e. 2.8 ± 1.8 h after delivery), 4 d and 7 d postpartum for analyses of neutrophil activity and abundance of adhesion molecules using flow cytometry (Jain et al., 1991; Smits et al., 1997). Number of bacteria (*E. coli* and *S. aureus*) phagocytised/neutrophil was greater ($P < 0.01$) for cows in the SO at 4 d postpartum associated with a greater ($P < 0.05$) intensity of H_2O_2 produced/neutrophil at 4 d and 7 d postpartum in cows fed SO fat supplement (**Figure 3**). Neutrophil abundance of

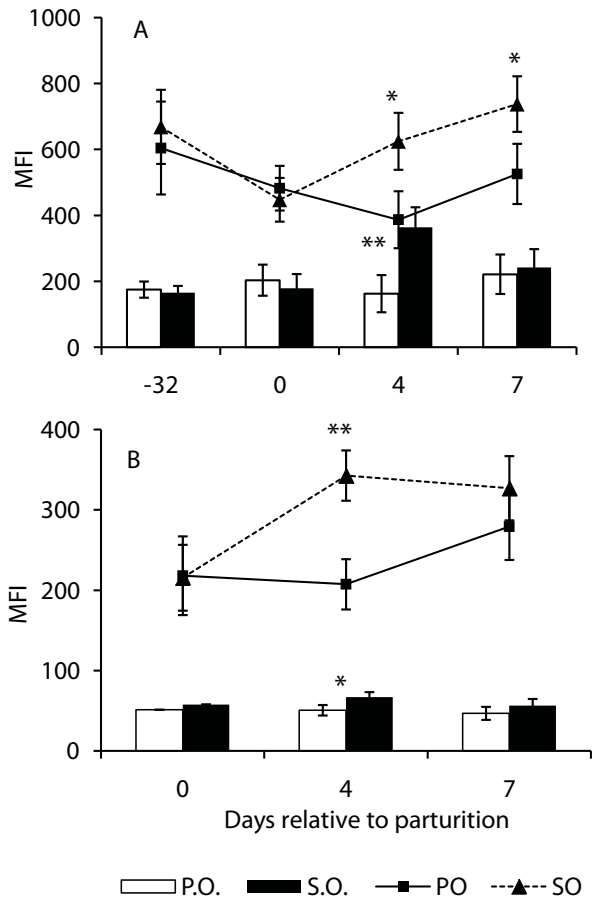

Figure 3. Least squares means (±S.E.) of neutrophil mean fluorescense intensity (MFI) for number of bacteria phagocytised per neutrophil (bars), and for intensity of H_2O_2 produced per neutrophil (lines) in whole blood stimulated with *E. coli* (A) or *S. aureus* (B). Cows were supplemented with palm oil (PO; n=23) or safflower oil (SO; n=24) during the transmition period. *P<0.05 and **P<0.01.

Table 2. First and second services pregnancies per AI at 32 d and 60 d after insemination and pregnancy loss [% (n=)]for experimental diets.

	Diets				Diet contrasts[1] (P value)		
	PO-PO	SO-PO	PO-FO	SO-FO	C1	C2	C3
1st service							
D32	38.7 (107/276)	35.8 (96/268)	39.1 (103/263)	35.8 (89/248)	NS	NS	NS
D60	33.7 (92/273)	29.7 (79/266)	37.0 (97/262)	32.8 (81/247)	NS	NS	NS
Loss	11.5 (12/104)	15.9 (15/94)	4.9 (5/102)	7.9 (7/88)	NS	< 0.05	NS
2nd service							
D32	27.7 (43/155)	26.7 (41/154)	30.3 (44/154)	43.3 (65/150)	NS	< 0.05	= 0.10
D 60	21.0 (38/152)	22.5 (34/151)	27.3 (39/143)	41.3 (62/150)	NS	< 0.01	< 0.05
Loss	5.0 (2/40)	10.0 (4/38)	7.1 (3/42)	4.6 (3/65)	NS	NS	NS

[1]Contrast are C1 (transition diets [PO-PO + PO-FO vs. SO-PO + SO-FO]), C2 (breeding diets [PO-PO + SO-PO vs. PO-FO + SO-FO]) and C3 (interaction of diets [PO-PO + SO-FO vs. PO-FO + SO-PO]). PO (palm oil; EnerGII); SO (safflower oil; **Prequel 21); FO** (fish oil; StrataG**). All fat supplements were manufactured as calcium salts by Virtus Nutrition, LLC (Corcoran, CA, USA) and supplemented at 1.5 percent of dry matter. NS = non-significant.**

L-selectin (arbitrary units) was increased (P < 0.01) after parturition (752.75) and was greater (P < 0.05) at 4 d and 7 d postpartum for SO (1 205.3 and 1 134.2; S.E. = 96.2) compared with PO (862.5 and 892.8; S.E. = 95.8) supplemented cows, respectively. No effects of diet or day were observed in the abundance of β_2-integrin.

Neutrophil cytokine production and FAs profiles were measured in sub-samples of cows sampled at enrollment and at 35 d postpartum (n = 26) which was the last day of PO and SO feeding for the sub-sample of cows. Neutrophils were isolated from whole blood and incubated in media, with or without lipopolysaccharide (LPS), at 37 °C in a 5% CO_2 incubator for 18 h (Sohn et al., 2007). Mean concentrations of TNF-α and IL-1β in supernatants of isolated neutrophils stimulated with LPS were greater (P < 0.01) for cows supplemented with SO (106.97 pg/ml and 1.45 ng/ml) compared with PO (63.25 pg/ml and 0.67 ng/ml), respectively. Concurrently, neutrophil LN content of the FAs, although numerically greater, was not significantly greater (P = 0.19) in cows fed SO (23.23%) than with PO (20.61%). The predominant FAs in the neutrophils were linoleic, stearic, palmitic, oleic and erucic acids which comprised approximately 72% of all FAs. The ratio n-6 (C18:2 + C22:4): n-3 (C18:3 + C20:5 + C22:6) of FAs tended (P = 0.07) to be greater for cows fed SO (9.16 ± 0.73) compared with PO (7.16 ± 0.73) supplements.

Blood samples were collected from PO (n = 15) and SO (n = 17) cows daily from parturition to 10 d postpartum and continued thrice weekly until 35 d postpartum for analyses of plasma concentrations of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) and acute phase proteins (i.e. haptoglobin [HP] and fibrinogen [FB]), respectively. Plasma concentrations of PGFM were not affected by transition diets except for d 4 and d 7 postpartum, in which a greater (P < 0.05) concentration was detected for the SO (2 809 ± 310 pg/mL and 2 667 ± 314 pg/mL) compared with PO (2 081 ± 325 pg/mL and 1 443 ± 325 pg/mL) diets, respectively. Plasma concentrations of HP and FB were higher (P < 0.05) for SO (0.034 OD and 248.8 mg/dL, n = 17) compared with PO (0.02 OD and 205.3 mg/dL, n = 15).

Although feeding SO improved aspects of innate immunity (i.e. neutrophil function and acute phase response), SO (n = 562) and PO (n = 554) cows had similar frequency distributions of mucupurulent (10% and 14.4%) and purulent (30.4% and 28%) cervical discharges evaluated once between 8–10 d postpartum.

Collectively, feeding a linoleic fatty acid enriched diet, beginning in the close up ration pre-partum, changed fatty acid profiles of tissues placing the cow in a 'pro-inflammatory state'. Such a state involves a lower threshold for initiation of an inflammatory response and increased sensitivity of cells upon stimuli. Inflammation is the first step for initiation of an immune response.

Cows at 43 d postpartum began a Presynch protocol with two injections of $PGF_{2\alpha}$ injected 14 d apart. The Ovsynch protocol was initiated 14 d after the second injection of $PGF_{2\alpha}$. of the Presynch with a GnRH injection (100 µg) followed 7 d later by an injection of $PGF_{2\alpha}$ and a final injection of GnRH 56 h later. Timed AI for first service was performed 16 h after the second GnRH injection of the Ovsynch protocol. All cows received a controlled internal drug-releasing device (CIDR) containing 1.38 g of progesterone at 18 d after the first TAI followed 7 d later by removal of the CIDR device and an 100 µg injection of GnRH. At 32 d after first TAI, cows were examined for pregnancy by per-rectum ultrasonography to identify presence of an embryo and an embryonic heart beat. Non-pregnant cows were injected with 25 mg of $PGF_{2\alpha}$ and then injected with 100 µg of GnRH 56 h later. A TAI was performed 16 h after the last GnRH for the second service. Cows were examined for pregnancy by per-rectum ultrasonography at 32 d after second service. All cows diagnosed pregnant after first and second services were re-examined by per-rectum ultrasonography at 60 d after insemination to determine pregnancy losses.

Pregnancy per AI, pregnancy losses, and cumulative proportion of pregnant cows after two services were analysed using predetermined statistical contrasts to test the effects of the transition diets (PO-PO + PO-FO vs. SO-PO + SO-FO), breeding diets (PO-PO + SO-PO vs. PO-FO + SO-FO) and the interaction of transition and breeding diets (PO-PO + SO-FO vs. PO-FO + SO-PO) accordingly with the experimental feeding design described above.

Transition, breeding and interaction of diets did not affect pregnancy per AI at 32 d and 60 d after TAI for first service (**Table 2**). However, pregnancy loss from d 32 – d 60 after the first TAI was less (P < 0.05) in FO compared with PO supplemented cows during the breeding period. For second service, breeding diet (i.e. PO or FO) altered (P < 0.05) the 32 d estimates of pregnancy per AI and a tendency (P < 0.10) for an interaction was detected between transition and breeding diets. The increase in d 32 pregnancy/AI caused by FO was greater in cows fed the SO transition diet, whereas there was no increase in pregnancy/AI in cows fed the PO breeding diet regardless of transition diet. Both breeding diet and a transition by breeding

diet interaction ($P < 0.05$) were detected for the 60 d pregnancy/AI response in which FO stimulated pregnancy rate/AI but the response to FO was greater in cows fed the SO transition diet (**Table 2**).

Neutrophil cytokine production and profiles of FAs were measured in a sub-sample of cows (n = 28) at 85 d postpartum at a time when cows were fed the breeding diets (i.e. PO or FO) for approximately 55 d. Mean concentration of TNF-α, but not IL-1β, in supernatants of isolated neutrophils was less ($P < 0.01$) for cows supplemented with FO (42.55 pg/mL and 0.6 ng/mL) compared with PO (82.68 pg/mL and 0.78 ng/mL) in response to LPS, respectively. Concurrently, The neutrophil content of EPA (1.5% and 0.30%), DPA (C22:5n-3; 3.48% and 2.33%) and DHA (1.65% and 0.11%) FAs were greater ($P < 0.01$) in cows fed FO compared with PO diets, respectively. Consequently, the ratio of n-6 (C18:2 + C22:4): n-3 (C18:3 + C20:5 + C22:6) FAs was less ($P < 0.01$) in cows fed FO (3.75) compared with PO (8.48). These responses indicate that at the time of conducting inseminations, neutrophils available to reproductive tissues were under a greater anti-inflammatory response, which may complement the immune-suppressive effects of the conceptus in early pregnancy.

Feeding of Organic Selenium to Improve Reproductive Performance

During the immediate postpartum period, the cow's immune system is challenged severely (Goff, 2006), and the innate and humoral defence systems are reduced. Selenium (Se) has long been associated with immunity. Cattle supplemented with Se-yeast had an 18% increase of Se in plasma in comparison to supplementation with inorganic sodium selenite (Weiss, 2003). The state of Florida, USA, as well as many areas of the world, is Se deficient in a subtropical environment, and lactating dairy cows are exposed to a seasonal period of heat stress that impacts reproductive performance and health. Furthermore, heat stress increases oxidative free radicals, and Se in the form of selenoproteins can function as an antioxidant that may benefit reproductive function.

Yeast converts Se to selenoamino acids, particularly selenomethionine, which are not destroyed by ruminal microbes and can be incorporated by the cow into a variety of selenoproteins. An experiment was conducted that fed organic selenium (Se; Se yeast [SY; Sel-Plex®, Alltech]) during the prepartum to postpartum periods (Silvestre et al., 2006a and b; 2007). Objectives were to evaluate effects of organic Se on P/TAI at the first and second postpartum services, uterine health, immune status and milk yield during the summer heat stress period. Cows were assigned (23±8 d prepartum) to diets of organic Se (Se-yeast [SY; Sel-Plex®, Alltech; n = 289] or inorganic sodium Se [SS; n = 285]) fed at 0.3 ppm (DM basis) for > 81 d postpartum. Rectal temperature was recorded each morning for 10 d postpartum. Vaginoscopies were performed at 5 d and 10 d postpartum. Cows were programmed for TAI to first and second service using presynchronisation-Ovsynch programmes followed by a resynchronisation comprised of an Ovsynch beginning at 20d - 23 d after first service. An ultrasound pregnancy diagnosis was conducted at 27d – 30 d after first TAI. Strategic blood sampling determined anovulatory status at Ovsynch and ovulatory response after TAI to first service. The PR at second service was determined by rectal palpation at ~42 d postpartum. Blood was sampled for Se (n = 20 cows/diet) at –25, 0, 7, 14, 21, and 37d postpartum.

Plasma Se increased in SY compared to SS-fed cows (0.087 > 0.069±.004 µg/mL; $P < 0.01$). Milk yield (35.6 kg/d for 81 d), and frequencies of retained foetal membrane (9.7%), mastitis (14.4%), anovulation (17.7%), and synchronised ovulation after TAI (82.5%) were not affected by diets or reproductive programme. Diet failed to alter first service PR at ~30 d post AI (SY, 24.9% [62/249] and SS, 23.6% [62/262]) or pregnancy losses between ~30 d and ~55 d post AI (SY, 39.3% and SS, 37.1%.) These low pregnancy rates and high embryonic losses are typical of cows managed during the summer heat stress period of Florida. Diet did indeed alter second service PR (SY, 17% [34/199] vs SS, 11.3% [24/211]; $P < 0.03$). The benefit of SY on second service pregnancy rate is very interesting. We hypothesise that cows of the SY group were better able to reestablish an embryo-trophic environment at second service following either early or late embryonic losses. For example, cows presented for second service may not have been pregnant to the first service by 30 d at the ultrasound diagnosis or were pregnant and underwent embryonic loss and required a second service. Indeed P/TAI to the second service for cows that had lost an embryo was 22.7% (5/22) for the SY versus 4.2% (1/24) in the control or SS group ($P < 0.09$); P/TAI to the second service for cows that were not diagnosed pregnant at first service and were re-inseminated did not differ (16.3% [29/177] for SY versus 12.3% [23/187] for SS.

Diet altered frequency of multiparous cows detected with > one event of fever (rectal temperature > 39.5 °C; SY, 13.3% [25/188] < SS, 25.5% [46/181]; $P < 0.05$) but the SY effect was not observed in primiparous cows which had a much higher frequency of fever (40.5%). Vaginoscopy discharge scores, measured at 5 d and 10 d postpartum, were affected by SY and SS diets ($P < 0.05$), respectively: clear (47.1% vs 35.0%), mucupurulent (43.4% vs 47.8%) and purulent (9.3% vs 17.1%).The frequency of cows with a purulent-fetid discharge was reduced and proportion of cows with a clean discharge was increased. This is additional support that feeding the organic selenium (i.e. SY) improved the uterine environment.

Innate immunity (i.e. neutrophil function) was determined by phagocytic and oxidative burst capacity of neutrophils in whole blood using a dual colour flow cytometric method. Samples were collected from a sub-sample of 36 cows at –26 d postpartum and 40 cows at zero, 7, 14, 21 and 37 d postpartum and analysed for neutrophil function. Adaptive immunity (ability to induce an antibody response) was monitored with anti-IgG to ovalbumin (ovalb) following vaccination with ovalb antigen (1 mg [i.m.]) dissolved in an *E. coli* J5 endotoxaemia preventive vaccine at -60 and –22±6 d postpartum (day of initiating SY [n = 38] and SS [n = 47] diets) and again at parturition (d zero) with ovalb dissolved in PBS with Quil-A adjuvant. Serum samples were collected on d of immunization and at 21d and 42d thereafter.

Percentage of gated neutrophils that phagocytised *E. coli* and underwent oxidative burst did not differ between dietary groups at –26 d postpartum (44.6±4.6%). For subsequent samples, a diet x parity x day interaction was detected ($P < 0.05$); namely, SY improved neutrophil function at parturition in multiparous cows (42±6.14% vs 24.3±7.2%) and at seven, 14 and 37 d postpartum in primiparous cows (53.9 vs 30.7%, 58.6 vs 41.9%, and 53.4% vs 34.8%, respectively; pooled S.E. = 6.8%). Neutrophil function was suppressed in primiparous cows at the time of parturition and not restored until seven – 14 d postpartum. In contrast, the multiparous cows did not have a restoration in neutrophil function until 14 d – 21 d postpartum. Organic Se improved phagocytosis and killing activity of neutrophils in both multiparous and primiparous cows. However, the primiparous cows seemed to be more responsive in that SY stimulated neutrophil function throughout the first 21 d postpartum whereas, SY stimulation in multiparous cows was evident on only the d of parturition.

Anti-IgG to ovalb did not differ between dietary groups at –60 and –22 d postpartum (0.18±0.01 and 0.97±0.04 OD). However, a diet x parity interaction was detected; IgG concentration did not differ between diet groups in primiparous cows across all d of sample collection (1.37±0.08, 1.43±0.07; $P > 0.10$), but it was higher

in SY cows at 21 d and 42 d postpartum (1.91 ± 0.1 > 1.24 ± 0.07, 1.44 ± 0.7 > 0.99 ± 0.07; P < 0.01). Thus our measurement of adaptive immunity was improved in multiparous dairy cows in response to SY but not in primiparous cows. Our findings indicated that feeding Se as organic Se (Se-yeast, Sel-Plex®), beginning at 26 d prepartum, elevated plasma Se concentrations, increased neutrophil function at the time of parturition, improved immuno-responsiveness in multiparous cows, and increased second service PR during summer in an environment that is Se deficient.

The ingredient β-carotene also has been fed twice daily as an antioxidant during the summer period of heat stress (Arechiga et al., 1998a). The β-carotene (Hoffmann-LaRoche Inc., Nutley, NJ) was added to the TMR as part of the vitamin premix at the morning feeding. The β-carotene premix was formulated to contain an additional 880 mg β-carotene /kg and was fed at a rate of approximately 0.45 kg/cow/d (400 mg β-carotene /d/ cow). The premix was assayed and found to contain a mean of 1 113 mg β-carotene/kg (i.e. intake of approximately 500 mg β-carotene /d/ cow). In cows that received supplemental β-carotene, beginning at 10–15 d postpartum and continued for at least a 90-d period postpartum, the percentage of cows pregnant at 120 d postpartum was greater (35.4% > 21.1%; $P < 0.05$). Furthermore, β-carotene feeding increased milk yield. Not only does β-carotene serve as an antioxidant but it is comprised of two retinol molecules. The PPAR transcription factor heterodimerises with retinol binding protein; retinol is a ligand for the retinol binding protein. Thus the molecule β-carotene interacts with fatty acid ligands to regulate various intracellular processes within the endometrium and other tissues.

CONCLUSIONS

Multi-scenarios of management can improve reproductive performance of lactating dairy cows during seasonal periods of thermal stress. Implementation of various extensive to intensive heat abatement systems to improve productivity depends on the severity of the local environment. A system that sustains a skin temperature at or below 35 °C avoids reductions in milk yield. Intensive cooling to completely eliminate heat stress results in close to normal fertility indicating that short periods of hyperthermia compromise fertility. Upgrading local *Bos indicus* breeds with *Bos taurus* dairy cattle benefits production with the optimal percent *Bos taurus* being dependent upon severity of the local environment. Genetic variance for heat tolerance exists among bulls of the Holstein breed such that more heat tolerant bulls have daughters with higher pregnancy rates. Indeed heat tolerant genes are being identified, such as the slick hair gene, which improves heat tolerance and productivity. *Bos indicus* embryos are more heat tolerant and offer the potential of introducing possible heat tolerant genes into *Bos taurus* breeds.

Various reproductive technologies can improve seasonal reproduction such as: timed embryo transfer of normal embryos or embryos treated with factors (e.g. IGF-1) that improve fertility; implementation of timed AI programmes in dairy heifers and lactating dairy cows to successfully implement a seasonal breeding programme to minimise heat stress, or to sustain an acceptable level of fertility in summer with dairy heifers, or to manage lactating dairy cows on pasture to coordinate seasonal availability of pastures with early lactation and inseminations prior to the heat stress period. A timed insemination reproductive management programme resulted in comparable fertility to the use of bulls in natural service during a seasonal period of heat stress, when excellent management and compliance were used in both systems.

Inclusion of nutraceuticals such as specific fatty acids (e.g., linoleic acid and eicosapentaenoic fatty acids), and organic selenium are able to regulate postpartum immune function and improve subsequent health, production and fertility. The above technological approaches provide an array of alternatives for producers in different social-economic and environmental locations to improve productivity and fertility of dairy cattle.

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Climate Change in Context: Implications for Livestock Production and Diversity

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ABSTRACT[2]

Climate change is predicted increasingly to affect the livestock sector in the coming decades, with potentially harmful consequences for production and for livestock genetic diversity. This paper considers mostly the indirect effects of climate change. It is expected that livestock production systems will face more frequent disastrous events and that higher temperatures will, in the absence of adaptive measures, increase physiological stress in livestock — with negative consequences for production. Tropical breeds are often well adapted to high temperatures, but the wider diffusion of such breeds or their incorporation into breeding programmes is restricted by the limited extent to which they have been characterised and improved in structured breeding programmes, trade constraints and the difficulty involved in introducing a new breed if it possesses only one advantageous trait. It is, nonetheless, concluded that given the unpredictability of climate change and of the general development of the livestock sector, conservation of adapted breeds is important. The disruptive effects of climate change on breeds' agro-ecosystems of origin may mean that increasing attention has to be given to *ex situ* conservation. Exchange mechanisms are needed to ensure that if international transfers of genetic resources are required as part of adaptation strategies, they can take place efficiently and equitably.

Keywords: *climate change, animal genetic resources, ecosystem.*

INTRODUCTION

Challenges for Food Security in the Next Decades

Agricultural development in the next 30 years will present unprecedented challenges. Globally, major increases in crop and livestock production are needed to feed a population of around 9 billion people in 2050. Agricultural production would need to increase by 70% by 2050 to raise average food consumption and to cope with a 40% increase in world human population. Compared with 2005/07, this requires an additional production of one billion tonnes of cereals and 200 million tonnes of meat annually. Approximately half of the total increase in grain demand will be for animal feed. Despite the absolute increase, growth in overall agricultural production will decelerate as a consequence of the slowdown in population growth and because a growing share of population will reach medium to high levels of food consumption (Bruinsma, 2009).

Eighty percent of cattle, buffalo and small ruminants are located in developing countries (2001–2003); however, they produce only 41% of global milk, 51% of global beef, and 71% of small ruminant meat (Steinfeld et al., 2006). Between 1980 and 2007, global beef output per animal grew at 0.4%/year, milk at 0.3%, pork at 0.8% and poultry at 1.1% (FAO Statistical Database [FAO-STAT]). These general trends mask high variation in productivity between species and livestock production systems, both within and between regions. The differences are larger in ruminants than in monogastrics for which industrial systems prevail in both developed and developing regions, with 55% of pork, 68% of eggs and 74% of poultry meat globally coming from industrial systems (FAO, 2003; Steinfeld et al., 2006). The most revolutionary change in the meat sector is in poultry; its share in world meat production increased from 13% in the mid-1960s to 28% in 2003.

The most important supply drivers over recent decades were cheap grain and cheap energy, technological change, especially in genetics, feeding and transport, together with a policy environment, including incentives, favourable to intensive production (FAO, 2010a). The most important demand drivers were increasing incomes, urbanisation and changing consumption patterns. In developing countries, where almost all world population increases take place, consumption of milk and meat has been growing at about 4–5%/annum in the last few decades. *Per capita* consumption of poultry increased more than threefold between the mid 1960s and 2002 (FAO, 2003). This 'livestock revolution' (Delgado et al., 1999), however, is concentrated in a few countries, particularly China and Brazil, mainly because of lack of development and income growth in many countries. FAO (2010a) projects that given the consumption growth in the past by China and Brazil this push will not play the same role in the future. Therefore, growth in *per capita* meat consumption in developing countries is likely to slow down in the next decades.

Livestock currently use 30% of the earth's entire land surface. This is mostly permanent pasture; but 33% of global arable land is used to produce livestock feed. The sector also accounts for about 8% of global water use, mainly for irrigation of feed crops. However, in arid areas, water consumed directly by animals or for product processing

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can represent a considerable share of total water use. Furthermore, the sector is a large producer of greenhouse gases (GHG), accounting for eighteen percent of GHG emissions, as measured in CO_2 equivalent — via enteric fermentation, land use and land-use change (directly for grazing or indirectly through production of feed crops) and manure management (FAO, 2010a).

The sector is also changing in regard to its contribution to poverty alleviation and income growth. The dichotomy between large numbers of small-scale livestock keepers and pastoralists, and intensive large-scale commercial livestock production is growing. While traditional livestock systems contribute to the livelihoods of 70% of the world's rural poor, increasing numbers of large-scale operations with sophisticated technology based on internationally sourced feed and animal genetics, cater for the rapidly growing markets for meat, milk and eggs, and midsize family farms risk being squeezed out of expanding formal markets. Generally, this goes hand-in-hand with shifts from multifunctional to commodity-specific products, local to globally integrated markets and from scattered to clustered production. While livestock provide multiple roles and functions for the livelihoods of the poor, the same poor are especially vulnerable to environmental hazards and zoonotic diseases (FAO, 2010a).

At the same time, resource competition increases, for example through the decreasing availability of and competition for land and water (including from other land uses such as production of biofuels, urbanisation and industrial development). Poor soil fertility and reduced access to fertiliser, overgrazing and deforestation, and loss of wild and agricultural biodiversity are other challenges. Many countries, especially in Africa and small countries in Asia and Latin America are already struggling to adapt to current environmental degradation and climate variability. Climate change will exacerbate the existing challenges faced by the livestock sector. And while farmers have always adapted their production systems to changing climatic and environmental conditions, the speed and complexity of current changes are expected to outpace the adaptive capacity of a broad range of food production systems.

Trends in Animal Genetic Diversity

Genetic diversity is a critical basis for food security and rural development. It enables stock to be selected in response to changing market conditions or societal needs, new knowledge of human nutritional requirements, changing environmental conditions, and new or resurgent disease threats. Only 37 mammalian and avian species used for food and agriculture are reported in FAO's Domestic Animal Diversity Information System (DAD-IS), and five species (cattle, sheep, goats, pigs and chickens) provide the majority of animal source food. Genetic improvement is estimated to contribute between 50% (Shook, 2006) and 80% (Havenstein et al., 2003) to overall productivity increase, and countries with structured commercial breeding programmes far exceed the production output/ animal of the rest of the world (FAO STAT).

Breeds can be categorised as local (reported by only one country) or transboundary (reported by several countries) (FAO, 2007a). The latest assessment identifies 7 040 local breeds and 1 051 transboundary breeds (FAO, 2009a). About two-thirds of reported breeds are currently found in developing countries. Local breeds are usually based in grassland-based pastoral and small-scale mixed crop-livestock systems with low to medium use of external inputs. Especially in developing countries, they deliver a wide range of products and services – each at a low level of output – supporting the livelihoods of their keepers. They are usually not phenotypically or genetically well characterised and their adaptedness to specific environments is not well understood. The improvement of a small proportion of local breeds is targeted by structured breeding programmes and even fewer are included in conservation programmes. Some of these breeds are very localised (FAO, 2007a). In contrast, international transboundary breeds (defined as those breeds present in more than one region of the world) have spread globally for use in high external input, often large-scale, systems. They provide single products for the market at high levels of output. They are usually well characterised and their genetic improvement is supported by efficient, sometimes global, structured breeding programmes. Genebanks exist, often as back-ups to regular artificial insemination programmes.

While countries are interdependent with regard to animal genetic resources, due to the few centres of domestication and the subsequent global spread of species and breeds, trade flows in genetic material in the past 50 years were not evenly distributed (Valle Zárate et al., 2006). Gollin et al. (2008) estimated that more than 90% of exports originate from developed countries, with a 30% share of international trade from developed to developing countries in 2005. They found that the wealthier developing countries are importers of genetics, while the poorest countries are not engaged in any international trade in animal genetic resources. Most of the exported genetic material is from animals suited to high-input production systems, and the exporting countries are free of zoosanitary restrictions (Hiemstra et al., 2007).

These exchanges of animal genetic resources contribute to productivity increases in developing countries. Productivity gains are high in pigs and poultry, species for which the genetics, the husbandry and feeding technology of intensive production are easily transferable internationally, less so in more complex ruminant systems. Ludena et al. (2007) thus predict a degree of technological convergence in non-ruminant production, but divergence for ruminant production between developing and developed countries.

Such convergence may have implications for livestock diversity. Today, most of the livestock in developed countries (and increasingly so in developing countries) is kept in more or less controlled conditions; this is particularly true for pigs and poultry. As a consequence of the uniformity of environmental conditions, fewer breeds are needed. About 9% of all reported breeds are extinct and 20% are currently classified as being at risk; the loss of within-breed diversity is not known. High selection pressure within commercial breeds may lead to a narrowing genetic base. For another 36% of breeds the risk status is unknown (FAO, 2009a). FAO (2007a) indicates that breed loss and risk in the past century was highest in regions that have the most highly specialised livestock industries with fast structural change and in the species kept in such systems, with more than one third of cattle, pig and poultry breeds already extinct and currently at-risk (FAO, 2009a). Economic and market drivers also made up 26% of all responses on threats to animal genetic resources in FAO's questionnaire survey (FAO, 2009b).

CLIMATE CHANGE

To assess climate change and food security, FAO (2008a) used a comprehensive definition of climate change that considers changes in long-term averages for all essential climate variables, e.g. changes in the climate system, including the drivers of change, the changes themselves and their effects. Climate change is already affecting all four dimensions of food security: food availability, food accessibility, food utilisation and food systems stability (FAO, 2009c). The consequences of climate change will be most acute in developing countries, where the increase in food demand is expected to be greatest, where demand for livestock products will continue to grow faster than production and where climate changes are projected to be greatest. As described above, climate change is, however,

just one of the many environmental, economic and social factors affecting the evolution of livestock production systems and related breed diversity. Many of the environmental changes that are already occurring as a result of human activities and those that are likely to occur in the future as a result of climate change are incremental, but they are cumulative and may eventually materialise in environmental crises. The IPCC has warned of 'tipping points' where damage due to climate change occurs irreversibly (Lenton et al., 2008). If societies are aware of the complex interrelated incremental changes, they may be able to adjust policies and production practices accordingly and minimise impacts. Within limits, some ecosystems will likely be able to adjust to incremental changes. Climate change will also expose inappropriate land use practices of farmers and livestock keepers that may have been hidden or tolerated by nature during periods of favourable rainfall. However, if the rates of change are too rapid for agricultural practices to adjust, then societies will face disruptions in the delivery of ecosystem services (MEA, 2005).

Livestock contributes to and will be affected by climate change; the sector is therefore crucial for mitigating, and adaptating to, climate change. Producers will have to cope with both slow climatic changes and more frequent extreme weather events. Land-use changes for the production of feeds (mainly deforestation, 35%), as well as emissions from manure (31%) and enteric fermentation (25%) are the largest contributors to GHG emissions from the livestock sector (FAO, 2006a); they are therefore the primary targets for mitigation measures. As water cycles continue to change, the concept of 'virtual water' (water that is used to produce products that are exported to, or imported by, other countries) will become more relevant.

In addition to the Intergovernmental Panel on Climate Change (IPCC) reports describing the predicted impact of climate change on ecosystems and agriculture (IPCC, 2007; Parry et al., 2007; Easterling et al., 2007), several papers provide a general overview of the expected impact of climate change on livestock production (Adams et al., 1998; Feenstra et al., 1998; Smit and Skinner, 2002). Other papers model changes in production systems and species composition under climate change (Seo and Mendelsohn, 2007 and 2008), poverty impact (Thornton et al., 2007; Jones and Thornton, 2009) or projections of methane (CH_4) emissions from African livestock (Herrero et al., 2008). De La Rocque et al. (2008) provide an overview of the effects of climate change on animal diseases. Hoffmann (2010b) provides a review of responses in animal genetic resources management and breeding.

Climate change will affect the products and services provided by agricultural biodiversity. However, the role of agricultural biodiversity in the resilience of food systems still needs to be assessed, and it needs to be properly integrated into strategies for adaptation to and mitigation of climate change (Gitay et al., 2002; FAO, 2008d). Assessments that estimate that 15–37% of terrestrial species are at risk of extinction due to climate change (Thomas et al., 2004; IPCC, 2007) do not take into account their value for food and agriculture (Lane and Jarvis, 2007). Jarvis et al. (2008) modelled 16–22% extinction risks for wild relatives of some food crops[3] and a reduction of over 50% in the range sizes of most of them. However, the low resolution of available data and the complexity of livestock production systems and weather patterns make it difficult to model the effects of climate change spatially or by production system. In addition, the domestication of several livestock species within one region implies that these species, and the breeds that were developed from them, have similar environmental envelopes. On the other hand, many breeds of the major livestock species are today globally distributed, and their geographic distribution is overlaid by different production systems. Data on most breeds' spatial distribution and adaptation traits are scarce (FAO, 2008b). This makes breed-level modelling of climate change impacts very difficult. FAO is currently working on georeferencing the spatial distributions of breeds.

This paper aims to shed light on the likely sensitivity of breed diversity to climate change in combination with other drivers, the production and ecosystems breeds depend upon, and the goods and services they supply. First, the direct and indirect effects of climate change and secondly, their potential impact on livestock production and on breed diversity are discussed. Traditional local and commercial international transboundary breeds are taken as extremes on a continuum of breed management. Some policy implications are discussed.

Ecosystem Changes

Ecosystem changes resulting from climate change are relevant to livestock production because of the land dependency of most production systems and the close interaction between livestock genetic resources and other agricultural biodiversity, including parasites and microbes (Mitchell, 2003; Hudson et al., 2006; Lafferty et al., 2008; Rosenzweig et al., 2008; Lafferty, 2009). Water, feed and forage are the most important inputs for livestock production. The overall and relative availability of these resources may be affected by ecosystem changes that are accelerated by climate change. However, at present the effects of direct human pressures such as non-sustainable practices, infrastructure development and fragmentation of rangeland ecosystems appear to be greater than those directly attributable to climate change. Anthropogenic landscape modification, in turn, has created obstacles to species migration in response to climate change. All those pressures combined, together with continued human population growth and concentration, are expected to result in larger areas being affected by disturbances such as fire, the effects of insects and diseases, and soil and vegetation degradation. The implications in the livestock sector are more complex due to, among others, the higher trophic level of animals as compared with plants.

Climate change will lead to evolutionary responses at species level. It will affect species distribution and interaction and lead to changes in communities and habitats. Shifts in species distribution are already being observed, with expansion at high altitudes and high latitudes and contraction at low altitudes and low latitudes. Tropical species (e.g. of insects) are expanding into temperate areas (Dukes and Mooney, 1999; Epstein, 2001) as a result of many drivers, one of which is climate change.

Evolutionary responses are faster in species with short generation intervals, large populations and high reproductive rates. Narrowly adapted and endemic species that rely on a particular environment are more likely to be disadvantaged by changes to their home environments than generalist species that can survive in a variety of different environments. Generalist or colonising species may take advantage of climate change to expand their ranges. This may be particularly significant for weed infestation or the spread of disease, because initially at least, invasive species are more likely to be generalists than specialists. One example is the highly pathogenic avian influenza (HPAI) virus, which changed its host range and infection pathways (Webster et al., 2006; Slingenbergh et al., 2010).

Marginal ecosystems (e.g. high altitude or arid systems) are often home to very endemic, specialised species that are considered ecologically valuable; these are also the systems particularly vulnerable

3 peanut (*Arachis*), potato (*Solanum*) and cowpea (*Vigna*).

to climate change (IPCC, 2007). In addition to the high number of adapted livestock breeds (FAO, 2006b), at least 30% of the world's cultivated plants and innumerable uncharacterised rangeland fodder species originated in drylands (UNCCD, 2005); these constitute a precious genetic stock for future agriculture. In the Near East, 90% of the region's breeds are kept in drylands. In Africa, this share is 56%, 42% in Asia and 19% in Latin America[4]. The distribution of some domesticated species is completely (camelids, yaks) or mainly restricted to drylands. More than 70% of breeds of ass, around 50% of sheep and goat breeds, and 30% of cattle and horse breeds reported are adapted to drylands (FAO, 2006b). It can be assumed that breeds adapted to drylands will be affected by natural resources degradation linked to climate change rather than temperature or precipitation change *per se*.

It is likely that ecosystem components will disintegrate and reassemble in a new ecological state (McCarty, 2001; Lovejoy, 2008). When usually interacting species have different phenological and evolutionary responses, differential range expansions may lead to new species relationships and trophic encounters (plant/herbivore, pathogen/host, predator/prey) and new communities. Such new encounters will lead to a reassessment of what is meant by 'invasive species' (Hoffmann, 2010a). Exchanges between cultivated and natural habitats, such as domestic and wild fowl systems, already occur and may further change with climate change (Gilbert et al., 2007 and 2008; Slingenbergh et al., 2010); this may include pathogen-host shifts as observed in HPAI (Webster et al., 2006).

The expected increases in rates of disturbance may facilitate successional change, providing a mechanism for ecosystem readjustment, but may also lead to a transient or permanent loss of biodiversity which in turn may adversely affect the function of the ecosystem. However, the loss of specific species does not necessarily lead to a loss of ecosystem function, as other species with the same function may occupy the vacant niche. Changes in plant community composition in response to climate change may be substantial in some areas and will probably lead to changes in water and nutrient cycling. In arid ecosystems particularly, the more than 20-year time lags complicate the attribution of multiple causes to vegetation changes (Valone et al., 2002). There are examples in which land-use practices including grazing had a bigger impact on arid ecosystems than climate (Wittig et al., 2007) and others in which the reorganisation of the ecosystem was triggered mostly by the effect of climate (Brown et al., 1997; Curtin, 2002).

Establishment of species in new locations depends not only on temperature and precipitation, but also on ecological parameters such as soil characteristics and vegetation composition in the target area, competition, disease pressure, reproductive behaviour, and the migratory capacity of the species. Current vegetation and disease vector models do not take into account the complex ecological relationships and non-linear processes that determine whether a species that migrates to remain in its ecological equivalent zone can actually establish itself where it arrives. Anthropogenic factors add another layer of complexity. Location-specific projections are therefore not easy, especially for agricultural ecosystems consisting of a combination of natural and managed biodiversity.

4 The figures refer to camelids (Bactrian camel, dromedary, llama and alpaca), cattle and yaks, goats and sheep, asses and horses.

Host-pathogen Interactions and Disease

About 75% of the new diseases that have affected humans over the past decade were caused by pathogens originating from animals or from products of animal origin. The link between disease risks from various pathogens and climate change has been increasingly recognised. Together with other factors such as land-use and habitat changes, the movement of human and animal populations and drug and pesticide resistance, climate affects vectors, pathogens, hosts and host-pathogen interactions from the level of cellular defence to that of the habitat (Epstein, 2001). The life cycles of parasites, such as seasonal presence, reproduction and transmission, overlap with the life cycles of their final and intermediate hosts. Hoberg et al. (2008) provide an overview of predicted responses of complex host-pathogen systems to climate change. Climate change may affect the spatial distribution of disease outbreaks, and their timing and intensity. In addition to long-term climate effects, short-term weather events affect the timing and intensity of disease outbreaks. Outbreaks of African horse sickness, peste des petits ruminants, Rift Valley fever, bluetongue virus and anthrax are triggered by specific weather conditions and changes in seasonal rainfall profiles (De la Rocque et al., 2008).

Extreme weather events or other short-term changes in the host-vector-pathogen interface may generate clusters of disease outbreaks. The predicted reduction in the availability and quality of water will increase the risk of water-borne diseases for humans and livestock. In addition to diseases affecting the animal itself, a new range of pests and diseases will impinge on crop and forage species, thus affecting the quantity and quality of livestock feeds.

Climatic effects on host-vector and host-parasite population dynamics are more pronounced in high latitudes and high altitudes, where rising minimum temperatures increase the growth potential of many parasite populations (Rogers and Randolph, 2006). Parasites may appear earlier in the season and go through more generations than they do today. The predicted temperature increase will further the geographic expansion of vector-borne infectious diseases (e.g. Rift Valley fever, bluetongue and tick-borne diseases) to higher elevations and higher latitudes and affect the transmission and course of the diseases. The bluetongue virus was able to establish itself successfully in new ecosystems by using a temperate vector instead of its traditional African-Asian *Culicoides* vector. The possible impact of bluetongue on endemic sheep breeds in the UK is raised by Carson et al. (2009).

Expansion of the range of a pathogen or vector does not necessarily result in wider disease transmission (De la Rocque et al., 2008). For some diseases, the effects of risk factors, such as the movement of animals and changes in production systems, habitats and ecosystems, will remain more important than climate change. Additionally, the risk of vector-borne diseases will display great local spatial variation and may be more affected by the abundance of competent hosts than climate change (Rogers and Randolph, 2006; Randolph, 2008 and 2009).

Rapid spread of pathogens or even small spatial or seasonal changes in disease distribution may expose livestock populations that lack resistance or acquired immunity to new diseases, resulting in more serious clinical disease. The expected increased and often novel disease pressure will favour genotypes that are resistant or tolerant to the diseases in question. FAO (2007a) lists 59 cattle, 33 sheep, 6 goat, 5 horse and 4 buffalo breeds, mainly from developing countries, that are reported to be resistant or tolerant to various pathogens, however, the underlying physiological and genetic mechanisms are not well understood.

Beyond the individual-animal level, the contribution of genetic diversity in populations to the dynamics of pathogen transmission needs further investigation. Mathematical models (Springbett et al., 2003) and evidence from plants (Mitchell et al., 2002) indicate that high species diversity and high genetic diversity within populations affect both the probability of the occurrence of epidemics and their outcome. In the case of vector-borne diseases, highly diverse host communities show lower infection rates among vectors due to the presence of unsuitable hosts — a mechanism known as the 'dilution effect' (Morand and Guégan, 2008). This highlights the need to maintain biodiversity in agricultural production systems and landscapes (Slingenbergh et al., 2010).

Feed and Fodder

There is a high probability that by the end of the century average growing-season temperatures will exceed the hottest seasons on record, and that heat stress will limit increases in crop production or cause output to fall. Without adaptation measures, food security will be threatened, especially in already food-insecure regions (Cline, 2007; Schmidhuber and Tubiello, 2007; Lobell et al., 2008; Jones and Thornton, 2009; Fischer, 2009).

In many livestock production systems, feed costs are the largest element of production costs. A key difference among livestock species is their differing ability to use feed resources that cannot be used directly as human food. While in extensive systems, forage may make up the entire diet of ruminants, it is supplemented by concentrates in intensive dairy, beef cattle and sheep fattening systems. Conversely, the diets of monogastric livestock consist largely of cereals and oilseed residues. Rowlinson (2008) suggests that it is unlikely that climate change will alter the current range of feed ingredients of commercial diets where rations for all species include high-quality by-products, many of which are imported from a wide range of countries and can be exchanged in least-cost ration formulation.

The effect of climate change on feed-grain supply is difficult to predict, as it depends on many factors in addition to climate. However, the non-food sector's demand for feed inputs, especially for biofuel and other industrial uses, is expected to increase, thereby potentially exacerbating the impact of climate change-induced reductions in feed supply for the livestock sector (FAO, 2008d; OECD-FAO, 2009). Afforestation for C-sequestration purposes reduces the land area available for grazing. While second-generation biofuels will increase competition with ruminants, by-products of first-generation biofuels are commonly used as supplemental protein feeds for ruminants. However, the feed rations of monogastric species may not be able to adjust as readily to absorb the increased supply of biofuel by-products. The mainly unknown impact of climate change on aquatic ecosystems, together with ongoing overfishing, and increasing use of fishmeal in aquaculture — thereby competing with its use as livestock feed - will also require changes in feeding rations.

Climate change is likely to affect the quality and quantity of the forage component of ruminant diets. Together with the predicted increase in fires and changing grazing regimes it may modify the plant species composition (grass, herbs, shrubs, trees) or give room to invasive species (Epstein et al., 2002; Easterling et al., 2007). In general, increases in temperature, CO_2, precipitation and N deposition will result in increased primary production in pastures (Tubiello et al., 2007). As long as water requirements can be met, the projected increase in temperature in temperate regions may have benefits for early season growth and lead to shifts towards more productive forage species, allowing higher-output breeds to be kept. New feeds, browse species and improved rangeland management are already being introduced in developing countries to improve feed supply, however, with mixed success in adoption. The reductions in precipitation expected in many already semi-arid areas, particularly if combined with increases in rainfall variability, will affect vegetation growth rates and may reduce rangeland production. This in turn will lower the stocking rates and productivity of grazing livestock (Hein et al., 2009), and increase the vulnerability of the system, including via land degradation that further exacerbates overgrazing of remaining areas.

Animal production in grassland-based systems is related to the availability of young and protein-rich plant material. High temperatures tend to increase lignification of plant tissues and thereby decreases forage digestibility. Also the climate change induced predicted shifts of plant species and communities may also affect forage production and quality. Examples include the predicted shift from C3 to C4 grasslands and the increase in shrub cover in grasslands (Christensen et al., 2004; Morgan et al., 2007). C4 plants are more efficient in terms of photosynthesis and water use than C3 plants. C3 and C4 plants coexist in the tropics, but react differently to increases in temperature and CO_2, which may result in changes to the composition of rangeland vegetation. Besides various changes in ecosystem function (Easterling and Apps, 2005; Easterling et al., 2007; Tubiello et al., 2007), this has implications for forage supply. Firstly, because C4 perennial grasses tend to start growing later in the spring and to mature earlier than C3 perennials, seasonal forage quality becomes more variable and overall forage availability may decline (Liang et al., 2002). Secondly, C3 forage plants generally have higher nutritive value, but yield less, while tropical C4 plants contain large amounts of low-quality dry matter and have a higher C:N ratio. Ruminants fed subtropical C4 grasses emitted more CH_4 per unit of digested dry matter than those fed temperate C3 grasses (Ulyatt et al., 2002). Browse often contains higher protein concentration than grasses. The digestibility of natural browse can be lower than that of grasses due to secondary plant components (Illius, 1997), but higher in improved browse species (e.g. *Leucaena*). From range management and animal nutrition perspectives, it will therefore be important to maintain a balance between C3 and C4 grasses, legumes and shrubs. Well-managed rangelands also provide better soil C sequestration. FAO (2009d) provides estimates of the potential carbon storage and sequestration in pasture and rangelands in drylands and outlines the main land management measures for improving carbon cycling and grassland management.

Few ecosystem models predict the general impact of climate change on rangelands and livestock production (Hall et al., 1998; Christensen et al., 2004). Tietjen and Jeltsch (2007) found few models that aimed to understand rangeland system dynamics. They conclude that current models insufficiently reflect the impact of increased CO_2 levels and changes in intra-annual precipitation patterns on plant productivity — both crucial external drivers under climate change — and provide little guidance to livestock and land managers.

Adaptation to Climate Change and Related Drivers

Commercial and small-scale livestock keepers choose the species best adapted to current climatic and agro-ecosystem conditions within their social, economic and technology contexts. As all these are constantly evolving, they have been selecting and introducing breeds for centuries. It can be expected that the portfolio of breeds demanded by society will continue to change. Projecting a continuation of trends prevailing over the past decades, this will imply a growing dichotomy between livestock kept by large numbers of smallholders and pastoralists, and those kept in intensive large-scale commercial production - independent of climate change. Many stakeholders

do not yet perceive climate change as a problem for the management and conservation of livestock biodiversity. In a recent survey on threats to livestock diversity (FAO, 2009b), climate change was mentioned by only 1% of 1 335 respondents; moreover, it was only mentioned in the context of extensive land-based production systems. It was not mentioned at all by respondents to a separate questionnaire which sought the reasons why breeds classified as being at-risk in DAD-IS got into this state (ibid.).

There is general agreement that the direct effects of climate change will be of a similar nature in low- and high-external input livestock production systems, both of which will be affected by increased frequency of catastrophic events, disease epidemics and water scarcity, which will lead to physiological stress and productivity losses in the animals. Loss of animals as a result of droughts and floods, or disease epidemics related to climate change may increase (FAO, 2008a). If breeds are geographically restricted in their distribution (endemic) – as is the case for some local and rare breeds - there is a risk that they will be lost in localised disasters (Heffernan and Goe, 2006; Carson et al., 2009). The direct effects of climate change depend very much on the production and housing system with intensively raised livestock kept in protected or controlled environments and fed with supplements being less likely to be directly affected by climate change (Adams et al., 1998; Freitas et al., 2006). As such intensive systems have more access to technologies and capital, the effect for the high-output breeds kept may be buffered.

Although the direct effects of climate change on the animal are likely to be small as long as temperature increases do not exceed 3 °C (Easterling et al., 2007), projections suggest that further selection for breeds with effective thermoregulatory control and adaptive traits will be needed. The speed of selection depends on many genetic factors, but also on reproductive technology and biotechnology which can be differently applied among the main species (FAO, 2010c).

Adaptation traits are usually characterised by low heritability. In general, the genetic relationships between adaptative and productive traits and potential selection responses need further attention. While annual genetic gain in production traits has been 1–2% over the past 50 years, adaptation and disease resistance traits, most of them multi-locus, are more difficult to select for and often have antagonistic genetic relationships to production traits. However, many commercial breeding programmes today aim to improve production, longevity and functional traits simultaneously — for example in dairy cattle, pigs and layer chickens. Breeding for adaptation to climate change will not be fundamentally different from existing breeding programmes, but inclusion of traits associated with temperature tolerance in breeding indices, and more consideration of genotype x environment interactions to identify animals most adapted to specific conditions will become more important. Hoffmann (2010b) reviews breeding for improved heat tolerance, nutrition and disease stress.

The indirect effects of climate change such as ecosystem changes or the implementation of climate change mitigation measures will differ between production systems. According to models by Seo and Mendelsohn (2007 and 2008), livestock production in developing countries is sensitive to climate change. Their projections indicate that commercial production systems will be more vulnerable because their specialised nature makes it difficult for them to switch to other species. Conversely, they found net revenues of small farms will increase as temperature or rainfall levels rise due to their more diverse species portfolios, the ease with which they can shift between species and diversify, and their reliance on goats and sheep.

The indirect impacts of climate change may even exceed the direct impacts, they may lead to changes in the ranking of species and breeds and to regional shifts in the market. In low external input systems, changes in fodder quantity and quality and changing host-pathogen interactions in local agro-ecosystems will come into play. In general, changes in the amount of land area devoted to cropping (including fodder) relative to rangeland and their relative productivity will influence the balance between non-ruminant and ruminant production.

In intensive landless systems, the indirect effects will be more related to changes in availability and price of inputs such as feed, energy and water. In this context, use of specific feeds and differences in feed conversion ratios (FCR) between breeds will influence the proportion of commercial *versus* local breeds.

In both types of system, the effects of climate change mitigation measures may play a role, but affect them and the breeds they harbour differently. The measures aimed at mitigating the effects of land-use change from/for? feed production and manure management are more relevant to intensive production systems where they may increase production costs.

The implications of climate change adaptation and mitigation strategies on breed diversity will depend on the public goods targeted and the trade-offs between them (FAO, 2009c): the effects of mitigation measures aimed at reducing GHG emissions from enteric fermentation may differ from the effects of those targeting emissions from manure or those aimed at improving soil C sequestration, and would certainly differ from the effects of adaptation measures that aim to increase social equity. In general, scenarios for ruminants and monogastrics differ because of differences in the feed base that they utilise and in the GHG emissions associated with the production process. Livestock keepers, particularly pastoralists, make use of the livestock species and breed differences in diet selection, walking ability, heat tolerance and water requirements to produce the products and services they need from their animals. For example, feed intake and digestibility in dromedaries do not decline under heat stress (Guerouali and Wardeh, 1998). Among cattle, taurine breeds have a better FCR with high quality feed while indicus breeds generally deal better with low quality forage than do taurine breeds. Taking into account the direct effects of climate change together with changes in agro-ecological conditions and production systems, the models by Herrero et al. (2008) and Seo and Mendelsohn (2008) indicate that farmers will switch from beef and dairy cattle and chickens towards goats and sheep as temperatures rise.

Livestock can compensate for the expected lower fodder quality and shrub encroachment to a certain extent if they are able to select high quality diets from different plant components or vegetation communities. There is some evidence for breed differences in diet selection behaviour and browsing ability linked to different metabolic profiles (Blench, 1999; Bester et al., 2002; Jauregui et al., 2008; Fraser et al., 2009). Also a number of plants and unconventional feed resources are adapted to harsh conditions, such as poor soil and drought. Many traditional livestock farmers use multi-species and multi-breed herds to maintain high diversity in on-farm niches and to buffer against climatic and economic adversities (Hoffmann et al., 2001; Hoffmann, 2003; FAO, 2009e). Traditional knowledge and practices are therefore very useful for adaptation to climate change; this includes species and breed substitution. For example in West Africa, dromedaries replaced cattle, and goats replaced sheep following the Sahelian droughts of the 1980s. This species mix made better use of available feed resources: While cattle and sheep feed largely on herbaceous vegetation, dromedaries browse on shrubs and trees and goats use both strata. The use of browsing species has several advantages: browse is increasing in some environments due to overuse of the herbaceous and layer; browse tends to offer green forage also during the dry season, and there is less competition by other domestic species. Replacing cattle by dromedaries in traditional manure contracts has opened a new vegetation stratum for recycling

nutrients to the soil (Hoffmann and Mohammed, 2004). In addition, camel milk is a reliable source of food during dry seasons, and interest in its special properties is rising. Disease resistance also plays a role, and while species and breed displacement from the arid and semi-arid to the sub-humid zones in West Africa has been observed, extension into the humid zones, where disease pressure (especially trypanosomosis) is high, is still limited.

Degradation of the natural resource base for livestock production, especially overgrazing in grassland-based systems and pollution in intensive systems, are major future challenges (FAO, 2010a). Such environmental damage may exacerbate the impact of climate change and raise the costs of adaptation. For example, provinces of Western China recently imposed restrictions on grazing with the objective of reducing rangeland degradation (Zhang and Hong, 2009). Also reforestation measures aimed at C-sequestration tend to increase the value of land previously used for grazing, in addition to the already mentioned effects of increased demand for biofuel. This may further reduce the land area available for livestock production, thereby adding to the threats to local ruminant breeds and the livelihoods of their keepers.

In Africa already today most livestock are found in semi-arid environments. These systems are expected to expand at the expense of humid and temperate/tropical highlands systems. However, while Seo and Mendelsohn (2008) predict that ruminant numbers in rangelands will increase as long as there is sufficient precipitation to support vegetation growth, Herrero et al. (2008) model large shifts of livestock populations from rangeland-based grazing to mixed crop-livestock systems based on improved feeding of crop by-products. Jones and Thornton (2009) predict that livestock keeping will replace cropping in today's marginal mixed crop-livestock systems. The outcomes of those models imply different breed portfolios. Mixed crop-livestock systems, especially those close to markets, have increasingly used crossbreeding with high output breeds (e.g. dairying in East African highlands), but only local breeds are adapted to the harsh conditions in pastoral systems. It can be expected that local breeds kept by poor people will continue to play a role in marginal areas (Hoffmann, 2010c). Problems for their survival may occur if adapted higher performing breeds penetrate into the environments currently occupied by local breeds, or if climate change is faster than selection.

The availability and prices of high quality feedstuff and grain will affect the comparative advantage of different species and the distribution of high output breeds. If the present economic drivers including rising feed prices continue, superior FCR will grant monogastrics a comparative advantage over cereal-fed ruminants. High output breeds of all species selected for improved FCR and high yield will dominate the market production of milk, eggs and meat. These breeds will continue to out-compete local breeds, thereby increasing the threat of extinction for local breeds, especially of monogastric species (FAO, 2009a).

Increasing individual animal productivity, improving feed quality, optimisation of feed rations and feed additives, and reducing livestock numbers are the most immediate GHG mitigation options at the animal level. In general, CH_4 output from enteric fermentation in ruminants increases with the higher dry matter intake linked to high performance, but CH_4 emissions/unit product decrease as the proportion of concentrate in the diet increases. However, the production pathways of different animal products differ in their GHG emissions and this may influence the future emphasis given to different production systems, species and breeds. Intensive poultry production based on line hybrids has the lowest GHG emissions/kg meat, followed by pigs (Williams et al., 2006). Milk protein can be produced with less CH_4 emission than beef (Williams et al., 2006; FAO, 2010b). This may tend to disadvantage breeds kept in extensive grazing systems. In an intermediate GHG reduction scenario, dairying might become the major focus of cattle production due to low CH_4 emissions/kg milk, and beef may become a by-product of dairying. Dual-purpose breeds and crossbreeding may gain importance.

Local ruminant breeds, which have low output and therefore a higher GHG emission/kg of single product, are considered unproductive. Ruminants use forages that cannot otherwise be used by humans; this enables human communities to inhabit harsh areas where production of crops is impossible (Gill and Smith, 2008). However, the productivity equation does not usually take into account the multiple products and services provided by livestock in most smallholder and pastoral production systems. Because of the above mentioned differences in feed quality, productivity improvements in ruminants grazing more digestible temperate pastures will result in lower relative CH_4 reductions than in pasture-fed ruminants in the tropics (McCrabb and Hunter, 1999). However, less investment in research and breeding is targeted towards the many local breeds and forages in extensive tropical systems.

Carbon sequestration in grasslands may partially offset GHG emissions from other components of the production process and offers co-benefits of improved grazing management and related positive environmental effects (soil C sequestration, biodiversity) and a favourable impact on livestock productivity (Smith et al., 2007). It is therefore proposed to include soil C sequestration, which has the highest GHG mitigation effect in agriculture and to which improved grazing could significantly contribute, in a post-Kyoto Protocol mechanism (Smith et al., 2007; FAO, 2009d). Payments for environmental services, be it C sequestration, landscape management or biodiversity conservation would increase the chances of local breeds being maintained, and may favour the return of herbivore livestock species to forage-based feeding and land-based production systems. However, this needs a favourable political and economic environment.

Interdependence

Introducing new breeds into geographical areas or production systems is an adaptation strategy that livestock keepers have applied for millennia. If climate change exceeds the adaptive capacity of the currently used genetic portfolio, increased strategic crossbreeding with better adapted or higher performing breeds or insertion of specific genes through the use of biotechnology may occur. Climate change may thus increase the pressure to maintain wide access to animal genetic resources as countries will increasingly depend on better adapted or higher performing genetic resources from other countries to adapt their food and agricultural systems.

Such species or breed replacement processes may involve considerable costs and substantial investments in learning and gaining experience. Although in principle, a species or breed may replace the current one as single new components in a production system, more probably it will be introduced together with other components such as technology and knowledge (Ludena et al., 2007). FAO (2007a) provides a wealth of information on successful as well as failed introduction of breeds. In human-managed systems, 'establishment' of a new species or breed depends on how many components of the old production system can be transferred to the new system. This means that beyond the genetic material, the technologies and information supporting a new production system need to be available, accessible and affordable. In the USA, the introduction of new breeds has been successful when based on several production traits and when the private sector has been interested, while introductions aiming to take advantage of single traits have not proved sustainable (Blackburn and Gollin, 2008).

The heightened interdependence may increase the importance and value of specific genetic material and its associated knowledge, be it traditional or modern. The sharing of genetics and breeding-related knowledge generally declines the more specialised the knowledge associated with breeding and the more valuable the livestock species. This observation applies equally to commercial breeders of developed countries and pastoral breeders of developing countries. Knowledge about breeds and breeding in developed countries is increasingly privatised; commercial breeders keep their breeding lines and breeding programmes under tight control, including through the use of contracts, trade secrets or patents (Gura, 2007). The special knowledge needed for dromedary breeding and the maintenance of the breeds is still in the hands of the traditional pastoralists in the Sahel and in northern India where farmers buy dromedaries as draught animals (Hoffmann and Mohammed, 2004; FAO, 2009e). However, such specialist traditional knowledge in developing countries that may assist in targeted search for adaptive traits is increasingly under pressure due to the social changes linked with ongoing economic development and to climate change (FAO, 2009e).

The IPCC predicts large human population movements to adapt to climate change. The most widely accepted forecast for the number of 'environmentally induced migrants' by 2050 is 200 million (IOM, 2009). As humans migrate, they may for various social, economical and political reasons become disconnected from components of the agricultural ecosystems they currently manage. Livestock breeding and production systems are complex and knowledge intensive. They are often regionally stratified or integrated, with different human population groups having different roles and knowledge systems in the cycle — reproduction, growing, using the adults as dairy, meat or draught animals, etc. The vertical integration of developed-country production systems, for example the UK hill-lowland sheep system, is simple compared with that of systems in developing countries where different ethnic groups keep different breeds and have different roles in the cycle. As the climate changes, production systems will disintegrate and genetic resources will become uncoupled from traditional knowledge about their management. The HIV epidemic in Africa has demonstrated the detrimental effects of the loss of traditional knowledge in farming systems (Goe, 2005). Therefore, in order to increase the overall resilience of the global food and agricultural system in the advent of climate change, the associated knowledge for animal genetic resources, both commercial and local, should be documented and made widely accessible and transferable together with the genetics.

One can/may assume that specific traits of tropical adapted breeds may become important for climate change adaptation, leading to a reassessment of their values. In this case, it will be important to facilitate access to these resources and share the benefits from their use to ensure that adaptation efforts are not hindered by access restrictions by countries that happen to host such sought-after resources.

Hoffmann (2010b) argues that it is unlikely that climate change will reverse the current direction and trends of genetics exchange and make local breeds from developing countries more attractive to the international market. Reasons are developing countries' insufficient resources or capacity, resulting in a low level of characterisation or structured breeding programmes of the majority of local breeds. Additionally, the performance and technology focus on high output breeds indirectly reduce incentives for characterisation and selection within local breeds in developing countries for improved production or adaptation. Currently, only a few countries with well-developed research, extension and breeding and artificial insemination institutions have commercially relevant tropical cattle breeds and even fewer countries have commercially significant breeding programmes for adapted breeds of the other species.

Conservation

In nature conservation it is now argued that *in situ* strategies have to account for the fact that environmental conditions in species' historic ranges will change, and indeed are already changing (McCarty, 2001). Further to claims to facilitate species migration and maximising adaptation opportunities through the maintenance of intact ecosystems, this results in a review of *in situ* conservation areas, as protected habitats may be populated with new species, or rare species may migrate away from their protected habitats (ibid.). Thus, the role of *in situ* conservation areas as means to facilitate species migration and maximise adaptation opportunities by maintaining intact ecosystems needs to be reviewed. According to Jarvis et al. (2008), increased *in situ* and habitat conservation will be important for the survival of crop wild relatives whose range sizes are reduced by climate change. However, recognising that climate change may affect our food system quickly, they stress the urgent need to identify priority core species for collection and inclusion in genebanks.

Public germplasm collections such as for plant genetic resources do not exist for animal genetic resources where *ex situ* conservation is technically more challenging and ownership over the genetic resources is mostly private. Conservation measures for threatened breeds, which are a priority of the *Global Plan of Action for Animal Genetic Resources* (FAO, 2007b) have been established in some countries (FAO, 2007a). Data from 194 countries indicate that on average 7.5 breeds are at risk and 1.7 breeds are in conservation programmes (DAD-IS). Most conservation programmes are based in developed countries, with strong collaboration between genebanks and the animal breeding industry. In developing countries, the focus is currently on *in vivo* conservation with high variability in the quality of the programmes (ibid.), but in common with plant genetic resources, this may shift to *in vitro* conservation of animal genetic resources. Consequently, the sensitivity of *in situ* conservation programmes to the effects of climate change should be assessed and *ex situ* conservation measures taken if needed. As a first step in conservation, it is necessary to characterise animal genetic resources and subsequently to build inventories that include information on the spatial distribution of breeds and valuable breeding stocks. Then, conservation priorities have to be made (Boettcher et al., 2010). Cryo-conservation, from national to global, will depend on the infrastructure and resources available at each level and involve private genebanks held by breeding organisations or companies and public genebanks.

CONSEQUENCES FOR ANIMAL GENETIC DIVERSITY

The livestock sector is highly dynamic, and the need for increasing production while reducing the environmental footprint of livestock will continue to be major future challenges (Pelletier, 2008; FAO, 2010a). Climate change, with its slow but long-term effects, will be an additional factor affecting this dynamic (FAO, 2010a); it will further the need for resource-efficient livestock production and may thus intensify current trends with a growing dichotomy between livestock kept in extensive and those in intensive systems. However, the livestock community is not yet fully aware of its possible impact.

The direct effects of climate change will depend very much on the livestock production and housing system, with high output breeds in confined systems being better protected from natural adversities than breeds in extensive grazing systems. In systems where the rate of technology adoption is generally low, or in regions that have a limited capacity to adapt (e.g. sub-Saharan Africa),

the risk of breed displacement or loss may increase. Local breeds under traditional management are generally more adapted to harsh conditions and resilient to environmental changes than are high output breeds, but their adaptation to complex stressors is not well described, understood and valued.

Although many existing technologies will be crucial for climate change adaptation and mitigation, research gaps exist, especially with regard to the interactions between adaptation and mitigation. The outcomes of different scenarios and models imply different breed or species portfolios, making it difficult for decision makers to make rational choices. With the current state of knowledge it is not possible to predict whether climate change will be faster than natural or artificial selection. It would be an interesting research question to assess and model the likelihood, speed and impact of the various aspects of climate against genetic evolution of livestock and use the results to guide the need for interventions. The variable degree of confidence of projections, and the uncertainties related to climate change vulnerabilities and impacts are often regarded as a hindrance for immediate and concrete action, and FAO (2009c) argues that considerable precaution must be taken in policy making for food security under climate change projections.

In view of the uncertainty for future developments and climate change, the adaptive traits harboured in all breeds should be assessed and the use and non-use values of animal genetic resources be maintained. This applies equally to transboundary commercial and local breeds which fulfil different societal and ecological functions. Immediate needs for research and method development include:

- developing simple methods to characterise, evaluate and document adaptive and performance traits in specific production environments (FAO, 2008b), including the associated knowledge. For example, feeding and water intake behaviour and relationships between energy reserves, endocrinological parameters and breeds' reproduction performance, and improved methods to assess the use and non-use values of animal genetic resources;
- making breed inventories that including relevant spatial information, and monitor threats to breeds, be they caused by climate change or other drivers. This may entail some basic predictive modelling of future breed distribution and early warning systems;
- exploring possible synergies between plant and animal breeding (FAO, 2008c);
- developing methods for life-cycle assessments and include delivery of ecosystem services in the analysis, recognising and rewarding C sequestration and sustainable use of biodiversity in well managed rangelands with local breeds;
- strengthening agro-ecosystem modelling as a tool to improve understanding about the complex interaction between livestock genetics and adaptation with changes in the biophysical and socio-economic environment.

Developed and developing countries differ with regard to their genetic resources portfolios and the management of these resources. Most high output breeds are selected for the requirements of developed country markets and production systems. Most tropically adapted breeds exist as key resource in the livelihoods of rural people in developing countries, but these breeds and their keepers are often neglected. This undervaluation of many local breeds results in their not being well characterised and with few structured breeding or conservation programmes. The recent adoption of the *Global Plan of Action for Animal Genetic Resources* (FAO, 2007b) provides for the first time an internationally agreed framework for the management of animal genetic resources, and also proposes measures to support developing countries and small-scale livestock keepers in their endeavours (FAO, 2009e). Without strengthening capacity for breed characterisation, improvement and conservation in developing countries, climate change in combination with ever increasing demand for food may exacerbate current trends in the livestock sector and the divide between developed and developing countries (OECD-FAO, 2009). While *in situ* conservation is favourable due to its pro-poor effects, *ex situ* conservation programmes need to be developed to cater for habitat destruction and allow for emergency response. It will be necessary to improve adaptive traits in high output and performance traits in adapted breeds, and to facilitate wide access to genetic resources and enable transparent exchange of the associated knowledge in local and commercial breeds.

Developed and developing countries also differ in their adaptative capacity and in the expected interactions between climate change adaptation and mitigation. Due to their low capacity, developing countries will not implement adaptation measures solely as a response to climate change, but will promote closer relationships between climate change adaptation, pro-poor investment and development policies, allowing for co-benefits. Equally, they will exploit the other potential benefits of many measures aimed at reducing GHG emissions for improving the productivity and environmental integrity of agricultural ecosystems (Smith et al., 2007). Special care should be taken to ensure that climate change mitigation measures enhance systems' resilience; they should not reduce future adaptation options or undermine food security and rural livelihoods. The livestock sector, being a contributor to and affected by climate change on the one hand and a mainstay of the livelihoods of millions of rural poor on the other, has a role to play. Breeds, agricultural biodiversity and ecosystems must be an integrated part of general mitigation and adaptation efforts.

Pro-poor policies need to be developed that strengthen livestock keepers' adaptation strategies, their ecological knowledge and local institutions. Especially in marginal areas, land management for climate change mitigation and adaptation, biodiversity conservation and poverty alleviation should complement each other (UNCCD, 2005; FAO, 2007b, 2008c, and 2009d).

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Indigenous Cattle in Sri Lanka: Production Systems and Genetic Diversity

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ABSTRACT

Production status, farming systems and genetic diversity of indigenous cattle in Sri Lanka were evaluated using six geographically distinct populations. The indigenous cattle population of the country is considered as a nondescript mixture of genotypes, and represents more than half of the total cattle population of 1.2 million heads. Five distinct indigenous populations were investigated for morphological analysis, and four were included in evaluating genetic differences. Farming systems were analysed using a pre-tested structured questionnaire. The genetic variation was assessed within and between populations using 15 autosomal and two Y-specific microsatellite markers, and compared with two indigenous populations from the African region. Farming system analysis revealed that indigenous cattle rearing was based on traditional mixed-crop integration practices and operates under limited or no input basis. The contribution of indigenous cattle to total tangible income ranged from zero to 90% reflecting the high variation in the purpose of keeping. Morphometric measurements explained specific phenotypic characteristics arising from geographical isolation and selective breeding. Though varying according to the region, the compact body, narrow face, small horns and humps with shades of brown and black coat colour described the indigenous cattle phenotype in general. Genetic analysis indicated that indigenous cattle in Sri Lanka have high diversity with average number of alleles per locus ranging from 7.9 to 8.5. Average heterozygosity of different regions varied within a narrow range (0.72 ± 0.04 to 0.76 ± 0.03). Genetic distances between regions were low (0.085 and 0.066) suggesting a similar mixture of genotypes across regions. Y-specific analysis indicated a possible introgression of Taurine cattle in one of the cattle populations.

Key words: *indigenous cattle, farming systems, phenotype, genetic analysis, microsatellite markers, alleles.*

INTRODUCTION

Sri Lanka is an island in the Indian Ocean between the latitudes of 5° 55′ N and 9° 51′ N and longitudes of 79° 41′ and 81° 53′ E, covering an area of about 65 610 km^2, and lies in close proximity to the southeastern coast of India. The country´s economy is considered as agricultural, but the contribution of the sector to the gross national product is only 20%. The main contributors to the agricultural sector are from the plantation sub-sector and from field crops such as paddy rice. The contribution of livestock to the agriculture sector is only 8% (DCS, 2008). The livestock sector consists mainly of dairy cattle and poultry sub-sectors, the main emphasis of the former being to increase the availability of liquid milk within the country because around ten million Rupees is spent annually in importing milk products of which 96% is milk powder (DCS, 2008).

The total cattle population of the country has fluctuated during the past few decades but at present it is estimated at 1.2 million heads (DCS, 2008). Around 0.2 million heads are slaughtered annually, the majority of which are indigenous cattle (Silva et al., 2004). Indigenous livestock species constitute a major proportion of the total value of the animal genetic resources (AnGR) of Sri Lanka, but the value of indigenous cattle as a component of rural agriculture is diminishing for a variety of reasons which are common to many other countries in the region. Generally, indigenous cattle are evaluated only in terms of milk production as this is the main economic product. Heat tolerance and resistance to several endemic diseases make these indigenous breeds thrive better in local rural environments and production systems than other breeds of cattle.

These characteristics become prominent in the context of smallholder production systems, which are the predominant cattle rearing systems in the country and where resource levels are low (Ranaweera, 2007). In most instances, traditional cattle farmers, who follow set farming practices acquired from their ancestors, manage low input systems. It has been estimated that there are around 400 000 dairy farmers in Sri Lanka and 2.45 million people (70% of the estimated 3.5 million livestock dependent people) sustain their livelihoods from the dairy sector (Ibrahim et al., 1999; Ranaweera, 2007). Although estimates are not available, a considerable proportion of these dairy sector dependents rely on indigenous cattle. Based on the proportional geographic distribution of cattle, indigenous cattle and their crosses represent 60% of the total cattle population in the country (Ibrahim et al., 1999) but their contribution to milk production is marginal.

Indigenous cattle have long been identified as a separate category of cattle and have been used for several genetic improvement programmes in the past (Wijeratne, 1970; Tilakaratne et al., 1974). However, no systematic approach has been taken to identify and describe their phenotypic or genotypic differences, and as a result, the total indigenous population remains as a nondescript type of animal except for few breeds/types. The objective of this research was therefore to identify the production status, farming systems and genetic diversity of indigenous cattle in Sri Lanka.

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MATERIALS AND METHODS

Selection of Locations and Sampling Process

Sampling locations were selected to represent the general indigenous population of the country. Five geographically distinct indigenous cattle populations (NC – North Central, So – Southern, No – Northern, Tk – Thamakaduwa and Th – Thawalam) were investigated for morphological traits (**Figure 1**), and four (all except Th) were included in genetic diversity analysis. While the NC, So and No sampling locations represent the general indigenous cattle populations, the Tk and Th locations signify two cattle populations subjected to isolated breeding and selection for specific purposes over a long period. The respective farming systems were also evaluated to complete the requirement in developing conservation and utilisation strategies. Sampling was carried out based on the non-existence of artificial insemination facilities to assure the target populations were indigenous. The six populations were assumed to be genetically isolated from each other in the absence of a nomadic pattern of rearing and regular cattle migration. The farming systems were analysed by single visits to each location using a pre-tested structured questionnaire. Single visits were practised as there is no variation in farming system according to the period of the year. Morphometric measurements were taken during the visit. Blood samples were collected from the jugular vein for molecular genetic analysis.

Data Analysis

Farming system and morphometric data were compiled and analysed using Microsoft Access and Microsoft Excel software packages.

Molecular Analysis

DNA was purified from blood samples following a standard protocol (Sambrook and Russel, 2001) and all samples belonging to each population, were genotyped using 15 autosomal microsatellite DNA markers (AGLA293, ILTSTS023, ETH225, HEL01, ILSTS006, INRA032, MGTG4B, TGLA122, ETH152, INRA035, ILSTS050, BM2113, ILSTS005, CSSM66 and INRA005) and a Y-specific microsatellite marker (INRA 132), at the Biotechnology Laboratory of the International Livestock Research Institute (ILRI), Nairobi, Kenya. Molecular data were analysed using Microstat and DISPAN software packages.

RESULTS AND DISCUSSION

Farming system analysis revealed that indigenous cattle are reared as a traditional practice in all regions of the country under limited or no input environments. Since their low productivity masks their real contribution to rural livelihoods, the level of utilisation needs to be assessed taking the systems approach into consideration. This is an important aspect since the utilisation of cattle always is confounded by the attributes of respective farming systems (Silva et al., 2008). The contribution of indigenous cattle to total tangible income ranged from zero to 90% in different regions reflecting the high variation in the purpose of keeping indigenous cattle (**Figure 2**). Integration with crops, especially paddy rice, was the most common feature of the systems across the regions. Feeding and other management practices are not expensive and hence their contributions to total production costs are marginal. Feeding practices within indigenous cattle production systems varied depending on the resource availability within the system (**Figure 3**). Expenses connected with keeping the animals are limited to veterinary care and health management. However, in most cases, money is not spent on medicines unless these are essential, and use of indigenous medicine is common.

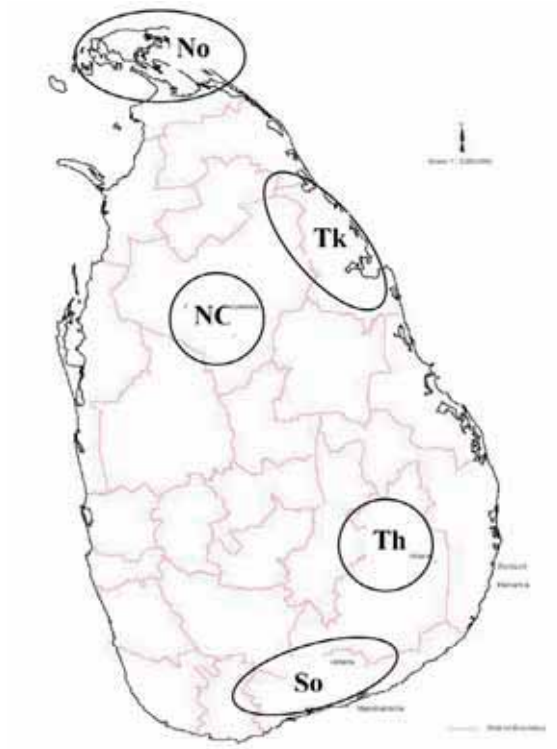

Figure 1. Sampling locations of indigenous cattle populations from Sri Lanka.

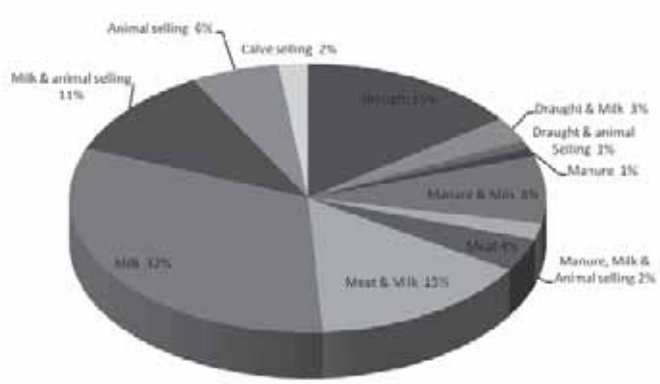

Figure 2. Purpose of rearing of indigenous cattle in Sri Lanka.

In this context, the present study confirms previous findings that the rearing of indigenous cattle is a profitable enterprise (Silva et al., 2008).

Morphometric measurements identified specific phenotypic characteristics resulting from geographical isolation and selective breeding. **Table 1** shows the morphometric measurements of indigenous cattle in general and in different populations. The comparison among different populations revealed a significantly higher ($P < 0.05$) heart girth, height at withers and body length in Tk and Th populations. These two populations have been selected and bred for the purpose of providing draught power. Morphological measurements showed that the indigenous cattle are small and compact, mostly similar to the Zebu type cattle found in the tropics (**Table 2**). These observations are comparable with earlier results obtained concerning the phe-

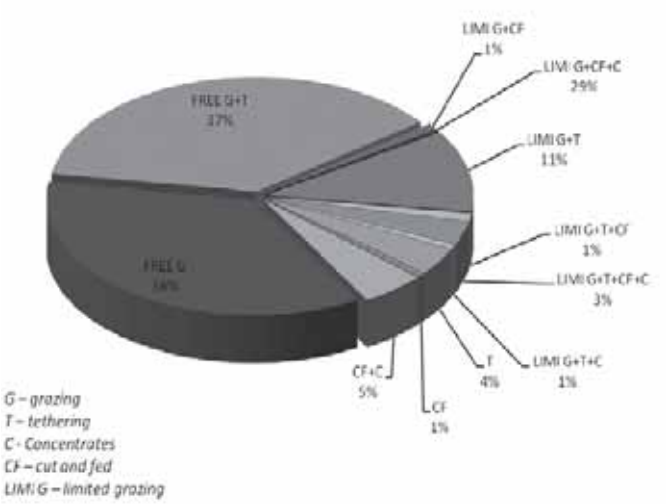

Figure 3. Variation of feeding practices of indigenous cattle in Sri Lanka.

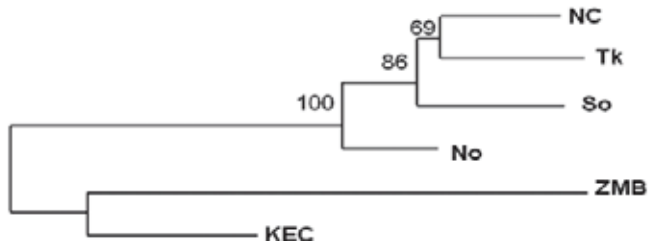

Figure 4. Phylogenetic tree showing the genetic distance among four indigenous cattle populations from Sri Lanka (NC, So, No and Tk) and two African populations (ZMB and KEC) (1 000 bootstrap).

Table 1. Morphometric measurements (cm) of different indigenous cattle populations from Sri Lanka.

Population[1]		Heart Girth	Height at withers	Hip width	Body length	Head length	Head width
NC	M	132 ± 09.7	101 ± 06.5	32 ± 3.2	103 ± 03.8	41 ± 3.1	16 ± 1.4
	F	132 ± 09.7	101 ± 06.4	32 ± 3.3	106 ± 08.8	41 ± 3.1	16 ± 1.6
So	M	125 ± 13.7	96 ± 09.7	30 ± 5.6	97 ± 12.2	38 ± 5.1	16 ± 2.7
	F	125 ± 13.2	96 ± 09.2	30 ± 5.5	97 ± 11.0	38 ± 4.9	16 ± 2.6
No	M	110 ± 18.9	93 ± 08.7	29 ± 4.2	97 ± 09.4	35 ± 3.7	12 ± 1.5
	F	111 ± 18.6	94 ± 08.5	30 ± 4.1	97 ± 09.2	35 ± 3.7	12 ± 1.5
Tk	M	150 ± 15.8	123 ± 16.0	33 ± 3.8	128 ± 33.2	41 ± 2.6	16 ± 1.7
	F	131 ± 13.0	118 ± 20.1	38 ± 5.0	109 ± 05.0	41 ± 3.0	14 ± 2.0
Th[2]	M	140 ± 12.3	112 ± 09.6	31 ± 4.6	116 ± 10.2	44 ± 4.1	14 ± 2.7
Overall	M	131 ± 19.1	100 ± 12.0	32 ± 5.6	105 ± 15.1	40 ± 4.4	15 ± 2.8
	F	131 ± 19.1	100 ± 11.9	32 ± 5.5	104 ± 15.1	40 ± 4.2	15 ± 2.5

[1] NC — North Central population; So — Southern population; No — Northern population; Tk — Thamakaduwa population; Th — Thawalam population; M — male; F — female.

[2] Only male population was available for measurements in pack animals.

Table 2. Morphological and production characteristics of indigenous cattle from Sri Lanka.

Morphological/production characteristics	Measurement/ Description
Coat colour	Black, brown (dark, light), gray, white and mixtures
Ear	Small / medium erect in horizontal orientation
Hump and top line	Small hump with straight top line
Head shape	Long and narrow with flat forehead
Hair type	Short and glossy
Gestation period (d)	250–290 d
Calving interval (months)	18 (10–27)
Age at first calving (months)	36 (18–60)
Milk yield (L/cow/d)	1.05 (0.5–1.2)
Lactation length (months)	3.5 (0.5–12)
Dry period (months)	3.0 (2–12)

Table 3. Number of alleles and allelic range from 15 autosomal microsatellite DNA markers in indigenous cattle from Sri Lanka.

Locus	No. individuals	No. alleles	Allelic range
AGLA293	207	16	216–244
ILTSTS023	207	12	170–202
ETH225	207	11	137–159
HEL01	207	9	101–117
ILSTS006	207	11	276–302
INRA032	207	13	162–204
MGTG4B	207	13	134–162
TGLA122	207	15	137–171
ETH152	207	7	189–203
INRA035	207	10	105–129
ILSTS050	207	11	140–162
BM2113	207	9	126–144
ILSTS005	207	7	180–194
CSSM66	207	15	178–206
INRA005	207	7	138–148

Table 4. Between population DA distance of indigenous cattle from Sri Lanka.

	NC	So	No	Tk
NC	1			
So	0.0723	1		
No	0.0772	0.0854	1	
Tk	0.0662	0.0773	0.0820	1

notypic characteristics of indigenous cattle (Tilakaratne, 1980 and 1984).

Genetic diversity analysis, based on genotyping of individuals using 15 microsatellite markers (**Table 3**), indicated that indigenous cattle in Sri Lanka have a high genetic diversity with the average number of alleles per locus ranging from 7.9 to 8.5. The average heterozygosity of different regions varied within a narrow range (0.72 ± 0.04 to 0.76 ± 0.03). As shown in **Table 4**, the genetic distances (Da distance) between regions were low (ranging between 0.085 and 0.066), suggesting a similar mixture of genotypes across regions despite the geographical isolation. However, two genetic clusters were visible though no relationship of those clusters was found between their distribution in different regions. The comparison of indigenous cattle in Sri Lanka with two other indigenous cattle types (KEC and ZMB) revealed that the indigenous cattle in Sri Lanka are genetically distinct (**Figure 4**). Introgression of Taurine cattle was evidenced in one of the cattle populations (NC) as suggested by the Y-specific microsatellite DNA analysis.

CONCLUSIONS

Indigenous cattle in Sri Lanka are reared in low input mixed crop farming systems and serve a variety of purposes. Based on the phenotypic evaluations, the Sri Lankan indigenous cattle could be described as a distinct population consisting of small compact non-specialised animals with little regional variation. Molecular investigations revealed high levels of diversity within populations and a predominant Zebu origin with subsequent introgression of Taurine cattle.

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Potential of Tropical Plants to Exert Defaunating Effects on the Rumen and to Reduce Methane Production

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ABSTRACT

This paper summarises the principal results obtained in Cuba concerning the potential of different tropical plants to exert defaunating effects in the rumen and to reduce methane (CH_4) production. The plants studied were *Sapindus saponaria, Morus alba, Trichanthera gigantea, Tithonia diversifolia, Gliricidia sepium Leucaena leucocephala, Stizolobium aterrimun* and *Arachis pintoi*. Grasses used as forage in the assays to obtain grass:foliage mixtures were *Pennisetum purpureum* Cuba CT-115 or Star grass (*Cynodon nlemfuensis*). The experiments were conducted using an *in vitro* system. Gases produced in the fermentation process were collected at intervals of 4, 8, 12 and 24 h and CH_4 production was determined by gas chromatography. Phytochemical analyses indicated the presence of tannins, saponins and others secondary compounds. *Enterolobium* and *Leucaena* had a high content of tannins and moderate levels of saponins. *Morus* contained moderate amounts of saponins. The inclusion of 15% *Leucaena* and *Gliricidia*, 20% *Sapindus* and *Arachis* as well as 40% *S. aterrimum*, negatively affected protozoal populations. The inclusion of 25% *Sapindus, Morus* and *Trichantera* foliages using *P. purpureum* as the pasture base reduced CH_4 production significantly. The results suggest that the use of trees and shrubs to supplement low quality forages seems appropriate for reducing CH_4 production and improving animal nutrition in tropical areas.

Key words: *tropical plants, defaunating effects, methane, phytochemical analysis, tannins, saponins.*

INTRODUCTION

The tropical zone includes 37% of plant lands. Many domestic ruminants live in this environment and their principal sources of feed are low quality pastures and forages with little or no supplementation. The efficiency of digestion in the rumen requires a diet that contains essential nutrients for fermentative micro-organisms. Lack of or deficiencies in these nutrients decreases animal productivity and raises CH_4 emissions/unit of product.

Methane emitted from livestock not only represents a loss of between two and 12% of the gross energy consumed by the animal (Johnson and Johnson, 1995), but contributes to global warming because it is a greenhouse gas (Bauchemin et al., 2008). Thus, mitigating CH_4 losses from cattle has both long-term environmental and short-term economic benefits.

Numerous strategies have been developed to reduce ruminal methanogenesis by means of chemical (Itabashi et al., 2000), and biotechnological methods (McAllister et al., 1996; Attwood and McSweeney, 2008) as well as by nutritional management, including through supplementation. It is well established that inclusion of concentrates in the diet reduces the proportion of dietary energy converted to CH_4 and improves animal performance (Bauchemin et al., 2008). However, the use of concentrates to mitigate CH_4 emissions from livestock is impossible to implement in many areas of the world due to the high price of cereals on the market and its competition with human feed.

There are naturally occurring compounds in some forages that appear to have anti-methanogenic properties, specially tannins and saponins. The use of trees and shrubs as ruminal manipulators, mainly to reduce protozoal population has been reported (Navas-Camacho et al., 1994; Hess et al., 2003; Galindo et al., 2005).

The enormous biological diversity within tropical trees and shrubs provides a bank of materials that can be used in animal feeding to improve productive efficiency and also as future strategies for CH_4 mitigation. The use of these plants in agro forestry or sylvopastoral systems could be an economically viable strategy in Cuba. Here, trees and shrubs have been evaluated with the objective of determining their nutritive value and effects on ruminal microbial fermentation and CH_4 mitigation, and other new plants are now being evaluated for these purposes under *in vitro* and *in vivo* conditions.

This paper summarises the principal results obtained in Cuba concerning the potential of different tropical plants for use as animal feeds, and regarding their possibilities for exerting defaunating effects on the rumen and reducing CH_4 production.

MATERIALS AND METHODS

Locations, Plants and their Preparation

The studies were carried out in areas around the Instituto de Ciencia Animal which is located at a northern latitude of 22° 53´ and a western longitude of 82° 02´, and at 92 m above sea level. The plants studied were *Sapindus saponaria, Morus alba, Trichanthera gigantea, Tithonia diversifolia, Gliricidia sepium Leucaena leucocephala, Stizolobium aterrimun* and *Arachis pintoi.* For the preparation of plant foliages, leaves with petioles and young stems were collected in a manner simulating animal selection according to the methodology of Paterson et al. (1983). The grasses used as forage to obtain mixes of grass:foliages were *Pennisetum purpureum* Cuba CT-115 and four-

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month old established or mature Star grass (*Cynodon nlemfuensis*). The foliages and grass forage were sun dried on a plate for 2–3 d until their humidity was reduced to between 20% and 25%; they were then ground to obtain a particle size of 1 mm.

Screening for Secondary Metabolites

Phytochemical screening was carried out and the presence of secondary metabolites detected as described by Rondina (1969). The experimental procedure was as follows: dry and milled material was macerated for 48 h in ethanol and refluxed for 4 h., which enabled separation into a part (Fraction A) with the remainder being evaporated to dryness. The dry extract was then diluted with HCl and the insoluble fraction diluted with dichloromethane; this constituted Fraction B. The acid solution was then neutralised with NH_4OH and extracted with dichloromethane, the organic phase constituting Fraction C. The liquid phase was extracted with a mixture of dichloromethane-ethanol, the solid phase constituting Fraction D and the liquid phase Fraction E. Starting with the raw material, Fraction F was obtained by extraction with boiling water.

The assays used to detect the secondary metabolites were:

- *Tannins:* in Fraction A by means of the Jello test
- *Triterpenoides and steroids*: in Fractions B. C and D by means of the Lieberman Bourchard reaction
- *Alkaloids*: in Fractions C and D using the Mayer and Dragendorff tests
- *Flavinoids*: in Fractions D and E, detected by the Shinoda reaction
- *Leucoantocianidins and catequins*: in Fractions D and E by means of the Rosenheim test
- *Saponins:* in Fraction F, by the foam test
- *Total polyphenols and condensed tannins (CT)*: condensed tannins were quantified according Terrill et al. (1992), and total polyphenols by the methodology of Makkar and Goodchild (1996).

Microbiological Studies

The effects of the most promising trees and shrubs on ruminal microbial fermentation were conducted by the technique of submerged tissue culture with mechanical agitation at 39 °C. Fermentation tubes with a capacity of 250 mL were placed in incubation baths. In each tube, 1.5 g of previously dried material was added containing Star grass (*Cynodon nlemfuensis*), the selected plant and 150 mL of filtered rumen liquid from two Holstein bulls fed Star grass and fitted with a simple rumen cannula.

The effect of condensed tannins on the ruminal protozoa population was similarly studied. The treatments consisted of: i) Star grass + ground maize and ii) Star grass + ground maize and condensed tannins (CT). Star grass was included at a rate of 0.75 g DM/tube and the maize at 0.25 g DM/tube. The amount of CT was equivalent to 5 mg/100 g DM. The condensed tannins were isolated from *L. leucocephala* foliage. Sampling of ruminal liquid were carried out before and at two and four h and the experiment was replicated eight times.

Effects on Methane Production

Seven treatments were studied: T1: grass forage (*Pennisetum purpureum* clon Cuba CT-115; T2: *Trichanthera gigantea* foliage, T3: *Sapindus saponaria* foliage; T4: *Morus alba* foliage; T5, T6 and T7 were foliage:grass mixes at ratios of 25:75 of *Trichanthera:Pennisetum, Sapindus:Pennisetum* and *Morus:Pennisetum*, respectively.

The technique of *in vitro* gas production (Theodorou et al., 1994), used Menke media (Menke and Steingass, 1988). Samples (500 mg ground feed) were added to 30 mL buffer/rumen fluid mixture (10 mL rumen/20 mL buffer) bottles of 100 mL (four replicates per treatment), sealed in anaerobic conditions and incubated in a water bath at 39 °C. Rumen fluid from two rumen-fistulated crossbreed Zebu steers fed on low quality Star grass and one kg of concentrate was strained before the morning feed through polyester cloth. Ruminal fluid was maintained under O_2-free CO_2.

The mixture of gases from the fermentation process was collected by displacing the volume in 20 mL syringes at intervals of 4, 8, 12 and 24 h. The gaseous content of the syringes was injected into a gas chromatograph, and the percentage CH_4 calculated by reference to a standard sample (purity 100%). Methane concentrations were then calculated by the general gases equation.

Microbial Analysis

Total viable and cellulolytic bacteria and cellulolytic fungi were counted according to the anaerobic technique of Hungate (1950). The culture medium of Caldwell and Bryant (1966) modified by Elías (1971) was used for total viable and cellulolytic bacteria. The culture medium for fungi included 100 000 IU penicillin and 0.1 g of streptomycin /100 mL of culture media. Protozoa were counted using a microscope after being preserved in 10% formaldehyde.

Statistical Analysis

Data were analysed using the SPSS system for Windows by a simple classification model for a completely random design. Differences between means were detected by the Duncan test (1955). In the studies of the effect of foliages on microbial populations, a factorial design was used to evaluate treatments.

RESULTS

The chemical composition of the plants evaluated is shown in **Table 1**. Protein levels of foliages ranged between 10.5% and 28.5%, the average being 20.8% which is higher than those observed in most tropical grasses. *Arachis* was the foliage with the lowest crude protein (CP) and highest neutral detergent fibre (NDF) content.

The phytochemical analysis (**Table 2**) indicated the presence of secondary compounds. All plants had variable amounts of tannins, saponins and other metabolites. *Enterolobium, Leucaena* and *Stizolobium* showed high content of tannins and medium levels of saponins. *Morus alba* showed high levels of saponins and triterpenes, while the content of secondary metabolites in *Tithonia* and *Gliricidia* was not very high.

The content of total polyphenols and condensed tannins in foliages (**Table 3**) shows the higher values of these compounds (5.84% and 1.80%, respectively) in *Leucaena*, while *Sapindus* was the plant with lowest values of condensed tannins.

In other assays, information from previous studies on these plants was used to establish the relationship between the concentration of condensed tannins and total polyphenols and the population of ruminal protozoa. It was found that there was a quadratic relationship between these variables:

Total polyphenols/protozoa: $y = 7.1915 – 171.72\ X + 2137.8\ X^2$, $R^2 = 0.99$

Condensed tannins/protozoa: $y = 45.523 – 1004\ X + 5921.3\ X^2$, $R^2 = 0.99$.

Table 1. Chemical composition plants (% DM).

Plant	OM	CP	NDF	ADF	Lignin
L. leucocephala	90.5	27.69	47.37	33.86	8.30
G. sepium	93.7	28.5	40.40	20.10	4.40
S. saponaria	83.78	16.3	50.90	33.2	9.40
E.cyclocarpum	88.20	14.8	55.80	35.80	8.30
S. aterrimum	92.42	25.69	46.33	29.18	—
A. pintoi	87.47	10.57	58.75	43.32	8.27
T. gigantea	78.15	19.83	45.34	28.37	6.11
M. alba	88.80	19.51	31.76	27.15	7.65
T. diversifolia	94.06	23.95	33.43	29.54	—
Mean	88.56	20.76	45.51	31.27	7.36
S.D.	5.09	5.81	9.16	6.05	1.56

Table 2. The phytochemical screening of plants.

Plants foliage	Tannins	Flavonoids	Saponins	Triterpenes	Esteroids	Antho-cianidins	Alcaloids
L. leucocephala	+++*	+	++	++	++	+	+++
Arachis pintoi	+	+	++	+	+	+	+++
S. aterrimum	+++	+	+	+	+	+	+++
E. cyclocarpum	+++	+	++	+	+	+	++
S. saponaria	++	—	+	+++	—	—	++
Gliricidia sepium	+	+	++	+	+	+	—
T. gigantea	++	—	—	++	ND	—	+
Morus alba	++	—	+++	+++	ND	—	+
Tithonia diversifolia	++	++	+	+	++	+	++

ND — not detected.

*+ indicates qualitatively the level of metabolite in the sample.

Table 3. Content of total polyphenols and condensed tannins (% DM).

Plants	Total polyphenols (%)	Condensed tannins (%)
L. leucocephala	5.84	1.80
G. sepium	1.92	0.30
S. saponaria	0.46	0.18
E. cyclocarpum	1.37	1.49
S. aterrimum	2.14	2.30
A. pintoi	1.18	1.19

Effect of Some Legume Trees and Shrubs on Ruminal Microbial Populations

Supplementation of low quality grasses (Star grass or *Pennisetum*) with levels ranging from 2.5% to 60% of DM of different tropical foliages produced a marked defaunating effect, as well as promoting a greater population of bacteria and ruminal cellulolytic fungi, which may increase ruminal cellulolysis, the degradation of the fibrous fraction and consequently feed intake (**Table 4**). Unexpectedly, *Enterolobium* did not exert defaunating effects at least under the experimental conditions in which the studies were conducted, but increased the ruminal cellulolytic fungi population.

Supplementing the diet with 20% *Tithonia diversifolia* reduced the methanogenic bacteria and protozoa populations. In addition, *S. saponaria* at a rate of 2.5, 5 and 10% of the DM in forage diets, did not increase cellulolytic bacteria populations, but activated viable cellulolytic fungi, leading to a significant reduction in protozoa.

Levels of substituting *L. leucocephala* for grass at up to 60% increased populations of ruminal cellulolytic bacteria from 2.92×10^7 to 6.83×10^7 cfu/mL. Likewise, this increased the population of total

Table 4. Effect of different levels of tropical foliages on ruminal microbial population.

Foliage/Star grass rate, % DM	Cellulolytic bacteria	Protozoa	Cellulolytic fungi	Methanogenic bacteria
L. leucocephala	10^6 cfu/mL	10^6 n/mL	10^6 cfu/mL	
0/100	0.46 (2.92) [a]	1.67 (46.4) [a]	1.54 (34.5)[a]	—
20/80	0.56 (3.67) [b]	1.66 (45.7)[b]	1.67 (47.3)[a]	—
60/40	0.83 (6.83) [c]	1.73 (53.4)[b]	1.99 (99.1)[b]	—
SE ±	0.28***	1.4***	4.7*	—
T. diversifolia[1]	10^6 cfu/mL	10^5 n/mL	10^6 cfu/mL	10^9 cfu/mL
0/100	1.40 (24.9)[a]	0.57 (3.75)[a]	1.42 (26.1)	1.65 (45.2)[a]
10/90	1.75 (55.8)[b]	0.51 (3.25)[a]	1.46 (28.9)	1.44 (27.5)[b]
20/80	1. 46 (29.2)[a]	0.18 (1.5)[b]	1.40 (25.2)	1.22 (16.8)[c]
SE ±	0.12*	0.02*	0.15	0.45**
G. sepium[1]	10^6 cfu/mL	10^5 n/mL	10^5 cfu/mL	—
0/100	0.8 [a] (6.3)	1.70[c] (45.7)	0.90[a] (7.4)	—
15/85	0.9 [a] (7.9)	1.05[b] (11.2)	0.92[a] (8.3)	—
30/70	1.14 [b] (13.9)	0.41[a] (2.6)	1.19[b] (15.5)	—
SE ±	0.14*	0.09***	0.3*	
E. cyclocarpum[1]	10^5 cfu/mL	10^5 n/mL	10^6 cfu/mL	
0 /100	0.41(4.49)	0.59(3.95)	0.24[a] (5.26)	—
15/85	0.58 (9.13)	0.56(3.62)	0.89[b](12.30)	—
20/80	0.67 (7.88)	0.69(4.99)	0.69[a](4.99)	—
SE ±	0.04	0.04	0.19*	
S. saponaria[1]	10^6 cfu/mL	10^6 n/mL	10^6 ftu/mL	
0/100	0.74 (5.50)	0.86[a] (7.24)	0.69[b] (4.90)	
2.5/97.5	0.66 (4.58)	0.73[b] (5.31)	0.76[ab] (5.75)	—
5/95	0.47 (2.95)	0.65[bc] (4.47)	1.05[a] (11.22)	—
10/90	0.69 (4.90)	0.58[c] (3.80)	1.07[a] (11.95)	—
SE ±	0.14	0.04***	0.11*	—
S. aterrimum[1]	10^7 cfu/mL	10^6 n/mL	10^7 cfu/mL	
0/100	1.55 (35.5)	1.52 (33.4)	0.76 (5.8)	—
20/80	1.39 (24.6)	1.19 (15.7)	0.97(9.3)	—
40/60	1.01 (10.2)	1.05 (11.2)	1.024 (18.6)	—
A. pintoi[1]	10^7 cfu/mL	10^6 n/mL	10^7 cfu/mL	
0/100	0.64 (4.4)	0.90 (8.0)	0.92 (8.3)	—
20/80	0.62 (4.2)	0.97 (9.3)	0.58 (3.8)	—
40/60	0.66 (4.6)	1.00 (10.1)	0.99 (9.8)	—

[a, b,c.] Means with different letters between columns differ at $P < 0.05$ (Duncan, 1955).

[1] Data transformed according to $\log\sqrt{x}$ where x = total colony count. Original values between parentheses.

* ($P < 0.05$); (** $P < 0.01$); (***$P < 0.001$).

viable bacteria and cellulolytic fungi in the rumen. The presence of this plant in the feed reduced ($P < 0.001$) the protozoal population.

S. aterrimum is another plant that negatively affects the population of protozoa in the rumen. The inclusion of this legume reduced the population of ruminal protozoa in proportion to its level of inclusion in the diet. Also, cellulolytic bacteria were affected negatively. *Arachis pintoi*, on the other hand, did not modify ruminal bacteria populations, but it also affected the populations of total protozoa.

Effect of Condensed Tannins from *L. leucocephala* on Populations of Ruminal Protozoa

A study was conducted to evaluate the effect of condensed tannins extracted from leaves and petioles of *L. leucocephala* on the population of ruminal protozoa. The results (**Table 5**) suggest that tannins exert a reducing effect on total populations and that both *Entodiniomorphid* and *Holotrichia* protozoa are modified in the presence of these metabolites.

Table 5. Effect of the condensed tannins extracted from *L. leucocephala* on the population of ruminal protozoa (10^6 cells/mL).

Treatments	Control (time in h)			Condensed tannins (time in h)		
	0 h	2 h	4 h	0 h	2 h	4 h
Total protozoa	64.75	81.0	126.5	62.5	31.0	24.5
Entodiniomorphid	62.63	77.38	120.0	60.50	29.63	23.63
Holotrichia	2.38	4.13	6.5	2.00	1.38	1.63

Table 6. Methane emissions and dry matter degradability in the experimental diets with tropical plant foliage and *Pennisetum purpureum* forage.

Treatments	Methane production		
	mL	mL/kg DM	g/kg DM
Pennisetum	13.48^{a}	26.18^{a}	16.96^{a}
Sapindus	7.32^{c}	14.01^{cd}	9.10^{de}
Trichantera	5.62^{c}	10.82^{d}	7.01^{e}
Morera	7.52^{c}	13.76^{cd}	8.93^{e}
Sapindus:forage 25:75	9.13^{c}	17.02^{bc}	11.37^{d}
Morus:forage 25:75	10.30^{ab}	19.10^{b}	12.40^{b}
Trich.:forage 25:75	8.02^{c}	18.02^{bc}	11.60^{d}
ES± Sign	1.20*	1.52**	0.94**

a, b, c, d. Means with different letters between columns differ P < 0.05 (Duncan, 1955).

* (P<0.05); ** (P<0.01).

When the effect of *Sapindus Saponaria, Morus alba* and *Trichanthera gigantea* on CH_4 production in *in vitro* conditions was evaluated, CH_4 production (mL/kg DM) produced by the grass was higher than that obtained with foliages or its mixes with *Pennisetum* (**Table 6**). Methane values for *Trichanthera*, *Sapindus, Morus* were, however, similar. Foliage/*Pennisetum* mixtures produced more CH_4 than foliages alone, but values were lower than for grass, showing the anti-methanogenic effects of these plants.

DISCUSSION

To develop new feeding strategies with the aim of mitigating CH_4 emissions to environment and to improve the productivity of ruminants, the most promising approaches are those which suppress the microbes involved in methanogenesis without affecting fibre-degrading bacteria (Soliva et al., 2003).

In tropical conditions, there are many possibilities available for manipulating the rumen microbial ecosystem to achieve CH_4 reductions using trees and shrubs with high nutritive value and naturally secondary compounds, principally tannins and saponins that appear to have defaunants and antimethanogenic properties. The use of these plants in feeding strategies could be advantageous and cheaper if appropriate technologies are developed.

In general, tannins and saponins were found in all the foliages studied. These suppress CH_4 emissions, reduce rumen protozoa counts, and change rumen fermentation patterns (Hristov et al., 1999; Hess et al., 2003; Galindo, 2004; Hu et al., 2005).

When the correlation between polyphenols and condensed tannins present in tropical plants and protozoal population were studied, it was found that there was a quadratic relationship between them. Galindo (unpublished data) found inverse relationships between the methanogenic population of ruminal bacteria and protozoal populations. The presence of these compounds in most foliages evaluated could explain the effects of reducing rumen protozoa counts and suppressing the CH_4 emissions recorded in the plants studied.

The polyphenolic fraction in *Leucaena* was greater than in other foliages (**Table 3**). Garcia et al. (2009) characterised the chemical composition of the foliage of 53 Cuban accessions from the *Leucaena* genus and found that the polyphenolic fraction ranged between 3.4% and 5.02% DM, i.e. lower than the lowest measured in this study (5.82%). However, the condensed tannins fraction reported by the same authors was higher than that obtained here (3.78% vs 1.80%, respectively). Different experimental conditions, plant species, chemical structures of the compounds, their biological activity, analytical methods used and other factors could all explain these differences, and clearly further work is required to explain such variations.

Supplementing low quality forage with *Gliricidia, Leucaena, Thitonia* and *Stizolobium* produced a marked defaunating effect as well as encouraging a larger population of bacteria and ruminal cellulolytic fungi. Such effects may increase ruminal cellulolysis and fibre degradation, and consequently could reduce CH_4 emissions. It is interesting to note that most plants reduced protozoal counts, increasing the cellulolytic microorganisms responsible for fibre degradation.

Leucaena is possibly one of most used plants for animal feed in Cuba. It is used as a protein biomass bank and in sylvopastoral and agroforestry systems with good results (Alvarez et al., 2006). In the present studies, *Leucaena* foliage had the highest levels of polyphenols and tannins. The condensed tannins extract exerts a decreasing effect on total rumen protozoa populations. Both protozoa *Entodiniomorphid* and the *Holotrichia* were modified in presence of condensed tannins, which proves, once more, that these plant metabolites act as defaunating agents though death of these microorganisms. Recent studies carried out in grassland of *L. leu-*

cocephala in association with natural grass mixtures indicated that ruminal protozoa were reduced in cattle when this tree was included in the system, independently of inclusion level (30% or 100% of the area, Galindo et al., 2007).

Methane production decreased with the inclusion of foliages in the diets. There is some experience with the use of plant foliages as defaunants, but results concerning effects on CH_4 production are scarce, although Woodward et al. (2001) found a depression of CH_4 emissions in sheep and dairy cows fed with the condensed tannin-containing legume *Lotus corniculatus*. Also, Soliva et al. (2003) reported that *S. saponaria* (fruits) reduced CH_4 emissions and counts of ciliate protozoa and that all three foliages studied (*Trichanthera, Sapindus* and *Morus alba)* reduced such emissions relative to grass forage when included in the diet at the rate of 25% DM.

CONCLUSIONS

The results suggest that as strategies of supplementation of low quality forage, the use of trees and shrubs seems be an adequate option to reduce CH_4 production and improve animal nutrition in tropical areas. With the exception of *Arachis* and *Enterolobium*, the plants examined here showed defaunating properties and had the potential to mitigate CH_4 emissions.

The contribution of such plants to improve the efficiency of the rumen and reduce CH_4 emissions is an attractive area of study and while their potential has been demonstrated in the laboratory, it is necessary to verify this in livestock production systems.

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Evaluation of Spineless Cactus (*Opuntia ficus-indicus*) as an Alternative Feed And Water Source for Animals during the Dry Season in Eritrea

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ABSTRACT

Throughout East Africa, animal feed resources fluctuate seasonally and are often of limited availability. Finding alternative feed resources that can sustain animal production during the long dry season is an essential need. Cactus is a drought-tolerant and succulent feed resource available throughout the year in Eritrea. This study was conducted to evaluate the effect of including increasing levels of spineless cactus in the diet of sheep fed urea-treated barley straw. Twenty four fat tailed Highland male sheep with mean live weight of 21.1 kg were randomly assigned to four treatments (T1-T4). Animals in T1 received urea (5%) -treated barley straw (UTBS) alone *ad libitum*, while those in T, T3 and T4 received *ad libitum* UTBS supplemented with 175 g, 350 g and 525 g of spineless cactus (dry matter [DM] basis), respectively. With increasing level of cactus, there were significant increases in DM intake ($P < 0.001$) and bodyweight (BWt) gain ($P < 0.05$), while water consumption decreased ($P < 0.001$). The highest DMI was found in the last two treatments (101.8 g/kg $BWt^{0.75}$d and 96.5 g/kg $BWt^{0.75}$d, respectively) as compared with the first two treatments (94.4 g/kg $BWt^{0.75}$d and 87.6 g/kg $BWt^{0.75}$d). Water intake was significantly decreased with the progressive increase in cactus intake. The highest BWt gain (51.9 g/d) was found when sheep received 350 g DM of cactus (T3), while the lowest was in the control diet (26.8 g/d). The metabolism data demonstrated that available energy intake (TDNI) was directly related to animal performance. In conclusion, feeding cactus with UTBS can significantly increase animal performance and feed intake, and reduced water intake.

Key words: *spineless cactus, urea-treated barley straw, feed intake, water intake, body weight gain.*

INTRODUCTION

Animal feed and water shortages are among the main constraints for the livestock sector in arid and semi-arid regions of East Africa. The major feed resources come from the rangeland pasture and crop residue, the quality and availability of which decreases rapidly following the rainy season. This fluctuating pattern of animal feed supply results in a pattern of gain and loss in animal growth and performance. In a country like Eritrea where feed shortage is such a serious problem, utilisation of multipurpose trees and shrubs that can cope with low and erratic rain fall, high temperature, poor soils, and required low energy inputs can serve as an alternative strategy to reduce the chronic animal feed and water shortage. Spineless cactus (*Opuntia ficus-indica*) possesses important characteristics for animal feed in drought-prone regions. This includes high DM yields, drought tolerance, nutritive value and palatability for animals (Tegegne, 2001). Spineless cactus is a fast growing xerophytic plant. Cactus has high water use efficiency due to its crassulacean acid metabolism (CAM) photosynthetic pathway (Nobel, 1995). This makes cacti an extremely important fodder in water-scarce semi-arid regions (Felker and Inglese, 2003). Cactus is a naturalised plant in Eritrea being well adapted to marginal land with poor soil fertility and the low, erratic rainfall conditions. Cactus remains succulent during the long dry season and can serve the animal as a source of feed and water during this period. Furthermore, cactus is suitable as human food, as fuel, for medical uses, as bee forage, and in rangeland rehabilitation projects (Barbera et al., 1995). Cactus pear plays a key role as a lifesaving feed both for human and animals especially in time of drought.

In Eritrea, the use of cactus for animal feed is currently limited to grazing and during the dry season. Cut and carry of cactus is practised during drought periods, but is not common. The cactus peels waste and surplus fruit contribute a substantial amount of feed to ruminants, especially to peri-urban dairy cattle

Although cactus pears have great potential in promoting a sustainable animal production system, knowledge in Eritrea is limited concerning its nutritive value, its utilisation as animal feed, and its role in animal performance. Farmers usually report that their animals get diarrhoea when fed high level of cactus during the dry season. Therefore the aim of this research was to assess the potential of spineless cactus as an alternative source of feed and water for ruminant animals fed poor quality roughage during the dry season.

MATERIAL AND METHODS

The experiment was carried out in the highlands of Eritrea, which have a semi-arid climate. A randomised complete block design was used to allocate 24 fat tailed Highland male sheep with initial mean live weight of 21.1 kg into one of two replications for four feed treatment groups (T1-T4), consisting of six animals per group. Animals in T1 received *ad libitum* amounts of urea (5% by weight) -treated bar-

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N.E. Odongo, M. Garcia & G.J. Viljoen (eds), Sustainable Improvement of Animal Production and Health. Food and Agriculture Organization of the United Nations, Rome, 2010: **55–58**

ley straw alone, while those in T, T3 and T4 received *ad libitum* urea-treated barley straw supplemented daily with 175 g, 350 g and 525 g of spineless cactus (DM basis), respectively. Diets were offered twice daily, aiming at 20% refusals. At the end of the feeding trial, four sheep were transferred to metabolic crates where digestibility trials were conducted for each diet over seven days of feeding and collecting. Data were analysed using standard analysis of variance (ANOVA) and general linear models (GLM) with GENSTAT statistical software.

RESULTS

Chemical Composition of Spineless Cactus Cladode

The chemical composition (proximate analysis) of the spineless cactus and urea-treated barley straw is presented in **Table 1** Spineless cactus cladodes were high in water and ash, but low in crude protein and crude fibre content. In this analysis, cactus had 65% more digestible energy than urea-treated straw.

Effect of Supplementation on Feed, Nutrient and Water Intakes

The performance characteristics of sheep fed urea-treated barley straw supplemented with increasing level of spineless cactus are presented in **Table 2**. The amount of cactus in this study was restricted; however, it was a highly palatable feed. Except for T, there was a highly significant ($P < 0.001$) increase in DMI with increasing spineless cactus levels in the diet (94.35, 87.57, 101.81 and 96.48 g/kg $BWt^{0.75}$d, for T1, T, T3 and T4, respectively), and as expected a comparable reduction in straw DMI. The highest DMI expressed on a metabolic weight basis (102g/kg $BWt^{0.75}$d) was found in sheep that received 350 g spineless cactus, and this was highly significantly higher ($P < 0.001$) than with the other treatments.

The crude protein intake of the animals was lower for T2 than for the either T1 or T3. When compared with the estimated NRC (1985) requirements (**Table 3**), these animals may have lacked an adequate protein intake.

Increasing the level (or proportion) of spineless cactus in the diet increased the energy density of the diet. Therefore, supplementation of cactus tended to increase energy intake (total digestible nutrient intake or digestible organic matter intake). In contrast, increasing the level (or proportion) of cactus decreased the CP density in the diet. Thus, supplementation of cactus tended to decrease CP intake. The DCPI was highest on diets T1 and T3, and lowest on diet T4.

Water consumption was significantly reduced ($P < 0.001$) with increasing intake of cactus (**Table 2**). Sheep in T1 consumed more water (2 L/d) than the other treatments (0.85, 0.51, 0.15 L/d for T, T3 and T4, respectively). Compared with only urea-treated straw (T1),

Table 1. Chemical analysis composition of experimental feed ingredients.

Nutrients (%)	Diets	
	Spineless Cactus	Treated Straw
Dry matter (DM)	12.9	69.42.5
Analysis on DM basis		
Crude protein (CP)	4.75	10.2
Ash	16.77	7.3
Crude Fibre (CF)	15.85	46.5
Ether extract (EE)	0.88	1.1
Nitrogen free extract (NFE)	61.6	34.8
Gross energy (MJ/kg)	13.22	8.0

Table 2. Performance of sheep supplemented with increasing level of cactus.

Parameters	T1	T2	T3	T4	LSD	SE
Initial BWt (kg)	21.25	21.25	21.08	21.25	NS	-
Final BWt (kg)	23.67b	24.25b	25.67a	25.50a	0.83	0.26
Weight gain (g/d)	26.80b	33.30b	51.90a	47.20a	12.26	
Cactus DMI (g/d)	0	175	350	525	-	
Total DMI (g/day)	987.2c	905.2d	1080.1a	1062.2b	3.65	8.94
Total DMI (g/kg $BWt^{0.75}$d)	94.35b	87.57c	101.81a	96.48b	3.42	4.05
Water Intake (L/d)	1.98a	0.78b	0.57c	0.18d	0.03	0.04
DOMI (g)	541.80	504.80	667.80	656.30	-	
DCPI (g)	61.20	51.80	61.50	42.60	-	
TDNI (g)	542.00	588.20	672.00	663.10	-	

LSD — least significance difference; DOMI — digestible organic matter intake; DCPI — digestible crude protein intake; TDNI — total digestible nutrient intake; SE — standard error.

Means with different superscripts (^{a} – ^{d}) in the same row differ significantly ($P < 0.001$).

Table 3. Crude protein intakes (CPI in g/d) of sheep (20–25 kg weight) compared with estimated NRC (1985).requirements.

CPI determined in this study	Treatment	T1	T2	T3	T4
	Observed	97.80	82.79	98.28	68.08
CPI estimated from NRC (1985)	25g ADG	92.5	92.5	92.5	92.5
	50g ADG	100.5	100.5	100.5	100.5

ADG — average daily gain; CPI — crude protein intake.

sheep in T2 drank of 50% less water/kg feed intake and sheep in T4 approached no water consumption.

Effect of Supplementation on Animal Performance

The BWt gain was significantly ($P < 0.05$) higher for sheep on T3 and T4 compared with the control diet (T1). The highest gain (51.9 g/d) was found when sheep received 350 g DM of cactus (T3), while the lowest was in the control T1 diet (26.8 g/d). Although DMI of the sheep in the control was higher than that of sheep in T, sheep in the latter group performed better.

DISCUSSION

The high water and low CP content of spineless cactus cladodes found in this trial are similar to values reported by other authors (Nefzaoui and Ben Salem, 2001; Flachowsky and Yami, 1985) for cactus pear grown on poor soils. The protein content of cactus was below the general minimum of 7% CP required for normal microbial activity in the rumen. Therefore, animals fed with cactus-based diets need appropriate protein supplementation. This study confirms that urea treatment of low quality forage can be a suitable protein source. In semi-arid regions, where water is very scarce, the high water content of cactus can be considered as a benefit. To develop a feeding system using locally available feed resources, an abundant and cheap source of carbohydrate is very important (Preston and Leng, 1987). This makes spineless cactus highly valuable for its energy content (Felker, 1995).

Cactus is a highly palatable feedstuff and the higher total DM intake in T2 could be associated with the higher consumption and digestibility of cactus. In agreement with this result, Tegegne et al. (2005) found that total DM intake in sheep increased progressively from 77 kg $BWt^{0.75}$/d to 100g/kg $BWt^{0.75}$/d when cactus supplementation increases from zero to 60% in pasture and hay based diets. In the current study, the gradual decrease in DMI of urea-treated straw can be explained by the substitutive or associative effect of feed when replaced with a highly soluble source of carbohydrate. Such an effect was also reported by Njwe and Olubajo (1989) when they supplemented West African Dwarf goats fed fresh Guatemala grass (*Tripsacum laxum*) with increasing levels of cassava flower and groundnut cake. In this study, no digestive disturbances or ill health effects were observed even at the highest cactus inclusion rate which constituted about 50% of DMI. Previously, Ben Salem and coworkers (1996) observed that spineless cactus could be fed without any digestive disturbance to a level of up to 55% of the total DMI. This would help farmers to economise on their straw budget. The absence of a negative effect coupled with higher digestibility and water content would facilitate a rapid disappearance of cactus dry matter from the rumen (Nefzaoui and Ben Salem, 2001).

Water intake was high when sheep were fed urea-treated barley straw alone and this is in agreement with King (1983), who found that 2.2 L/d was consumed by sheep in East Africa. However, water intake decreased significantly with the progressive increase in cactus intake. This is consistent with pervious work (Ben Salem et al., 1996; Tegenge et al., 2005), reporting that water intake decreased significantly as the level of cactus intake increased in diets of low quality roughages. Sheep drank negligible amounts of water when cactus supplementation reached 525 g/d. In line with this finding, Ben Salem and coworkers (1996) indicated that sheep stop drinking water when cactus intake reached 600 g/d. In the tropics, the dry season is characterised by higher temperatures, decreased supply of water and higher herbage DM. Therefore, animals that are sustained on poor quality dry roughages require high amounts of water to facilitate digestion. Also, animals travel long distances to reach water points, spending more energy and losing BWt. During the drought season in East Africa, the distance traveled by small ruminants to watering points increased by between 43% and 52% (Ndikumana et al., 2002). Several authors have shown that spineless cactus can supply considerable water to the animals (Le Houerou, 1996; Sirohi et al., 1997). Also, De Kock (1980) reported that sheep could survive for up to 500 d without drinking water when allowed to consume unrestricted amounts of cactus. In countries where water is a vital resource during the dry season, the high water content of cactus could therefore play a significant role in mitigating drinking water shortage.

There was a significant improvement in BWt gain when urea-treated barley straw was supplemented with spineless cactus — a result in accordance with previous work in sheep (Tegegne et al., 2005) on supplementing urea-treated wheat straw fed with cactus. The higher performance of sheep in T3 and T4 as compared with those fed the control (T1) diet can be ascribed to the combined effects of the higher DMI of the sheep on the supplemented diets and the higher readily digestible carbohydrate content of spineless cactus. This result is in accordance with previous works (Tegegne et al., 2005; Tikabo et al., 2006) on supplementation of cactus to urea-treated crop residue fed sheep.

Although DMI in the control group (T1) was higher than in T, sheep in the latter group performed better. This could be attributed to the more readily fermentable carbohydrate intake associated with the inclusion of cactus in the latter group. The DM digestibility of urea-treated straw was higher when supplemented with cactus than when fed alone. Therefore, the value of cactus as a cheap source of energy for efficient utilisation of non-protein nitrogen is also important in improving the nutritive value of poor quality roughage. A 22% improvement in BWt was achieved in this study, which is quite significant since animals normally lose weight during the dry season although cactus is abundant and succulent in this season. However, a much greater improvement (72%) in BWt was reported when cactus is supplemented with by-pass protein source (Shoop et al., 1977), highlighting the importance of dealing with the deficiencies in nitrogen or CP content of cactus when developing feeding strategies.

The results of this experiment support previous work on using cactus as a cheap source of energy for efficient utilisation of non-protein nitrogen. Earlier, Shoop et al. (1977) suggested that the high level of soluble carbohydrates in cactus could be combined with ammonia- or urea-treated straw, as it could provide a readily available source of energy necessary for the efficient utilisation of the non-

protein nitrogen in the rumen. Tegenge et al. (2005) clearly showed that cactus pear could substitute for wheat bran at up to 40%, as long as it was combined with straw treated with urea.

The digestible nutrient intake data from the metabolism trial are in agreement with the feeding trail. Increasing spineless cactus supplementation of the urea-treated straw based diet increased the energy density of the diet (higher percentage TDN). Body weight gain was highly correlated with DM intake and its energy concentration; these results are consistent with the findings of others (Solaiman, et al., 1980; Moore et al., 1999;). The higher performance of sheep in T3 and T4 compared with those in T1 and T2 may be explained by the increased dietary energy made available through an associative effect i.e. the addition of cactus improves the total digestibility of the diet, including the digestibility of the straw. With increasing level of cactus in the diet, there was an increase in the energy concentration the diet, while the protein concentration decreased. Besides the slight improvement in performance of sheep in T, cactus supplementation at the lower level (175 g DM) did not result in a significant difference relative to the control diet (T1). This might be because, although T2 shows an improvement in energy, there is both a decreased percent dietary protein and lower daily digestible protein intake. In this case, the higher energy may not properly be utilised for better growth, because protein becomes limiting to animal performance.

Supplementation of 350 g DM spineless cactus (T3) seems to be the optimum supplementation rate. At this level, the animals are able to maintain protein intake, so that the animals can benefit from the extra energy and optimise growth. Therefore animals in T3 had a higher TDNI as compares with the other groups, illustrating a more optimal balance of protein and energy. Inclusion of cactus at a higher rate (T4) was still better than the first two treatments, indicating benefits from additional energy, but it may have begun to decrease due to the low protein intake.

CONCLUSIONS

Feeding cactus to sheep in combination with urea-treated barley straw can significantly increase feed intake and animal performance and reduce water intake. For diets based on urea-treated straw, the optimal inclusion rate for cactus is about a one third of the diet, or 350 g/d of cactus DM. Below this level, cactus reduced the protein content of the diet, so only a slight improvement was seen over urea-treated straw alone. Above this level, cactus could contribute a laxative effect to the diet, so that no further improvement was seen over the 350 g/d level. Therefore, utilisation of spineless cactus as an animal feed could play a vital role in promoting sustainable livestock production by providing an alternative feed as well as water for animals in dry areas.

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Effects of Supplementing Urine Treated Rice Straw with Concentrates on Productivity and Methane Emissions of Ongole Crossbred Cattle

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ABSTRACT

Cattle were fed rice straw treated with urine (1 kg urine/1 kg DM rice straw, and kept in the container for a week) ad libitum and a concentrate mixture (50:50) of wheat bran and beer cake. One group of cattle (C25, four animals) was fed the concentrate at 25% of estimated dry matter intake (DMI) at 3% of body weight, while the other four cattle (C75) received concentrate at 75% of estimated DMI for a period of six weeks. Daily DMI, methane (CH4) production and live weight gain (LWG) were measured. Dry matter intakes were similar in both groups (6.72 kg/d and 7.59 kg/d), but LWG of C75 (1.07 kg/d) was higher ($P < 0.05$) than that of C25 (0.47 kg/d). Methane production was similar in both groups (219.3 L/d vs 240.4 L/d and 29.29L/kg DMI vs 35.15 L/kg DMI for C25 and C75 respectively). However, when calculated per kg LWG, CH4 production was significantly lower ($P < 0.05$) than that of C25 (205.8 L/kg LWG and 967.2 L/kg LWG, respectively). The results suggest that better feeding not only increases productivity but also leads to significant mitigation of CH4 emissions. Feeding management should therefore be considered for controlling CH4 emissions in the animal industry.

Key words: *Ongole crossbreds, rice straw, supplementation, methane emissions.*

INTRODUCTION

Methane is a potent greenhouse gas, and therefore reducing emissions from livestock and enhancing animal production efficiency are effective strategies for mitigating global warming (IPCC, 1995). The production of ruminal CH_4 represents a pathway of carbon loss that reduces energetic efficiency. If the gross energy intake that is lost in generating CH_4 could be channeled into weight gain or milk production, the efficiency of production would improve (Howden and Reyenga, 1999). Manipulation of diets to enhance animal production (milk or meat) can reduce total CH_4 emissions into the atmosphere/unit of milk or meat produced (Johnson and Johnson, 1995; Kurihara et al., 1999). Therefore, the simplest strategy to improve animal production is improving feed quality (Leng, 1993).

Rice straw is the most abundant feedstuff in tropical countries but has low quality. It therefore needs to be improved and this can be achieved in several ways e.g. by ammonia treatment, through the fermentation process or by supplementation with better quality feeds. The first two methods need skillful farmers and are less amenable to adoption by small holder farmers than the last one. The easiest method to improve rice straw quality, especially its protein content is by urine treatment (Purnomoadi et al., 2005; unpublished). In that study conducted on buffalo, rice straw treated with urine increased feed intake but decreased chewing activity, suggesting that it would contribute to a better efficiency of energy utilisation for production. Supplementation with concentrates was reported to increase productivity (Dillon et al., 1997; Kennedy et al., 2003) as well as to reduce enteric CH4 production when conserved forages formed the basal ration (Moss and Givens 1995; Ferris et al., 1999; Purnomoadi et al., 2003). This showed that CH4 emissions from low quality tropical feedstuffs could be controlled by modified feeding management (Devendra, 1992).

Since most livestock in the world are raised by small holder farmers, efforts to improve feeding management should be based on local feed resources, including by-products of agricultural industries. Examples of such feedstuffs include beer cake that contains highly digestible fractions (Amari and Purnomoadi, 1996), and wheat bran which is the residue from wheat flour industries that contains highly soluble carbohydrates fraction. These feedstuffs could decrease CH_4 production (Zinn, 1994; Moe and Tyrell, 1979). This study investigated the effectiveness of by-product concentrate feedstuffs (a mixture of beer cake and wheat bran) in improving productivity and reducing CH_4 production in cattle.

MATERIALS AND METHODS

Experimental Animals

Eight cattle (average age 1.5 y, bodyweight [BWt] 240 kg) were fed rice straw treated with urine (1 kg urine/1 kg DM rice straw, and kept in the container for a week) and a 50:50 concentrate mixture of wheat bran and beer cake. The chemical composition of the feeds are presented in **Table 1**. The cattle were divided into two groups for feeding treatments. The first group (four animals) was allowed concentrate feed at 25% (C25 group) of estimated DMI at 3% BWt, while the other group was given concentrate feed at 75% (C75 group) of estimated DMI. Both groups were given rice straw treated with urine *ad libitum* and allowed free access to water. The cattle were fed the diets for six weeks, followed by a further two weeks during which time samples were collected for digestion studies and CH_4 measurements.

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N.E. Odongo, M. Garcia & G.J. Viljoen (eds), Sustainable Improvement of Animal Production and Health. Food and Agriculture Organization of the United Nations, Rome, 2010: **59–61**

Table 1. Chemical composition of feeds (%DM).

	OM	CP	EE	CF	NFE	GE, kJ/g
Rice straw	79.9	7.9	2.3	27.5	42.2	16.86
Concentrate*	94.3	20.6	6.3	14.7	50.2	20.68

OM — organic matter; CP — crude protein; EE — ether extract; CF — crude fibre; NFE — nitrogen free extract; GE — gross energy.

*Concentrate = 50:50 mixture of beer cake and wheat bran.

Experimental Parameters, Measurements and Data Analysis

Parameters measured were daily DMI, total digestible nutrients (TDN), daily CH_4 production and live weight gain (LWG). Dry matter intake was measured daily for six weeks by subtracting weights of feed offered and refused and multiplying by their DM contents which were determined by oven-drying at 135°C for 2 h. Crude protein intake was calculated by multiplying DMI with CP content in feed, while the TDN was determined from 7 d total collections. Liveweight gain was measured before and after six weeks of feeding the cattle on their respective diets

Methane measurement was done using the facemask method which was performed for 10 min at 3-h intervals over 2 d, following the description by Purnomoadi et al. (2003). In this method, a mask is connected to a CH_4 analyser (infra-red gas analyser, VIA-510, Horiba Ltd., Japan) equipped with an airflow meter (STEC SEF-6470, Horiba Ltd., Japan) for measuring total air volume (L/min). Data on CH_4 concentration and airflow were averaged and recorded automatically every 3 sec using a computer. The 2 d CH_4 production (L/d) measured was averaged to obtain daily CH_4 production.

Data were analysed using the *t*-test (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Feeding and Animal Production

Daily DMI, nutrient intake and LWG are presented in **Table 2**. Feeding the higher level of concentrate (C75) resulted in higher LWG (P = 0.0145), although DMI and DM digestibility were similar in both treatments. The better LWG of C75 than C25 resulted from the better nutrient intake in C75, especially protein and TDN (which fulfilled energy requirements). Protein intake in C75 was higher (P = 0.0006) than in C25 (1.28 kg/d and 0.90 kg/d, respectively). Similarly, TDN intake in C75 was higher (P = 0.0008) than that in C25 (3.39 kg/d and 2.20 kg/d, respectively). The high LWG in C75 (1.11 kg/d) was higher than that mostly reported for Ongole crossbred cattle (range 0.4 kg/d – 0.7 kg/d) under various feeding regimes with CP content up to 15% (Arifin et al., 1998; Amini, 1998; Purnomoadi et al., 2007).

Table 2. Daily dry matter intake, nutrients intake, and live weight gain.

	C25	C75	P
Initial LWt (kg)	281.1	288.4	
LWG (kg/d)	0.31	1.11	0.0145 *
DMI (kg/d)	6.72	7.59	0.1933
Rice straw (kg/d)	3.54	1.95	0.0003 **
Concentrate (kg/d)	3.18	5.64	0.00003 **
Nutrients intake			
CP (kg/d)	0.90	1.28	0.0006 **
Digestible CP (kg/d)	0.63	0.96	0.0002 **
TDN (kg/d)	2.20	3.39	0.0008 **
DM digestibility (%)	46.54	54.01	0.1112
TDN (%)	32.8	44.7	0.0083 **

* P < 0.05; **P < 0.01.

Table 3. Methane Production.

	C25	C75	P
Methane production			
L/d	240.42	219.34	0.6537
L/kg DMI	35.15	29.29	0.3524
L/kg LWG	967.17	205.77	0.0186 *
MJ/ kg LWG	13.72	6.21	0.0324 *

* P < 0.05.

Table 4. Methane production and the predicted value by Shibata's equation (1993) and Kurihara's prediction (1995) for hot conditions.

Treatments	Measured (L/d)	Predicted value (L/d)	
		Shibata	Kurihara
C25	240	237	243
C75	219	265	266

Methane Production

Daily total CH4 production (L/d) was similar in C75 and C25 (**Table 3**). This was an unexpected result. Generally, highly digestible diets yield lower emissions than poor quality diets (Johnson and Johnson, 1995). This phenomenon might be explained by the effect of rice straw which is a highly fibrous and poorly digested feed, resulting in a longer retention time in gastrointestinal tract, and hence greater CH4 production arising from fermentation. At higher levels of concentrate supplementation, microbial fermentation of straw might be activated resulting in a shorter retention time. However, this shorter retention time would not result in lower CH_4 production, since rumen distension lower it will stimulate animal to eat. This agrees with the statement of Shibata et al. (1993) that CH_4 production is highly correlated with DMI. In this experiment, the DMI was also correlated with DMI. The higher LWG in C75 was associated with lower (P = 0.0186) CH_4 production/kg LWG than in C25 (205.8 L/kg LWG vs 967.2 L/kg LWG respectively).

This study showed that supplementing a low quality diet such as rice straw with a high quality concentrate did not lower CH_4 production quantitatively, but if animal productivity such as LWG was taken into account, the level of mitigation achieved was significant. These results are similar to those obtained in our previous study (Purnomoadi et al., 2003) using soybean pulp to supplement Napier grass hay.

Measurement of CH_4 production in developing countries is lacking due to the high cost of equipment. Thus, prediction equations have been established such as those by Shibata et al. (1993) and by Kurihara et al. (1995) which were developed using mainly dairy cattle in Japan. Shibata's equation is $Y = -17.766 + 42.793X - 0.849X^2$;

where Y is CH_4 production (L/d), and x is DMI (kg/d) was established for thermoneutral conditions, while Kurihara's (Y=63.27+0.02678X; where Y is CH_4 production (L/d), and x is DMI (g/d)) was established for hot conditions (30 °C – 32 °C) and would presumably be more applicable to tropical areas like Indonesia. These equations were applied to predict CH_4 production in this study (**Table 4**). In comparison with the values measured, CH_4 production predicted by Shibata's differed by –3 L/d and +46 L/d for C25 and C75, while Kurihara's differed by +3 L/d and +47 L/d for C25 and C75, respectively. These results show that both equations more accurately predicted the C25 emissions than those associated with C75, suggesting that feed quality has an important influence on their predictive value.

CONCLUSION

A combination of rice straw treated with urine and wheat bran and beer cake can lead to significant productivity increases while mitigating CH_4 emissions from cattle in tropical climates.

ACKNOWLEDGEMENTS

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Effect of Supplementing Urea-Treated Sorghum Stover with Sesame Cake or Fishmeal on the Body Weight of Sheep and Cattle

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ABSTRACT

Feeding trials were carried out on-farm to examine the effect of supplementing urea-treated sorghum stover (UTSS) with sesame cake (SC) or fishmeal (FM) on the body weight (BWt) of sheep and cattle. Twenty one male sheep and nine Barka cattle were divided into three groups of seven sheep and three cattle in each trial. The trials were conducted at the same time, but on two different farms. The control diet consisted of UTSS fed *ad libitum* to both species of animals. The second and third diets were UTSS supplemented daily with SC or FM. The experimental period lasted for 90 d. Feed intakes and BWt were recorded regularly. The dry matter intake (DMI) in sheep was significantly different ($P < 0.05$) between the control and SC supplemented groups, but not between the other treatments. It was highest for the SC supplemented group at 847 g/head/d followed by the FM supplemented and control groups at 826 and 821 g/head/d., respectively. Sheep supplemented with SC had the highest ($P < 0.05$) body weight gain (BWtG) (134 g/head/d) followed by the group supplemented with FM (115 g/head/d). The controls had the lowest BWtG (66 g/head/d). In cattle, the group supplemented with SC had the highest ($P < 0.05$) total DMI (6.13 kg/head/d) followed by animals supplemented with FM (5.81 kg/head/d) and the controls (5.78 kg/head/day), which were not significantly different ($P > 0.05$) from each other. The BWtG of cattle fed urea-treated sorghum stover alone was 559 g/head/d. This increased to 650 g/head/d with FM and to 741 g/head/d with SC supplementation. In cattle, BWtG was not significantly different ($P>0.05$) between the treatments. Feed conversion was best on SC followed by FM supplementation in both species (6.92 and 7.70 for sheep and 8.28 and 8.93 for cattle respectively). It can be concluded that feeding UTSS alone or supplemented with small amounts of SC or FM can increase the live weights of cattle and sheep at a reasonable cost.

Key words: *urea treatment, sorghum stover, fishmeal, sesame cake, weight gain, feed conversion.*

INTRODUCTION

Feed is the most important input in livestock production and its adequate supply in terms of quantity and quality throughout the year is a pre-requisite for any substantial and sustained expansion in livestock output. Among the major constraints limiting the potential development of livestock production in Eritrea, inadequate feed has been identified as the crucial bottleneck. In most areas, especially during the dry period, livestock fed only on crop residues or the native pasture cannot even meet their maintenance requirements or they lose BWt. Most ruminants are consequently subjected to chronic under nutrition.

Low quality feeds such as sorghum and barley straws and stalks are staple feeds for ruminant livestock in the traditional subsistence farming systems of Eritrea. These feed sources contain insufficient nitrogen to provide the ammonia needed by rumen microorganisms for the efficient fermentative digestion of such feeds. However, there are appropriate technologies to improve the nutritive value of these residues. Urea treatment and correct supplementation with locally available supplements have been successfully used in many developing countries (Dolberg, 1992; Hector, et al., 1990; Chenost and Kayouli, 1997). Nevertheless, until recently sorghum stover was not properly collected and stored by farmers in the lowlands of Eritrea, instead being grazed while in the field.

The potential nutritive value of straw and stover or low quality forage cannot be exploited if the microbes in the animal's rumen do not receive the correct balance of nutritive elements for their efficient digestion. The objective of nitrogenous and energy supplements is therefore to ensure additional supply of nutritional elements to the animal to achieve a targeted performance level. Several positive results have been achieved from supplementing urea-treated sorghum stover with oilcakes and fishmeal (McDonald, et al., 1973; Maglad et al., 1984; Williams, 1984; Preston and Leng, 1987; Little et al., 1991). However, this approach has not yet been studied in Eritrea. As a result there is no reliable information on the application of supplementation methods to livestock in Eritrea, in particular to intensive and semi-intensive livestock production. The objectives of this study were therefore to examine the effect of feeding urea-treated sorghum stover supplemented with locally available protein sources (fishmeal or sesame cake) on the performance of local sheep, and to estimate the economic value of the treatments.

MATERIALS AND METHODS

Two trials involving sheep and cattle were conducted on-farm, the former within the Adi-Omer Mixed Farming Project and the latter at the Gash Setit Agro-Industry and Trade farm.

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Table 1. Feeds provided to the experimental animals.

Species	Feed 1 (Control)	Feed 2	Feed 3
Cattle	UTSS *ad lib*	UTSS *ad lib* + 576 g SC	UTSS *ad lib* + 432 g FM
Sheep	UTSS *ad lib*	UTSS *ad lib* + 80 g SC	UTSS *ad lib* + 60 g FM

UTSS — urea treated sorghum stover; SC — sesame cake (g DM); FM — fishmeal (g DM).

Table 2. Chemical composition of the feed ingredients (% DM).

Feed ingredient	DM (%)	Ash	CP	EE	CF	Energy (MJ/kg)
NTSS	87.00	12.60	6.25	0.72	32.50	9.35
UTSS	60.00	12.70	11.12**	0.88	29.67	9.83
SC	89.81	11.87	44.47	13.93	9.24	16.12
FM	89.87	27.95	59.15	5.44	1.93	12.90

NTSS — non-treated sorghum stover; DM — dry matter; UTSS — urea-treated sorghum stover; CP — crude protein; SC — sesame cake; EE — ether extract; FM — fishmeal; CF — crude fibre.

** — Estimated value (Chenost and Kayouli, 1997).

Table 3. Mean values for live weight change, feed intake, feed conversion and cost of feed by sheep fed urea-treated sorghum stover supplemented with sesame cake or fishmeal.

Parameters	Feed		
	1 (Control)	2	3
No. of animals	7	7	7
Initial BWt (kg)	20.643	20.243	20.343
Final BWt (kg)	26.557	32.286	30.671
Experimental period (d)	90	90	90
Daily gain (kg)	0.066 [a]	0.134 [b]	0.115 [c]
Daily DMI (kg)	0.821 [a]	0.927 [b]	0.886 [ab]
Daily treated sorghum stover intake (kg)	0.821	0.847	0.826
Daily supplements (FM or SC) intake (kg)	0.000	0.080	0.060
Feed conversion (kg feed/kg gain)	12.439	6.918	7.704
Cost of feed (Nfa/kg gain)	25.72	18.94	25.80

[a] [b] [c], Means in the same row not having common letters differ significantly ($P < 0.05$); Least significant difference (LSD) for LWG = 0.01758; LSD for DMI = 0.0833. Feed 2 — control + sesame cake; Feed 3 — control + fishmeal.

Animals and Feeds

The feeding trial was conducted on 21 growing male sheep from local breeds aged between six and seven months, and nine male Barka male cattle aged between three and four years. The experimental period lasted for three months and was preceded by two weeks of adaptation. The animals were divided into three treatment groups (three cattle and seven sheep in each treatment) after balancing for initial live weight and age. Each group was then randomly allocated into one of the three dietary treatments by drawing lots (**Table 1**). All the animals were fed on a DM basis. Five kg urea were dissolved in 60 L water and evenly sprinkled on layers of 100 kg of chopped sorghum stover. The stover was then stacked into a concrete pit and covered with a plastic sheet to maintain an air-tight condition for a period of at least two weeks. The diets in the two treatments with supplements of SC or FM were formulated to be iso-nitrogenous and were thoroughly mixed before being offered to the animals.

Measurements and Analysis of Data

Samples of the feeds were collected at regular intervals and analysed according to standard procedures (AOAC, 1984). The chemical composition of the diets is given in **Table 2**.

After 15 d of adaptation, BWt was measured before feeding and watering at intervals of 15 d. Dry matter intake was determined for each group from the difference between the daily weight of feed offered and feed refused.

The feeding trials were designed according to a completely randomised design (CRD). Mean LWtG and daily DMI were analysed using GENSTAT Release 12.2 (2003) Windows software.

RESULTS AND DISCUSSION

Effects of Urea Treatment on Composition of Sorghum Stover

Urea treatment of sorghum stover was effective in upgrading its nutritional value, particularly the CP content of straw which increased

by 77.9% from 6.25% to 11.12% following treatment (**Table 2**). This was similar to the results of Saadullah et al. (1981) where the CP content of sorghum stover increased from 2.2% to 11.9% when treated with five percent urea.

Sheep Trial

Results from the sheep trial are given in **Table 3** and in **Figures 1** and **2**. Urea-treated stover displayed all the expected characteristics. Animals were highly attracted to treated stover and readily consumed it. The increase in DMI following urea treatment (Table 3 and **Figure 1**) can be attributed in part to the softer texture, odour of ammonia and addition of nitrogen (N), which made it more palatable. These changes are known to produce a faster rate of digestion (Mbanya et al., 2005). The reason for the increased DMI in the supplemented group could be due to the N added by SC or FM which facilitates the cellulolytic process and increases the rate of digestion in the rumen. Chenost and Kayouli (1997) pointed out that the ultimate objective of adding supplements to low quality forages is to increase digestive processes in the rumen and thus increase intake. This can only be achieved by increasing fibre breakdown in the rumen.

The straw intake and LWtG obtained in the control group in this study were higher than those reported by others. Hadjipanayiotou et al. (1993) found that Awassi sheep fed untreated straw gained 73 g/d and this increased to 88 g/d with urea treatment, but the straw intake remained constant at 744 g/d. However, Jurgens (1997), showed that a 21 kg sheep consumed a total of 1 kg of feed daily, a value similar to the DMI recorded in this study.

Feed conversion was higher in the SC supplemented group than in the control and FM supplemented group.

Cattle Trial

Dry matter intake of treated straw by cattle in this study was 5.78 kg/d (**Table 4** and **Figure 3**). This value is similar to the daily average straw intake of 5.6 kg/d found in cattle in China (Tengyun, 2000).

The higher DMI of the SC supplemented group was due to the added SC and not to a higher consumption of the treated stover since intake of stover (5.56 kg/d) was less than that of the control group (5.78 kg/d). In fact, the added supplements reduced stover intake in both supplemented groups as there was no significant difference ($P > 0.05$) in stover intakes between the three groups. The results of DMI of cattle given in **Table 4** are comparable with the figures given by Jurgens (1997), who showed that mature cattle weighing around 290 kg consume 2.5% of their BWt (i.e. 7.25 kg/d).

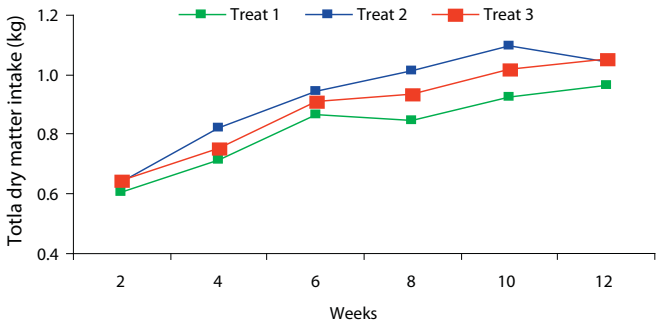

Figure 1. Dry matter intake of control and supplemented sheep (SED. = 0.0419); Treat 1 — control; Treat 2 — control + sesame cake: Treat 3 — control + fishmeal.

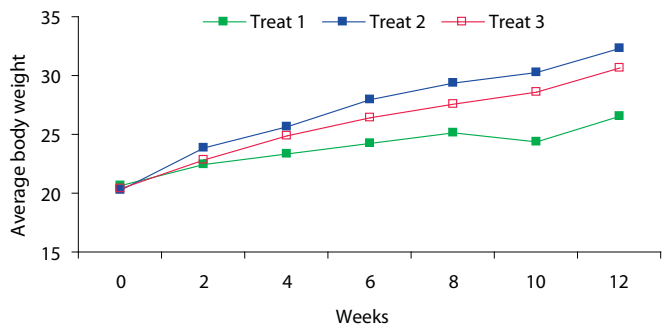

Figure 2. Changes in body weight of control and supplemented sheep (SED = 0.00807).

Table 4. Mean values for live weight change, feed intake, feed conversion and cost of feed by cattle fed urea-treated sorghum stover supplemented with SC or FM.

Parameters	Feed		
	1(Control)	2	3
No. of animals	3	3	3
Initial body weight (kg)	287.3	287.0	284.0
Final body weight (kg)	337.7	353.7	342.5
Experimental period (days)	90	90	90
Daily gain (kg)	0.559 [a]	0.741 [a]	0.650 [a]
Daily total DMI (kg)	5.780 [a]	6.133 [b]	5.807 [a]
Daily treated sorghum stover intake (kg)	5.780 [a]	5.557 [a]	5.375 [a]
Daily supplement (FM or SC) intake (kg)	0.000	0.576	0.432
Feed conversion (kg feed/kg gain)	10.34	8.28	8.93
Cost of feed (Nfa/kg gain)	19.92	23.43	29.54

[a b] Means in the same row not having common letters differ significantly ($P < 0.05$); LSD for LWtG = 0.3814; LSD for DMI = 0.3105. Treatment 2 — control + sesame cake; Treatment 3 — control + fishmeal.

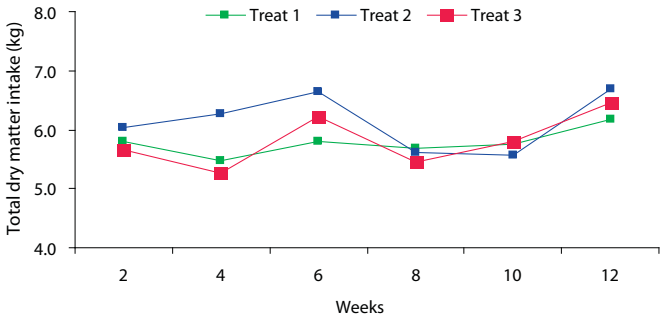

Figure 3. Weekly dry matter intake of cattle fed the control and supplemented diets (SED = 0.1562).

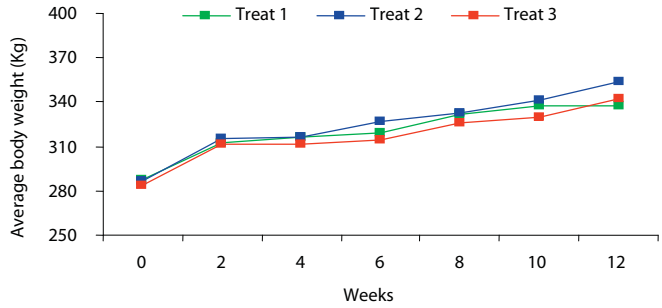

Figure 4. Weekly body weight change of cattle fed the control and supplemented diets (SED = 0.1374); Treat 1 — control; Treat 2 — control + sesame cake, Treat 3 — control + fishmeal.

In the control group, as a result of urea treatment which improved the nutritive value of the diet, animals not only maintained their weights but also gained more weight (559 g/head/d) than recorded in some other studies (**Figure 4**).

The literature reviewed by Chenost and Kayouli (1997) showed the improvement of DMI and weight gain of cattle fed 5% urea-treated straw. They found that mature cattle with an average BWt of 285 kg consumed 6.82 kg/d and gained 564 g/d. The figures obtained in this experiment (5.78 kg/d DMI and a gain of 559 g/d) were similar to the results of Chenost and Kayouli (1997), but higher than those obtained by Wongsrikeao and Wanapat (1985) on buffalo, which had a DMI of 4.75 kg/d and a BWtG of 261g/d. The values in this experiment were also higher than those obtained by Little et al. (1991), who recorded an average daily gain of 169 g in controls and 272 g and 271 g respectively when SC and cottonseed meal were used as supplements.

Fishmeal supplementation in cattle (432 g/head/d) did not result in any significant difference in BWt gain and DMI compared with the control group.

The daily intake of treated sorghum stover intake means the amount of treated sorghum stover consumed daily without SC or FM by the animals in all the groups. In this case the amount of feed consumed by the control group is the same as the daily total DMI. However, in feeding regimes two and three, the daily total DMI included SC and FM in addition to the daily treated sorghum stover intake. Therefore, the difference in the daily intake of treated sorghum stover was the result of the additional supplements (SC and FM in diets two and three respectively).

Although feed conversion was higher in the group supplemented with SC, (**Table 4**), both supplemented groups had higher feed conversions than the control. The feed conversion obtained in the control group was higher than that obtained by Khan and Davis (1981) who found conversion ranges of 13.5–28 kg feed /kg LWtG.

Economic Data from the Feeding Trials

There has to be a good economic reason for a farmer to feed treated straw or stover and /or to add supplements. The effects have to be evident. In this study, treating sorghum stover with urea for feeding cattle was as, and in the case of cattle, more economically advantageous for increasing daily LWtG and there was no economic reason to supplement cattle beyond urea treatment (**Table 5**). Also evident is that the cost of feed/kg LWtG was also lower for the SC supplemented groups than for the FM groups, the higher cost of FM supplementation being due to a combination of the relatively higher high cost of FM and the lower weight gain attained by the animals.

Preston and Leng (1987) indicated that the extent and rate of digestion of fibrous feeds are increased by a N supplement, which results in a greater DMI. This is reflected in the LWt changes recorded here.

DISCUSSION

There are different possible explanations for why SC supplemented animals had higher LWt gains than animals supplemented with FM. The increased total DMI recorded in SC supplemented animals could account for their higher LWt gains. Lindsay et al. (1982) indicated that the greater weight gain of supplemented animals was due to the increased intake of the basal diet. Another reason for the superiority of the SC could be related to its chemical composition. The SC used in this trial had lower ash, higher ether extract (crude fat)

Table 5. Cost summary of the sheep and cattle feeding trials.

	Cattle: cost of each treatment group			Sheep: cost of each treatment group		
	T1(Control)	Feed 2	Feed 3	T1(Control)	Feed 2	Feed 3
Total cost (Nkfa)	5 007.10	6 687.10	7 184.70	2 069.50	2 599.10	2 869.50
TCost (Nkfa/head/d)	18.55	24.77	26.61	3.285	4.13	4.55
BWtG (g/head/d)	0.559	0.741	0.650	0.066	0.134	0.115
TCost (Nkfa/kg gain)	33.18	33.43	40.92	49.77	30.82	39.57

BWG — body weight gain; TCost — total cost; total number of d — 90; Nkfa= Eritrean currency (US$ 1— 15 Nkfa).

T1 — control; Feed 2 — control + sesame cake, Feed 3 — control + fishmeal.

and higher energy content than the FM. These differences may be important in affecting DMI, and consequently LWtG. Meals that contain high amounts of ash are generally considered to be lower in protein quality and have a lower amino acid digestibility (Parsons, 2006; Shah and Muller, 1983). McDonald et al. (1995) suggested that a high ash content is one reason for the low energy content of a feed ingredient.

Depending on the diet, fat contributes approximately 7–10% of the digestible energy of rations (Preston and Leng, 1987). Dietary fat is converted to depot fat in the animal and stored in different parts of the body. The higher fat (EE) content of SC, which contains dietary long chain fatty acids (LCFAs), could therefore have contributed to the increased gain of the groups supplemented with SC. On the other hand, Sanderson et al. (2001) reported that FM supplementation increased rates of ash and crude protein gain, but had a small effect on fat gain.

Cattle supplemented with FM gained more weight than unsupplemented animals. This could be due to the fact that FM is well balanced in amino acids and has a high mineral and vitamin content (McDonald et al., 1973). Generally, FM has 20–30% of rumen degradable protein and about 70–80% of by-pass protein (Preston and Leng, 1987).

CONCLUSIONS

Urea treatment is a simple, cheap and applicable method to improve low quality roughages in Eritrea. Urea treatment improved the CP content of sorghum stover and increased the intake of animals. Animals fed urea-treated sorghum stover not only maintained their BWt, but also gained an appreciable and economically worthwhile amount of weight which was comparable with supplemented animals, particularly in cattle. The result of the experiment was similar for both species (sheep and cattle). Small supplementation with SC (8% of ration DM) as a protein source resulted in an increase in weight gain of cattle and sheep. The potential availability of SC is high in Eritrea and inclusion of this supplement in the diets of ruminant livestock significantly increased LWtG and reduced the cost of feed per unit gain of weight. Therefore, it can be concluded that feeding cattle only with urea-treated sorghum stover or supplementing it with small amounts of SC can lead to cost-effective weight gains. Nevertheless, further research with different levels of SC supplementation is required to determine the most economic level as is continuous evaluation of the quality of available FM. Also, long term on-farm trials on feeding urea-treated straw on milk production should be carried out to determine the practical advantages and disadvantages, as well as the economic returns from feed treatment and supplementation, including on crop residues like finger millet, maize stover and dried grasses. Strong extension linkages have to be developed to popularise urea treatment of straw, particularly in farming systems where there is wastage of crop residues.

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Effect of Replacing Dietary Soybean Meal with Tropical Legumes on the Performance of Lambs

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ABSTRACT

This study determined how supplementing bahiagrass hay (*Paspalum notatum* Flügge cv. 'Pensacola') with soybean (*Glycine max*) meal or warm-season legume hays affected intake, digestibility, and N utilisation by lambs. Dorper × Katadhin crossbred lambs (30.6±5.5 kg; n = 42) were fed bahiagrass hay *ad libitum* and supplemented with nothing (control), soybean meal, or hays of annual peanut (*Arachis hypogaea*), cowpea (*Vigna unguiculata*), perennial peanut (*Arachis glabrata*), pigeonpea (*Cajanus cajan*), or soybean. Legume hays were supplemented at 50% of diet dry matter (DM); soybean meal was supplemented at 4.25% of diet DM to match the average crude protein (CP) content (10.8%) of the legume hay supplemented diets. Cowpea, pigeonpea, and soybean were harvested at maturities that maximised DM yield and nutritive value, and peanuts were first cuttings. Diets were fed to six lambs per treatment for two consecutive 21-d periods. Supplementation with annual and perennial peanut, cowpea, and soybean hay increased ($P < 0.01$) DM intake versus control, but apparent DM digestibility was only increased ($P = 0.03$) by supplementation with either peanut. Nitrogen intake, digestibility, and retention were increased ($P < 0.01$) by supplementation particularly with annual or perennial peanut hay. Ruminal ammonia concentration was increased ($P < 0.01$) by all legume hay supplements versus the control. Microbial N synthesis and ruminally degraded organic matter (OM) were increased ($P = 0.03$) by perennial and annual peanut hay supplementation, but the efficiency of microbial synthesis was not different ($P = 0.52$) among diets. Annual and perennial peanut hays were the best supplements for the bahiagrass hay in this study.

Key words: *bahiagrass, digestibility, intake, nitrogen retention, supplementation, tropical legume.*

INTRODUCTION

Bahiagrass (*Paspalum notatum* Flügge) and bermudagrass [*Cynodon dactylon* (L.) Pers.] are the main forage grasses in Florida and much of the southern United States. The yield of these grasses is normally sufficient to meet intake requirements of most ruminant livestock during the grazing season; however, their quality is insufficient for growing or lactating ruminants due to low DM digestibility and CP concentration (Duble et al., 1971; Johnson et al., 2001). Because of rapidly escalating fertilizer and feedstuff commodity prices, ruminant feeding strategies that are less dependent on these inputs merit evaluation. Supplementing poor quality basal grass diets with legume forage has increased feed intake and diet digestibility by ruminant livestock (Minson and Milford, 1967; Getachew et al., 1994;). Legume supplementation improves N retention by the ruminant when grass diets that do not meet ruminant energy and N requirements are fed (Mosi and Butterworth, 1985; Matizha et al., 1997).

Alfalfa (*Medicago sativa* L.), the legume fed most commonly to livestock in the USA does not persist in the warm, humid climate of the Gulf Coast region (Prine et al., 1981). Perennial peanut (*Arachis glabrata* Benth.) is the main warm-season forage legume in Florida; however, it is sprig-planted and it takes 1–2 y to establish; it is therefore costlier to establish than seeded, warm-season legumes like cowpea [*Vigna unguiculata* (L.) Walp.], soybean [*Glycine max* (L.) Merr.], or annual peanut [*Arachis hypogaea* (L.)] (French et al., 2006). Effects of supplementing bahiagrass hay with these legume hays on animal performance are unknown.

This study determined the feed intake, digestibility, and N retention of lambs fed bahiagrass (cv. 'Pensacola') hay supplemented with soybean meal, or hays of perennial peanut (cv. 'Florigraze'), annual peanut (cv. 'Florida MDR 98'), soybean (cv. 'Pioneer 97B52'), cowpea (cv. 'Iron clay'), or pigeonpea [*Cajanus cajan* (L.) Millsp. cv. 'GA-2'].

MATERIALS AND METHODS

Forage Production

Legume hays were produced at the North Florida Research and Education Center in Marianna, FL, (31° N) and fed at the Department of Animal Sciences, University of Florida, Gainesville, FL. The legumes were harvested at the recommended maturity stage for maximising both DM yield and nutritive value i.e. when pods began to turn yellow for cowpea (NDA, 1997), pod setting for pigeonpea (Le Houérou, 2004), and stage R6 (pod with full size seed at one of the four uppermost nodes and completely unrolled leaves) for soybean (Coffey et al., 1995; Sheaffer et al., 2001). Established stands of perennial (4-y-old) and annual peanut (6-y-old; self reseeding) were harvested as first cuttings. Perennial and annual peanut, and cowpea were harvested to a stubble height of 10 cm, whereas soybean and pigeonpea were harvested to stubble heights of 20 and 40 cm based on previous recommendations for the respective forages (Romero et al., 1987; Le Houérou, 2004; Mislevy et al., 2005;). Cowpea, soybean, and pigeonpea were rolled into 200 kg round bales using a Vermeer 504 L baler (Vermeer Manufacturing Inc., Pella, IA). Annual and perennial peanut were stored as square bales (50 kg). An established stand of bahiagrass (11-y-old) was harvested as a 6-week regrowth to a stubble height of 8 cm and rolled into round bales. After at least 5

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months, each hay bale was chopped to approximately 8-cm particle length in a tub grinder (Roto Grind, model 760, Burrows Enterprises, Greeley, CO) to limit refusals.

Animals, Feeding, and Housing

Dorper × Katadhin cross ram lambs (n = 42) weighing 30.6±5.5 kg were stratified by weight and assigned randomly to seven treatments (six lambs per treatment per period) within each weight stratum. The experiment was a completely randomised block design with two periods (n = 12), each consisting of 14 d of adaptation and 7 d of measurement, and each lamb received a different diet in each period. Lambs were fitted with canvas faecal collection bags and housed in individual metabolism crates (0.5 × 1.5 m). Thirty-eight of the crates were also adapted for collection of urine. Water was provided *ad libitum* and 20 g of a mineral premix (Ranch House Trace Mineralized Salt, United Salt Corp., Houston, TX) was added to the diet of each lamb daily. Lambs were fed *ad libitum* (110% of previous day's intake) diets consisting of bahiagrass hay supplemented with nothing (control), soybean meal, or hays of perennial peanut, annual peanut, cowpea, pigeonpea, or soybean. Legume hays were fed at 50% of total diet DM, and soybean meal was fed at 4.25% of diet DM to match the average CP concentration (10.8% DM basis) of the legume hay diets. The bahiagrass and respective legume supplements were offered in the same feed trough at 0800 and 1500 h daily; the soybean meal was top dressed on the bahiagrass hay and fed at the same times.

Sample Collection

Samples (1 kg) of each hay and soybean meal were taken daily during the 7-d collection period, and daily orts were weighed and stored. Total faecal output was collected daily from each lamb, weighed, and a 10% subsample was frozen (–20°C) for subsequent analysis. The weight and volume of daily urine output was recorded. Sulphuric acid was added to subsamples of urine to ensure that the pH remained below 3.0, and the urine was stored (–20°C) for further analysis. Lambs were weighed and blood sampled by jugular venipuncture on d 0, 21, and 42. A Vacutainer tube (BD, Franklin Lakes, NJ) containing sodium heparin anticoagulant was used to collect 10 mL of whole blood from each lamb and the tubes were stored on ice and processed within 2 h. The blood was centrifuged at 1 920 g for 20 min at 4°C to separate the plasma, which was stored at –20°C until analysed. Ruminal fluid (100 mL) was collected from 28 lambs (four selected randomly per treatment) on the last day of period two by aspiration from orally-inserted stomach tubes at 0, 2.5, 5, 7.5 and 10 h after the morning feeding. A representative (100 mL) sample was analysed immediately for pH (Accumet, model HP-71, Fischer Scientific, Pittsburg, PA), acidified with concentrated H_2SO_4, centrifuged for 30 min at 4°C and 2 795 *g*, and frozen (-20°C) for subsequent analysis.

Chemical Analyses

Samples of hays, soybean meal, orts, and feces were composited by period and analysed for DM by oven drying at 105°C overnight, and for ash by combustion in a muffle furnace at 600°C overnight. Samples reserved for additional analyses were dried at 60°C for 48 h in a forced air oven and ground to pass through a 1 mm screen in a Wiley mill (Arthur H. Thomas Company, Philadelphia, PA). Total N was determined by rapid combustion using a macro elemental N analyser (Elementar, vario MAX CN , Elementar Americas, Mount Laurel, NJ) and used to calculate CP (CP = N × 6.25). Neutral detergent fibre was analysed using the method of Van Soest et al. (1991). Amylase and sodium sulfite were used for NDF analysis and the results were expressed on a DM basis. Apparent digestibility of DM, OM, N, and neutral detergent fibre (NDF) were calculated. Feed samples were analysed for acid detergent fibre (ADF) and acid detergent lignin (ADL) with the method of AOAC (1990). The ADIN concentration of the hays was measured with the elemental N analyser on ADF residues. The Van Soest et al. (1966) method and an ANKOM Daisy^{II} Incubator (ANKOM Technologies, Macedon, NY) were used to measure in vitro true DM digestibility (IVTD) of hays. Condensed tannin (CT) concentration of hays was analysed with the method of Terrill et al. (1992). Quebracho tannin (Unitán ATO, Buenos Aires, Argentina) was purified with Sephadex LH-20 (GE Healthcare Life Sciences, Piscataway, NJ) according to Asquith and Butler (1985) as modified by Hagerman (1994). Condensed tannin results were expressed as quebracho tannin equivalents.

Urine was analysed for N by rapid combustion with the elemental N analyser and for total purine derivatives (PD) as allantoin (Bochers, 1977). Xanthine, hypoxanthine, and uric acid were converted to allantoin using the procedure of Fujihara et al. (1987). Microbial protein supply to the small intestine was calculated from the urinary output of PD using the equation of Chen et al. (1992). Microbial efficiency was calculated as g of microbial N/kg of OM apparently digested in the total tract.

Ruminal fluid NH_3-N concentration was determined by an ALPKEM auto analyser (ALPKEM Corporation, Clackamas, OR) with an adaptation of the Noel and Hambleton (1976) procedure that involved colorimetric quantification of N.

Statistical Analyses

Data were analysed with PROC MIXED (SAS Inst. Inc., Cary, NC). The model for intake, digestibility, N excretion and retention, microbial protein parameters included dietary treatment, period, dietary treatment × period, and lamb (random variable). The model for ruminal fluid pH and NH_3-N included dietary treatment, time of collection (repeated measure), dietary treatment × time of collection, and lamb (random variable). Means were separated with a PDIFF statement. Significance was declared at $P \leq 0.05$.

RESULTS

Forage Chemical Composition

Dry matter concentrations were not different among hays, but OM concentration was less (P = 0.04) in perennial peanut than in all other hays (**Table 1**). As expected, CP concentration was least (P < 0.01) in bahiagrass hay. Among legume hays, CP concentrations were greater in annual and perennial peanut hays than in cowpea and pigeonpea hays. Concentration of NDF was greatest (P < 0.01) in pigeonpea hay followed by bahiagrass hay, and least in annual and perennial peanut hays. The greatest (P < 0.01) ADF concentration was in pigeonpea hay, and the least (P = 0.02) in perennial peanut hay. Lignin (*P* = 0.10) and ADIN (P < 0.01) concentrations were greater in pigeonpea hay than the other hays. *In vitro* true digestibility was greatest (P < 0.01) in perennial peanut hay followed by annual peanut hay. Bahiagrass hay contained less (P < 0.01) IVTD than all legume hays except pigeonpea hay which contained the least (P < 0.01) IVTD. Condensed tannin concentrations were low in all hays. Extractable CT concentration was greatest (P < 0.01) in perennial peanut hay followed by cowpea hay. Bound CT concentration was greatest (P < 0.01) in perennial and annual peanut hays followed by pigeonpea hay.

Table 1. Chemical composition and *in vitro* true digestibility of hays fed to lambs.

Item	Bahiagrass	Annual peanut	Perennial peanut	Cowpea	Pigeonpea	Soybean	SEM[1]
DM, %	91.1	91.0	90.8	91.5	91.8	91.6	1.8
OM, % DM	94.5^{a}	92.4^{b}	90.8^{c}	92.6^{b}	94.7^{a}	93.8^{ab}	0.53
CP, % DM	8.1^{d}	14.7^{ab}	15.2^{a}	11.7^{c}	12.2^{c}	13.5^{b}	0.40
NDF, % DM	73.8^{b}	46.2^{e}	43.3^{f}	62.2^{c}	78.6^{a}	59.0^{d}	1.0
ADF, % DM	39.8^{cd}	37.8^{d}	32.1^{e}	48.7^{b}	60.2^{a}	42.8^{c}	1.3
ADIN, % N	15.1^{b}	7.1^{e}	6.5^{e}	13.4^{c}	25.4^{a}	9.1^{d}	0.43
ADL, % DM	6.2^{b}	7.9^{b}	6.7^{b}	9.5^{b}	17.1^{a}	9.6^{b}	1.1
IVTD[2], % DM	50.7^{d}	71.4^{b}	77.2^{a}	57.9^{c}	35.1^{e}	57.4^{c}	1.1
Total CT[3], % DM	0.46^{d}	2.68^{b}	3.82^{a}	1.03^{c}	1.13^{c}	0.20^{d}	0.14
Extractable CT[3], % DM	0.12^{cd}	0.16^{cd}	1.56^{a}	0.56^{b}	0.26^{c}	0.05^{d}	0.07
Bound CT[3], % DM	0.34^{cd}	2.52^{a}	2.26^{a}	0.57^{c}	0.87^{b}	0.15^{d}	0.11

^{a,b,c,d,e,f} Within a row means without a common superscript letter differ ($P < 0.05$).

[1] SEM values reflect the variation of samples collected daily and composited within each of two periods (n = 2).

[2] *In vitro* true DM digestibility (IVTD); [3] Condensed tannin.

Table 2. Performance indices (DM basis) of lambs fed bahiagrass hay diets supplemented at 50% of DM with warm-season legume hays or at 4.25% of DM with soybean meal (SBM).

Item	Bahia-grass	SBM	Annual peanut	Perennial peanut	Cowpea	Pigeonpea	Soybean	SEM
Total intake, g/d								
Dry matter	665^{ef}	726de	975^{b}	1105^{a}	803^{cd}	612^{f}	864^{c}	29
Organic matter	629^{ef}	685de	911^{b}	1034^{a}	752^{cd}	579^{f}	811^{c}	28
Neutral detergent fibre	500^{e}	522de	594^{b}	654^{a}	558^{cd}	468^{e}	583^{c}	19
Digestibility, %								
Dry matter	58.5^{cd}	60.3^{c}	64.3^{b}	67.8^{a}	58.8^{cd}	56.3^{d}	60.7^{c}	0.9
Organic matter	60.6^{c}	61.4^{c}	65.4^{b}	68.7^{a}	59.7^{cd}	57.5^{d}	61.7^{c}	1.0
Neutral detergent fibre	60.8^{abc}	60.8^{abc}	57.9^{cd}	62.2^{a}	56.6^{d}	58.7^{bcd}	58.9^{bcd}	1.0
N utilisation indices								
N intake, g/d	8.8^{e}	12.1^{d}	17.7^{b}	21.3^{a}	12.9^{d}	11.8^{d}	15.6^{c}	0.54
Fecal N output, g/d	4.8^{d}	5.1^{d}	6.5^{b}	7.3^{a}	5.8^{c}	5.2^{d}	6.4^{b}	0.20
Urinary N output, g/d	2.1^{c}	2.7^{b}	4.3^{a}	3.6^{ab}	2.5^{c}	2.6^{c}	4.0^{a}	0.35
Retained N, g/d	2.0^{d}	4.2^{c}	7.0^{b}	10.5^{a}	4.6^{c}	4.1^{c}	5.1^{c}	0.54
N digestibility, %	46.5^{e}	56.8^{cd}	62.4^{b}	66.8^{a}	54.0^{d}	55.6^{cd}	58.1^{c}	1.1
PD[1] output, mmol/d	7.4^{bc}	6.3^{c}	10.1^{a}	11.0^{a}	6.2^{c}	7.2^{c}	9.7^{ab}	1.0
Microbial N, g N/d	6.4^{bc}	5.5^{c}	8.7^{a}	9.5^{a}	5.4^{c}	6.2^{c}	8.4^{ab}	0.91
DOM [2], g/d	385^{e}	437de	587^{b}	681^{a}	469^{cd}	336^{f}	500^{c}	21
Microbial efficiency, g microbial N/kg of DOM	16.5	12.8	15.1	13.2	11.9	18.4	17.0	2.30
Rumen NH_3-N, mg/dL	2.5^{c}	3.7^{bc}	6.6^{a}	7.0^{a}	6.1^{a}	5.5^{ab}	5.5^{ab}	0.70

[1] Urinary purine derivatives; [2] Apparently digested organic matter.

Within a row means without a common superscript letter differ ($P < 0.05$).

Intake and Digestibility

With the exception of pigeonpea hay, legume hay supplementation increased intake of DM, OM, and NDF (**Table 2**). Intakes of DM, OM, and NDF were greatest (P = 0.04) in lambs supplemented with perennial peanut hay, followed by annual peanut hay, and they were least (P = 0.04) in lambs consuming bahiagrass hay alone or pigeonpea hay compared with those consuming other legume hays. Intakes of DM, OM, and NDF were not improved by addition of soybean meal. Digestibilities of DM and OM were greatest (P = 0.03) when diets were supplemented with perennial peanut hay, followed by annual peanut hay. Addition of the other supplements did not affect digestibility of DM or OM except that pigeonpea hay supplementation reduced (P = 0.04) OM digestibility. Digestibility of NDF was greater (P < 0.01) in lambs fed bahiagrass hay alone, soybean meal or perennial peanut hay than in lambs supplemented with cowpea hay.

Nitrogen Utilisation

Nitrogen intake was increased (P < 0.01) by supplementation regardless of supplement type and it was greatest (P < 0.01) in lambs fed perennial peanut hay, followed by (P < 0.01) annual peanut hay. Faecal N output was greatest (P < 0.01) in lambs fed perennial peanut hay, followed by annual peanut and soybean hays, and it was least in lambs fed bahiagrass hay alone, soybean meal, or pigeonpea hay. Urinary N excretion was greater (P = 0.04) in lambs fed perennial and annual peanut or soybean hays than in those fed bahiagrass alone, bahiagrass plus cowpea hay, or bahiagrass plus pigeonpea hay. Nitrogen retention and digestibility were increased by supplementation and the greatest (P < 0.01) values occurred in lambs fed perennial peanut, followed by annual peanut. Purine derivative excretion and microbial N production were greater (P = 0.03) in lambs fed perennial and annual peanut hays than those fed all other diets except soybean hay. Apparent digestible OM intake was greatest (P < 0.01) in lambs fed perennial peanut hay, followed by annual peanut hay and it was least in those fed pigeonpea hay. Microbial efficiency was not affected by supplementation.

Ruminal Fluid pH and NH_3-N

No interactions between treatment and time occurred. Ruminal pH was not different among dietary treatments. Ruminal ammonia-N concentration was greater (P < 0.01) in lambs fed annual and perennial peanut and cowpea hays, than those fed bahiagrass alone or soybean meal.

DISCUSSION

The nutritive value of the bahiagrass hay was similar to that reported by Kostenbauder et al. (2007) and the NDF, ADF, and CP concentrations of perennial peanut were similar to those reported for the Florigraze cultivar (Romero et al., 1987). The CP concentration of cowpea was similar to that reported for Iron clay cowpea grown in Florida (Higuera et al., 2001), but the NDF, ADF and ADL concentrations were almost twice as great as those of Iron clay cowpea harvested at an earlier maturity stage (canopy close) in Texas (Muir et al., 2001). The CP concentration and IVTD of pigeonpea were much less than those reported (20% and 49–55% IVDMD, respectively) for similar early-maturing cultivars that were harvested earlier (50% flowering) and cut at a greater stubble height (0.6 m; Alexander et al., 2007). The CP concentration of soybean was similar to those reported for other cultivars (Seiter et al., 2004) but the NDF and ADF concentrations were greater.

Perennial and annual peanut hays had greater IVTD than other legume hays because they contained less NDF and ADF. Although the values were not identical, the ranking of forages by IVTD was the same as that by *in vivo* apparent DM digestibility, indicating that the IVTD method is suitable for comparing these warm-season legume forages. All forages contained low concentrations of CT. Condensed tannins reduce forage quality at concentrations of 6% of DM or greater (Waghorn et al., 1994), but concentrations of 2–4% of DM usually result in improved forage nutrient utilisation by ruminants (Min et al., 2003 and 2005). Condensed tannin concentrations were consistent with those reported previously for soybean (Reddy et al., 1985), annual peanut (Karchesy and Hemingway, 1986), cowpea (Baloyi et al., 2001), pigeonpea (Alexander et al., 2007), and perennial peanut (Valencia et al., 2007) forages.

Legume hays contained less CP than anticipated based on concentrations of CP in the standing plants (Foster, 2008) primarily because of leaf shatter during harvest and chopping, indicating that harvest management practices that minimise such losses are critical for preserving the quality of the hays. Nevertheless, supplementation with all legume hays, except pigeonpea, increased DM and OM intake**,** though only supplementation with annual and perennial peanut hay also increased DM and OM digestibility. Moore et al. (1999) reported that supplements decreased voluntary forage intake when the forage TDN:CP ratio was < 7 with a few exceptions such as when basal forage intake was > 1.75% of bodyweight (BWt) as for our bahiagrass diet.

The intake responses in this study typify effects of legume forage supplementation to poor quality basal grass diets (Said and Tolera, 1993). The reticulate venation of legume leaves confers less resistance to ruminal degradation than the parallel venation of grass leaves (Frame, 2005). Consequently, legumes are degraded more easily and rapidly by ruminal microbes than grass leaves. In addition, lesser structural carbohydrate concentrations in legumes versus grasses contribute to the faster degradation and passage rates of legumes (Waghorn et al., 1989; Wilson, 1994; Jung and Allen, 1995; Dewhurst et al., 2003). Collectively, these factors increase feed intake due to the decreased rumen fill resulting from faster degradation and passage rates (Mertens, 1973; Reid et al., 1988). Relative differences in DMI and digestibility among legume hay supplemented diets reflect partly the structural fibre concentrations and morphological characteristics of the legumes. Annual and perennial peanut had less NDF and ADF than the other legume hays; consequently, they were more digestible. Pigeonpea hay had greater NDF, ADF, and ADL concentrations because of its thick, woody stems, which probably decreased DM and OM intake and OM digestibility. As in other studies (Mir and Mir, 1993: Haddad, 2000; Mupwanga et al., 2000), legume supplementation did not increase NDF digestibility partly because legumes had more lignin than grasses (Wilson, 1994).

Legume hay supplementation increased N intake because of the greater CP concentrations of the legumes versus bahiagrass, as well as the greater DMI of most of the legume-grass diets by lambs. Nitrogen retention increased accordingly because all supplements increased N digestibility and most decreased the proportion of intake N lost as urine. Legume supplementation increased ruminal NH_3-N concentrations because it increased N intake relative to the control diet and most of the protein in legumes is in the form of soluble protein or RDP (Broderick, 1995). Legume supplementation ensured that ruminal NH_3-N concentrations exceeded the recommended concentration (5.0 mg/dL; Satter and Slyter, 1974) for maximising microbial N synthesis. Microbial N synthesis was only increased by supplementation with annual or perennial peanut diets, partly because they provided more energy (apparently digested OM) for microbial growth (Clark et al., 1992) than other supplements.

Soybean meal supplementation increased N intake and retention, reflecting the greater N concentration of soybean meal; however, the small amount of soybean meal that was fed was not sufficient to significantly improve other measures of digestion. Supplementation with N from legume hays or soybean meal increased N intake, digestion and retention, indicating that supplementation may improve the performance of lambs on bahiagrass diets. As a consequence of the relatively small amount of soybean meal supplemented, annual and perennial peanut hay supplements were more effective than soybean meal supplementation at improving N intake, digestion, retention, and microbial N production. Due to these positive effects on N metabolism, perennial peanut and annual peanut hays were the best legume hay supplements for the lambs although soybean and cowpea hays also showed some promise. Pigeonpea hay was the least desirable supplement because it did not improve DMI and it reduced OM digestibility. Pigeonpea should be harvested at greater stubble heights for use as a legume supplement, though this would reduce biomass yields. Future research should determine the optimal inclusion rates of perennial peanut, annual peanut, cowpea, and soybean hays in the diets of growing lambs and beef calves.

CONCLUSION

Annual and perennial peanuts were the best supplements and pigeon pea was the worst supplement for the lambs in this study.

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Methane Emissions by Livestock in India and Mitigation Strategies

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ABSTRACT

In India 92% enteric methane (CH_4) is emitted by cattle and buffalo, about 7% by sheep and goats and only a very small fraction (less than 1%) is attributed to other ruminants and non-ruminant herbivores like yak, mithun, pig, horse, mules, ass, etc. Therefore the strategies to be adopted to mitigate CH_4 emissions by livestock are primarily centred around cattle and buffalo. Keeping in mind the agricultural practices in India, the major mitigation techniques which might have some scope for practical application include replacement of non-productive or low productive animals with superior livestock, improving the quality of feeds offered to animals and the use of plant secondary metabolites which are present in many tropical plants. After screening more than 150 plant extracts, it was found that *Terminalia chebula, Sapindus mukorossi, Populus deltoides, Foeniculum vulgare, Syzygium aromaticum, Allium sativum, Psidium guajava, Mentha piperita* and *Eucalyptus globulus* were capable of inhibiting methanogenesis and ciliate protozoa in an *in vitro* gas production test. Although these plant extracts exhibited more than 50% inhibition in *in vitro* experiments, the same plants/plant extracts either showed no effect or a very poor effect in *in vivo* experiments. Probable reasons include the different concentrations of plant secondary metabolites used in *in vivo* and *in vitro* experiments and large variations in the chemical composition of different accessions of the plant products. Therefore, detailed experiments are needed to optimise the doses of plant secondary metabolites required to produce significant inhibition of CH_4 emissions without adversely affecting animal performance.

Key words: *methane emissions, tropical plant extracts, secondary metabolites, gas production test, open circuit respiration calorimetry.*

INTRODUCTION

India has 226.1 million cattle, 96.9 million buffaloes, 59 million sheep and 124.5 million goats, 18.5 million pigs, 0.9 million each of camels and donkeys, 0.8 million horses/ponies and a small number of yaks, mithuns, mules etc. (FAO, 2006), which account for 11.95% of the global livestock population and produce 12.45% of the total enteric CH_4 emissions. If emissions from livestock excreta are included, this proportion is reduced to 10.76% of the global emissions because waste management in western countries (especially USA and Europe) is by anaerobic processes which produce more CH_4 whereas solid waste management is practised in India which is an aerobic process producing lower CH_4 emissions. Although the situation does not appear to be so alarming, keeping in view the future commitments of the Kyoto Protocol, mitigation of CH4 emissions is essential to protect the environment. By doing so, better feed conversion efficiency can be obtained as a bonus.

The CH_4 emission factors for different categories of animals under Indian conditions are much lower than those calculated by the IPCC (2001 and 2006). Based on studies conducted at the Indian Veterinary Research Institute (IVRI), Izatnagar, India, the CH_4 emission factors (CH_4 emission, kg/h/y) for cattle, buffalo, sheep and goat vary between 25.6–47.6, 28.9–52.7, 2.6–4.1 and 3.3–4.3, respectively (**Table 1**). The variations might be due to different body weights of the animals, types of feed and individual behaviour (high or low CH_4 producer) of the animals. These CH_4 emission factors have been calculated on the basis of experiments conducted under conditions where animals are fed according to prescribed feeding standards, but the majority of animals at the farmers' door are fed much lower quality nutrients. Therefore, the estimates of CH_4 emissions calculated above are an over estimates to the tune of 20–25%.

Ruminants in India are fed primarily on a diet rich in cellulose, hemicellulose and lignin. Unfortunately, ruminants do not produce any enzyme which can hydrolyse these polymers into monomers for further utilisation as a source of energy. Therefore, these animals depend upon microbes which live in the gastrointestinal tract in an ecto-symbiotic relationship with the animal and help to bioconvert these polysaccharides into a usable of energy i.e. volatile fatty acids. This bioconversion process is very complex, being accomplished by the synergistic activities of different groups of microbes present in the reticulo-rumen e.g. bacteria, protozoa, fungi, archaea and bacteriophages.

The major substrates to be fermented in the reticulo-rumen consist of structural (cellulose, hemicellulose) and non-structural (soluble sugars, starch and pectin) carbohydrates, which are hydrolysed to mono- and disaccharides by microbial activity as an initial step in fermentation of feed. These monomers and dimers of sugars are absorbed by the microbes and partially oxidised to volatile fatty acids (VFAs [acetate, propionate, butyrate, lactate, valerate and other iso-acids]) and yield energy for their survival and growth. In the process of microbial fermentation under anaerobic conditions, reduced cofactors like NADH and NADPH are produced. For re-use in the anaerobic system, these reduced cofactors are oxidised to NAD+ and NADP+ by electron transfer to terminal acceptors other than oxygen. As CO_2 is already available in the rumen, CH_4 generation is preferred over the other hydrogen sinks (e.g. SO_4, NO_3, fumarate etc.) and therefore a significant portion of energy is wasted in

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Table 1. Methane emissions from Indian livestock (data from IVRI, Izatnagar).

Animal	BWt (kg)	Methane (L/h/d)	Methane (%GE)	Methane (kg/h/y)
Sheep	13–44	10–15.7	3.0–12.7	2.6–4.1
Goat	20–32	12.6–16.5	5.2–7.5	3.3–4.3
Cattle	220–360	98–221	3.6–8.4	25.6–47.6
Buffalo	200–501	111–202	3.9–9.0	28.9–52.7

the form of CH_4 (Johnson and Johnson, 1995) as it cannot be oxidised further in the rumen under anaerobic conditions. As the global warming potential of CH_4 is 23 times greater than that of carbon dioxide (IPCC, 2001), it is essential that its level in the atmosphere is kept within safe limits.

This paper describes studies to evaluate different strategies to control CH_4 emissions from the animals while promoting economic and eco-friendly livestock production.

STRATEGIES TO MITIGATE METHANOGENESIS

Replacement of Non-productive Animals

Non-productive or low productive animals should be replaced with either high producing indigenous cattle/buffalo or high producing crossbred cattle. This is not an easy task to accomplish in Indian conditions as non-productive and low producing animals cannot be removed immediately due to the ban on cow slaughter. However, this might be achieved slowly by replacing existing poor performers with high producing cattle. If a target is set to increase the number of high producing cattle from the present figure of 23 million to 46 million within the next five years, excretion of wastes and CH_4 would increase by only 3.2% and 1.7% respectively, but excreta and CH_4 produced/unit of livestock productivity would be considerably reduced.

Dietary Manipulations

Energy level in the diet and the type of roughage used (wheat straw, paddy straw or sugarcane bagasse) have significant effects on the extent of methanogenesis, while the level of protein does not appear to be important if it meets the minimum requirement of the animals (Chaterjee et al., 2006). Sugarcane bagasse caused the highest production of CH_4/unit digested dry matter (DM) and wheat straw the minimum, while CH_4 generation was intermediate with paddy straw as the roughage source. Sugarcane bagasse and paddy straw as substrate produced 11% and 4% more CH_4 than wheat straw. The *in vitro* evaluation of oilseed cakes revealed minimum CH_4 production with caster bean cake and karanj seed cake (20–21 ml/g DM), maximum with soybean cake (31 ml/g DM) and intermediate production with mustard, cotton and groundnut cakes. However, reductions in CH_4 production were accompanied by lower *in vitro* degradability which might be due to the presence of antinutritional factors in these two cakes (Kumar et al., 2007).

Improvement in the digestibility of lignocellulosic feeds with different treatments also results in lower methanogenesis by livestock. Wheat straw treated with urea (4 kg urea/100 kg wheat straw) or urea plus calcium hydroxide (3 kg urea + 3 kg calcium hydroxide/100 kg wheat straw) and stored for 21 d before feeding, reduced significantly the CH_4 emissions by sheep (Sahoo et al., 2000).

Beauchemin and McGinn (2006) reported that adding canola oil at the rate of 4.6% DM intake (DMI) inhibited CH_4 emissions by 32% and as a percent gross energy intake by 21%, but the decreases were attributed primarily to reduced feed intake and lower total digestibility of feed, especially the fibre component. On the other hand, in a study conducted by Cosgrove et al. (2008), infusion of a blend of linseed and sunflower oils (3:1) in the rumen of eight-month old wether sheep at the rates varying from 1.2% up to 6.2% of DMI did not affect CH_4 emissions, DMI and the concentrations and proportions of VFAs. There was, however, a loss of 7.5% gross energy in the form of CH_4 which was attributed to the presence of long chain fatty acids in these oil plants.

Fumaric acid is a precursor of propionic acid during feed fermentation in the rumen and may act as an alternative hydrogen sink. Its inclusion in the diet increased total VFA concentration, increased propionate proportion and decreased the acetate:propionate ratio (Beauchemin and McGinn, 2006), but the levels required to inhibit methanogenesis to a significant extent may cause a drop in pH which might adversely affect feed fermentation. Wallace et al. (2006) reported that encapsulating fumaric acid in a shell of hydrogenated vegetable oil prevented a fall in pH, but retained its ability to inhibit methanogenesis. Free fumaric acid (10% in the ration) and an equivalent amount of encapsulated fumaric acid decreased CH_4 emission by 49% and 75% respectively compared with control sheep. In wether lambs there was a CH_4 emission (g/d) which decreased linearly with increasing dose of fumaric acid (zero to 10% of the diet), but when expressed in terms of g/kg DMI, emissions were similar in all groups. Interestingly there was also a linear increase in rumen pH with increasing dose of fumaric acid, a finding which contrasts with other studies (Molano et al., 2008).

Plant Secondary Metabolites

Plant secondary metabolites (saponins, tannins, lignins, essential oils etc.) have anti-microbial activities to protect plants against invasion by microbes. This property has been exploited for controlling undesirable microbes in the rumen. Initial screening experiments have indicated that extracts of saponin-rich plants like *Sapindus mukorossi, S. saponaria* and *Acacia concinna* (Hess et al., 2004; Agarwal et al., 2006; Patra et al., 2006b), essential oil-rich plants like *Allium sativum, Coriandrum sativum, Eucalyptus globulus, Foeniculum vulgare, Mentha piperita, Ocimum sanctum, Populus deltoids* and *Syzygium aromaticum* (Busquet et al., 2006; Patra et al., 2006c; Agarwal et al., 2009) and tannin-rich plants like *Bergenia crassifolia, Emblica officinalis, Peltiphyllum peltatum, Populus deltoides, Quercus incana, Rheum undulatum, Terminalia belerica, Terminalia chebula* and *Vaccinium vitis-idaea* (Patra et al., 2006a and b; Kamra *et* al., 2006; Jayanegara et al., 2009) are examples of products which exhibited antimethanogenic and antiprotozoal activities. However, some of them also had adverse effects on feed degradability and nutrient utilisation by the animals.

Based on the results of *in vitro* screening experiments, a few plants and some mixtures of plants were selected for inclusion in

Table 2. Effect of plant secondary metabolites on *in vivo* CH_4 emissions and feed DM digestibility.

Plant	Methane inhibition (%)	DM Digestibility (%)	Animal	Reference
Mixture of *Allium sativum, Syzygium aromaticum, Foeniculum vulgare* and *Mentha piperita* (3 g/100 kg BWt)	No effect	No effect	Buffalo	Agarwal et al., 2007
Terminalia chebula (1% DMI)	24.0	11.3 (+)		
Allium sativum (1% DMI)	11.9	11.1 (+)	Sheep	Patra et al., 2008
Terminalia chebula and *Allium sativum* (0.5% each of DMI)	23.5	10.6 (+)		
Allium sativum and *Mentha piperita* (1% and 0.1% of DMI on alternate d) (Mix 1)	7.0	No effect	Buffalo	Verma et al., 2009
Mixture of three plants (2% of DMI on alternate days) (Mix 2)	12.0	No effect	Cattle calves	Unpublished
Mixture of three plants (1% of DMI/d) (Mix 3)	11.0	No effect		
Mixture of three plants (2% of DMI/d) (Mix 3)	15.5	No effect	Buffalo calves	Chaudhary et al., 2009
Mixture of three plants (3% of DMI/d) (Mix 3)	27.8	No effect		
Canola oil	32.0	Reduction	Growing beef cattle	Beauchemin and McGinn (2006)
A blend of linseed and sunflower oils (3:1) (Infused at 1.2% - 6.2% DMI)	No effect	No effect	sheep	Cosgrove et al., 2008

the diets of ruminants to study their effect on *in vivo* CH_4 emissions using open circuit respiration calorimetry. A mixture of *Allium sativum, Syzygium aromaticum, Foeniculum vulgare* and *Mentha piperita* (oil) in the ratio of 2:1:2:1 respectively, was fed to buffaloes at the rate of 3 g/100 kg BWt (**Table 2**). Each plant individually inhibited *in vitro* methanogenesis, but in *in vivo* studies there was no effect on CH_4 emissions, VFAs, microbial profiles and nutrient digestibilities (Agarwal et al., 2007). This might be due to insufficient levels of plant secondary metabolites being fed to the animals, since the level of secondary metabolites present in the additive and the nature of diet are some of the important factors influencing animal responses (Calsamiglia et al., 2007).

Terminalia chebula, Allium sativum and a mixture of the two, when fed to sheep at the rate of one percent DMI, decreased CH_4 production (L/kg digestible DMI) by 24%, 11% and 23.5%, respectively, but CH_4 energy losses as a percentage of digestible energy intake decreased (P = 0.08) in the groups fed *T. chebula* and the mixture compared with the control and *A. satium* fed groups (Patra et al., 2008). Both these plants separately and as a mixture caused an improvement of between 10.6% and 11.3% in feed DM digestibility (**Table 2**). *T. chebula* is a rich source of tannins (4.89% DM), whereas *A. satium* is rich in essential oils. The data indicated that *T. chebula* was more effective than garlic. The low *A. sativum* activity might be explained by the instability of allicin, the main secondary metabolite responsible for the antimicrobial activity of *A. sativum*.

Murrah buffaloes fed a wheat straw and concentrate mixture (50:50) and supplemented with a feed additive (a mixture of *Allium sativum* [1%] and *Mentha piperita* oil [0.1%] of DMI, [Mix 1]) on alternate d reduced CH_4 emissions by seven percent (L/kg DMI), but these reductions were attributed to lower DMI (Verma et al., 2009). There was no adverse effect on rumen fermentation pattern, enzyme and microbial profiles. In another experiment, a mixture of three plants (Mix 2) fed to calves on alternate d at the rate of 2% DMI caused a 9.4% fall in CH_4 emissions and BWt gain was 8.7% (448 g/day vs 412 g/d) higher than in controls, with no adverse effect on digestibility of nutrients (unpublished data).

In the authors' laboratory, a mixture of three plants (Mix 3) fed to buffalo calves at the rate of one, two and three percent of DMI, resulted in a dose-dependent inhibition of CH_4 emissions (L/kg digestible DM) without affecting DM digestibility at any feed additive levels (Chaudhary et al., 2009). Volatile fatty acid and fibre degrading enzyme activities were not affected, although there were a few changes in rumen microbial profiles as estimated by real time-polymerase chain reaction (RT-PCR), but these were not responsible for any significant change in rumen fermentation.

CONCLUSIONS

Experiments conducted so far indicate that the type of diet, feed additive, roughage level and type, the nature of the oil cake including their residual fatty acids are some of the important factors which affect rumen fermentation and CH_4 emissions by livestock. Therefore, it is very important to formulate diets by selecting ingredients which are poor CH_4 producers but are easily available to the farmer.

The results of *in vivo* experiments indicate that plants containing secondary metabolites which show promising results in *in vitro* experiments, do have a potential as rumen modifiers for controlling CH_4 emissions by ruminants. The levels used in *in vitro* experiments are usually very high and may not be usable in *in vivo* experiments. There is an interaction between feed additive and the diet fed to the animal. Therefore, levels of plant additives have to be standardised for different diets for practical application, in order to obtain significant inhibition of methanogenesis without adversely affecting nutrient utilisation.

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Correlation Between Milk and Blood Urea Nitrogen in High and Low Yielding Dairy Cows

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ABSTRACT

A study was carried out in two dairy farms (Olocuilta and Los Conacastes) in the central region of El Salvador. Sixty Holstein cows were grouped according to milk yield and days in milk: high yielding (HY, 30–90 d in milk) and low yielding cows (LY, >180 d in milk). The objective of the study was to evaluate the effect of milk yield and time after feeding on milk and blood urea-nitrogen (BUN) concentration, and to establish a correlation between these two parameters. On Olocuilta, HY cows had the highest BUN and milk urea nitrogen (MUN) concentrations. Blood urea nitrogen least squares concentration was 12.77 mg/dL and 13.98 mg/dL for the LY and HY cows, respectively; while the MUN average concentration was 12.30 mg/dL and 14.82 mg/dL for the LY and HY cows, respectively. BUN and MUN concentrations were similar at 30 min, one and two h post-feeding but by four h post-feeding BUN concentrations had decreased and were significantly lower than those of MUN ($P < 0.05$). On the other hand, in Conacastes the highest values were found for the LY group. BUN least square concentration was 11.22 mg/dL and 9.12 mg/dL for the LY and HY cows, respectively; while the MUN average concentration was 10.18 mg/dL and 8.83 mg/dL for the LY and HY cows, respectively. The reason for these differences seems to be related to protein balance. For instance on the Los Conacastes farm, protein balance was negative in the HY group (-88 g/d) while on Oloculita farm the balance was positive. The correlation between BUN and MUN for the Olocuilta farm had a regression coefficient of 0.84, and a correlation (r^2) of 0.7543. For Los Conacastes these values were 1.04 and 0.9017, respectively. It should be noted that BUN and MUN concentrations were better correlated at 30 min, one h and two h after feeding and that the correlation decreased at four h post-feeding due to a drop in BUN concentration. It is concluded that BUN and MUN concentrations are not related directly to milk yield but with the protein balance. There was a high correlation between BUN and MUN concentrations; hence, either of these parameters can be used to monitor protein nutrition in dairy farms.

Key words: *Holstein cows, milk yield, time of feeding, blood urea nitrogen, milk urea nitrogen, protein nutrition.*

INTRODUCTION

Dairy cows need appropriate quantities of protein for milk production and for this reason diets are supplemented with additional protein. However, excess protein can negatively affect production and reproduction, and pollute the environment (Sonderegger and Schurch, 1977; Peabody, 2004). When an excess of degradable protein relative to energy is present in the rumen, the concentration of rumen ammonia increases and elevates rumen pH (Gómez and Fernández, 2002). Some of the ammonia liberated in the rumen cannot be fixed by the microorganisms; this excess is absorbed and taken into the blood. The liver converts ammonia to urea which is excreted by the animal in the urine (Garriz and López, 2002). High concentrations of urea reflect an excess of protein in the diet, which can adversely affect fertility (Ropstad and Refsdal, 1987; Melendez et al., 2000; Nousiainen et al, 2004). An urea-nitrogen concentration higher than 20 mg/dL of milk suggests an excess protein supply in the diet which can decrease production and cause fertility problems (Ferguson and Chalupa 1989; Hojman et al., 2004). This also makes the diet more expensive and increases nitrogen excretion to the environment (Jonker et al., 1998). Measurement of urea nitrogen in blood and milk has been proposed as a tool to monitor protein nutrition (Ferguson et al., 1993; Hof et al., 1997).

Blood urea gets transported into milk and therefore urea is a normal constituent of milk (Ferguson, 2002; Acosta and Delucchi, 2002). The relationship between the levels of (BUN) and MUN in dairy cattle depends on the degradability of the different protein sources and nitrogen compounds (Acosta and Delucchi, 2002). Since it is both simpler and less stressful to take milk than blood samples (Acosta and Delucchi, 2002; Acosta et al., 2006), measurement of MUN is an easy method for determining BUN levels and for assessing the protein and energy supply in the diet. By determining MUN levels, milk producers could be advised on the appropriateness of different diets for providing a proper protein to energy ratio.

The main objective of this investigation was to establish correlations between BUN and MUN concentrations in dairy cows at two levels of milk production i.e. high and low, and at different times after feeding.

MATERIALS AND METHODS

Animals and Feeding

The study was carried out from December 2006 to July 2007 on two dairy farms: Ranch Olocuilta and Ranch Los Conacastes located in the central region of El Salvador. The herds had more than 80% of Holstein genetic make up and were producing more than 15 kg milk/cow/d. The cows were kept and managed in free stall barns. The feed offered consisted of forage (silage or green grass) and concentrate. Thirty cows were selected on each farm. Fifty percent of the cows on each farm were between 30–90 d in milk (high production) and the other 50% were > 150 d in milk (low production).

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Collection and Analysis of Milk

Milk and blood samples were taken at 30 min, one, two, and four h after feeding. Blood samples were taken from the jugular vein into vacutainer tubes without an anticoagulant. Milk samples (50 mL) were obtained directly from the udder.

After clotting, blood samples were centrifuged at 7 000 rpm for 15 min., and sera collected and stored in cryovials at -20°C for later analysis. BUN concentration was determined by means of the Liquicolor Enzymatic Colorimetric Test (Human ®,Damstad, Germany). A total of 240 samples were analysed.

MUN concentrations were determined in the clear medium obtained after precipitation of proteins using trichloroacetic acid for 15 min and then centrifuged at 4 000 rpm for 10 min and after filtering the supernatant and diluting it to 1:100 with distilled water. Urea nitrogen was analysed using the technique described by Merck® (Darmstat, Germany). Urea standards were prepared at concentrations of 1–5 ppm. A graph relating urea concentration (ppm) and absorbance was drawn to obtain the equation $y = ax + b$; in which x represents the urea concentration and y represents the absorbance. Urea (mg/dL = (*Absorbance-b*) /*a*. The urea level in mg/dL was multiplied by 4.16 to obtain MUN.

Feed Analyses

Feeds were analysed for crude protein (CP), neutral detergent fibre (NDF) and acid detergent fibre (ADF). Information on the characteristics of the cows and the proportions and composition of ingredients was entered into the NRC Dairy Cattle Program (NRC, 2001) to obtain reports on the protein and energy balances of the high and low producing cows.

Statistical Analyses

The effect of milk yield (high and low) and time after feeding (0.5, one, two and four h) on BUN and MUN concentrations were analysed using repeated measures analysis and the MIXED procedure of SAS (SAS Institute, Version 9.1.3, 2006). Variables for which variance analysis were significant at $P < 0.001$, were subjected to a test of comparison using the Student's t-test. Data were also subjected to linear regression to find the correlation and regression coefficients between BUN and MUN. In this case the GLM procedure of SAS was used.

RESULTS AND DISCUSSION

Protein Balance

On Olocuilta, the protein content in the diet was higher for the high yielding cows than for the low yielding and the same pattern was found for the protein balance (**Table 1**). On Conacastes, the protein percentage was similar for the two groups, while protein balance was positive in the low yielding cows but negative in the high yielding cows. These results showed that high producing cows are not necessarily those that receive more protein or have a more positive protein balance.

Blood Urea Nitrogen (BUN)

High yielding cows on Olocuilta had higher average values of BUN than low yielding cows (13.94 vs 12.75 mg/dL, $P < 0.05$). These levels are within the acceptable range of 10–20 mg/dL (Ferguson et al., 1993), and reflect the fact that the protein levels in the diet were adequate. On Conacastes on the other hand, it was found that high producing cows had lower values of BUN than low producing cows (9.09 vs 11.20 mg/dL, $P < 0.05$); this could be explained by the negative protein balance in high yielding cows.

Milk Urea Nitrogen (MUN)

The pattern of MUN was similar to that of BUN. On Olocuilta, high yielding cows had higher ($P < 0.05$) values of BUN (average 14.99 mg/dL) than those of low yielding ones (average 12.26 mg/dL) throughout the sampling period (**Figure 1**). However, for both productive states the values were within the normal range (10–20 mg/dL).

On Conacastes, higher mean values for MUN were obtained in the low yielding group (10.15 mg/dL). The MUN for the high yielding group was 8.91 mg/dL. It has been reported that MUN concentra-

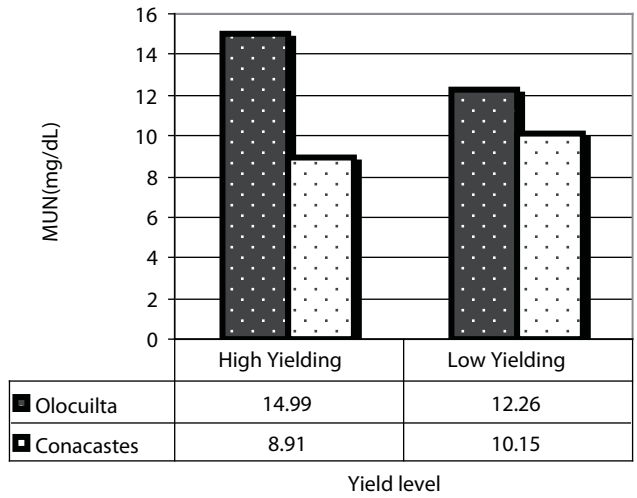

Figure 1. Milk urea nitrogen (mg/dL) for high and low yielding dairy cows on two farms.

Table 1. Milk production, nutritional composition of the diet and protein balance on two farms.

	Low yielding group		High yielding group	
	Olocuilta	Los Conacastes	Olocuilta	Los Conacastes
Milk yield, kg/d	16.50	11.40	22.25	19.00
NDF (% DM)	42.30	42.20	41.40	44.30
NE_l (Mcal/kg) DM)	1.48	1.55	1.47	1.57
Crude protein (% DM)	15.90	13.80	16.40	13.70
Crude protein balance (g/d)	428	401	537	–88

tions usually fall at the beginning and the end of lactation rather than in mid lactation. Probably these observed variations respond more to changes in the nutrient demand during the postpartum period than to changes in the diet (Acosta and Delucchi, 2002).

Time after-Feeding

Urea concentrations were compared in blood and milk at different times after feeding. For the Olocuilta farm these values ranged from 12.8 to 13.8 mg/dL (**Figure 2**). BUN and MUN values were similar for high and low producing cows at 30 min, one and two h after feeding. However, by four h post-feeding, BUN concentrations had decreased and were lower than those in milk ($P < 0.05$). This difference could be due to changes in absorption or production of urea that are quickly reflected in blood levels but not in previously synthesised and stored milk.

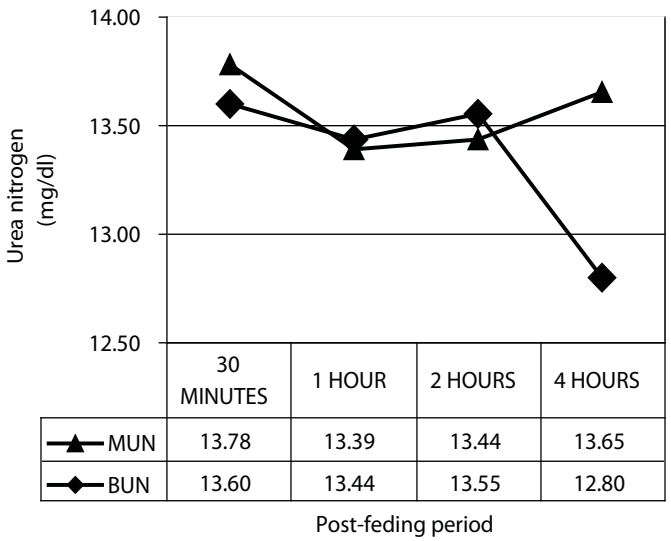

Figure 2. BUN and MUN concentrations at different times after feeding dairy cows on Olocuilta farm.

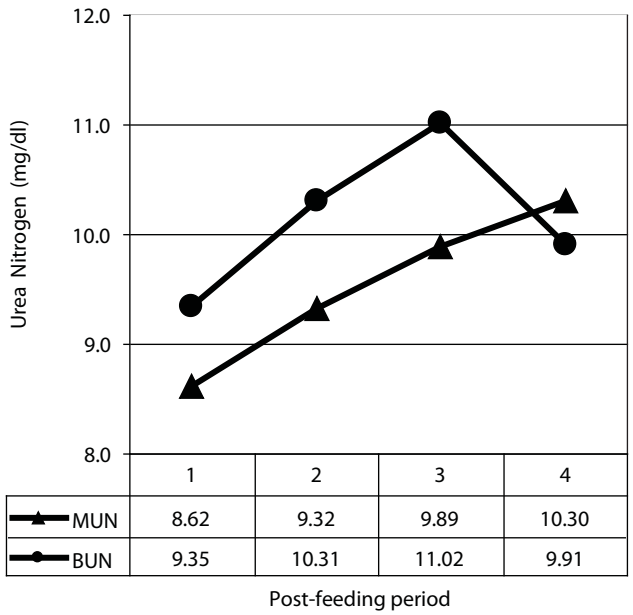

Figure 3. BUN and MUN concentrations at different times after feeding dairy cows on Conacastes farm.

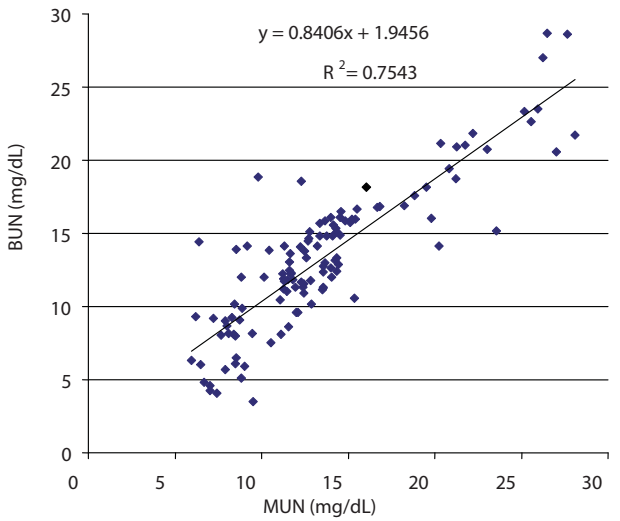

Figure 4. Correlation between BUN and MUN concentrations for dairy cows in Olocuilta farm.

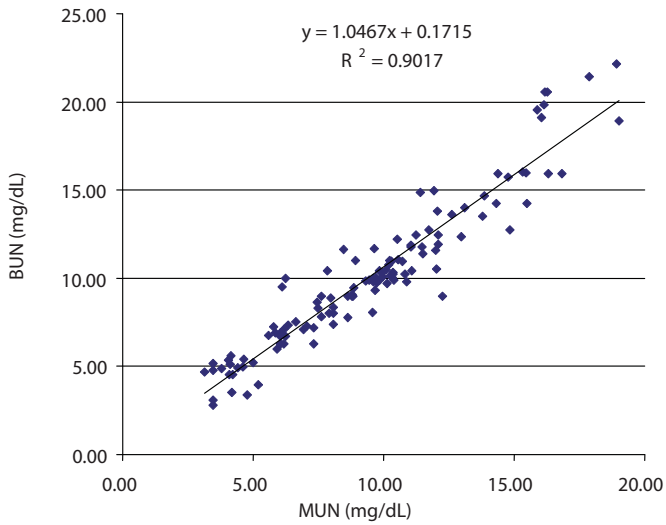

Figure 5. Correlation between BUN and MUN concentrations for dairy cows on Los Conacastes farm.

On Los Conacastes farm, BUN concentrations tended to be higher than those of MUN (**Figure 3**) and as observed on Olocuilta farm, a decrease was observed in concentration at four h post-feeding. However, BUN and MUN concentrations were statistically similar at all times after feeding. Butler (1998) reported elevations of BUN at four and eight h after feeding a 19% CP total mixed ration. However, the CP content in the present study was lower (**Table 1**).

Noticeable was how BUN values increased up to three h post-feeding and then started to decrease while MUN values increased steadily. These results suggest that MUN measurements have an advantage over BUN since milk samples can be taken at any time

after feeding. In other words, MUN values are better for predicting the nutritional status of the animals.

Correlation between BUN and MUN

Figure 4 shows the correlation between BUN and MUN concentration considering the values at all time periods after feeding. For Olocuilta farm, the regression coefficient between BUN and MUN was 0.84 (slope of the curve), while the correlation was 0.7543 (r2) (**Figure 4**). It should be noted that BUN and MUN concentrations were better correlated at 30 min, one, and two h after feeding.

The relationship between BUN and MUN for animals on Conacastes farm is shown in **Figure 5**. In this case the correlation was 0.90 (r^2) and the regression coefficient was 1.04. The values for MUN and BUN were almost identical (9.53 mg/dL and 10.15 mg/dL, respectively).

When all blood and milk determinations were compared using the Student´s test the association between both methods was high ($P < 0.05$), and when averages were compared they are almost the same for MUN (13.53 mg/dL) and BUN (13.35 mg/dL). Therefore, either measurement can be used to determine protein status using urea nitrogen although milk sampling is both simpler and causes no stress on the animals. Nonetheless, BUN values tend to decrease when sampling four h post-feeding, while that variation was not seen when determining MUN concentrations.

It has been established that urea balances quickly with other body fluids including milk, and that a relationship between BUN and MUN can be calculated (Broderick and Clayton, 1997). MUN values represent 83–98% of BUN values and hence by dividing MUN by 0.85 a good estimate of BUN can be obtained (Arias and Nesti de Alonso, 1999). The results obtained in this study are in accordance with this statement.

When comparing BUN concentration for the two farms, the values differed significantly ($P < 0.05$) with means being higher in farm Olocuilta (13.34 mg/dL vs. 10.14 mg/dL).

CONCLUSIONS

The determination of urea in milk is a useful tool for monitoring protein nutrition in dairy cows since is a reliable technique and highly correlated with BUN. In the nutritional management of dairy cows diets should be balanced based on the analysis of the feedstuff to have a good approach of the balance of the nutrients in the animal. For determinations of BUN, it is better to carry out the samplings before two h after feeding; for MUN, sampling can be carried out until four h after feeding without significantly altering the values.

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Use of Sodium or Calcium Salts of Fatty Acids as Sources of Energy in Buffalo Rations during Late Pregnancy

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ABSTRACT

Thirty pregnant buffaloes expected to calve within 60-75 days were divided into three groups balanced by bodyweight and expected date of parturition. The first group received a control ration consisting of concentrate diet (75% concentrate feed mixture with 25% yellow corn) plus berseem (*Trifolim alexandrinum*) hay and rice straw. In the second and third groups, yellow corn was replaced with either sodium salts of fatty acids (Na-SFA) or calcium salts of fatty acids (Ca-SFA). The content of acid ether extract (AEE) in Ca-SFA was lower than that of Na-SFA, while TFA's in Ca-SFA were higher. Degradability rates of dry matter (DM), organic matter (OM), crude protein (CP), effective degradability (ED) and potential degradability (PD) decreased with the ration containing Na-SFA. Undegradable values of DM, OM and CP increased with adding Na-SFA compared with adding Ca-SFA or the control diet. Digestion coefficients of DM, OM, CP and cell wall constituents (CWC) were lower with feeding the ration containing Na-SFA compared with that containing Ca-SFA, while no significant differences were found between the control and Ca-SFA-containing rations. Values for total digestible nutrients (TDN) and digestible crude protein (DCP) were reduced ($P<0.05$) with the ration contained Na-SFA compared with Ca-SFA. Feed intake was not affected by feeding rations containing Na-SFA or Ca-SFA, but bodyweight (BWt) was higher after feeding rations containing Ca-SFA or Na-SFA compared with the control. pH values, propionic acid and free fatty acids (FFA's) in the rumen were higher ($P<0.05$) when feeding the ration containing Na-SFA compared with that containing Ca-SFA or the control, while total volatile fatty acids (TVFAs), acetic, Ac:Pr ratio and NH3-N were significantly decreased. Adding Na-SFA in the ration decreased glucose and total protein concentrations in blood compared with Ca-SFA or in the control. Concentrations of albumin, globulin and their ratio were not affected with feeding rations containing either Na-SFA or Ca-SFA while levels of total lipid (TL), triglyceride and FFAs were higher ($P<0.05$) with feeding rations containing fat than with the control ration.

Key words: *Buffaloes, soapstock, fatty acids, feed intake, bodyweight, pregnancy.*

INTRODUCTION

Diets based on bulky feed resources are unsuitable for pregnant animals if they are not supplemented with a high proportion of concentrates (Mahouachi et al., 2004). It is expected that added fat is generally favourable to foetal development, mammary adipose tissue and subsequent milk production, especially for late pregnant buffalo. Soapstock (sodium salts of fatty acids, Na-SFA) is the waste generated in the mill during refining of crude oil when sodium hydroxide reacts with the free fatty acids in the oil (Khattab et al., 2001). Significant amounts of soapstock are produced from the processes of refining seeds for oil, and while these by-products are potentially harmful to the environment, they are by-products which are potentially available as dietary fat sources (Shain et al., 1993). Soapstock as fatty acids has a higher inhibitory effect on rumen microbes than in the form of triglycerides (Wu et al., 1993). Adding either 2.5 or 5% dietary soapstock on a DM basis to the diets of beef cattle tends to decrease rumen digestibilities of CP and crude fibre (Perry and Weatherly, 1976).

The aim of this study was to examine the impacts on rumen functions and performance of adding Na-SFA or Ca-SFA as an energy source instead of corn grains to the rations of late pregnant buffaloes.

MATERIALS AND METHODS

Palm oil and sunflower oil soapstocks were air dried and the resulting lumps were then broken in a hammer mill and mixed 1:1 on a DM basis with other concentrate ingredients in a granular form of 3 mm diameter or converted to calcium salts of fatty acids (Ca-SFA) according to El-Bedawy et al. (2005).

Animals and Diets

Thirty pregnant buffaloes in their third lactation and that were expected to calve within 60–75 d were divided into three groups according to BWt and expected date of parturition and penned in ventilated sheds. Three concentrate portions of the diets were formulated and pelleted in a feed mill, Diet 1 consisted of a 75% concentrate feed mixture (CFM) containing 29% cottonseed meal, 26% yellow corn, 35% wheat bran, 6% molasses, 3% limestone and 1% common salt, and 25% yellow corn. In Diets 2 and 3, either Na-SFA or Ca-SFA replaced 100% of corn energy, respectively. The diets were formulated and adjusted biweekly according to changes in BWt by adding berseem hay and rice straw to cover animal requirements according to NRC standards (1988).

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Digestibility Trials

Digestibility trials were carried out at the end of the experimental period using three replicates and applying the acid insoluble ash (AIA) technique suggested by Van Keulen and Young (1977). Acidified ether extract (AEE) was determined by a modified method described by (Abo-Donia et al., 2003). Samples of feeds or residues, faeces and urine were subjected to proximate analysis (AOAC., 1990). Fibre fractions as neutral detergent-fibre (NDF), acid detergent fibre (ADF) and acid detergent-lignin (ADL) were determined according to Goring and Van Soest (1970). Hemicellulose and cellulose were calculated as the difference between NDF, ADF and ADL. Gross energy (GE) of feed and faeces were determined using a Gollen Kump ballistic bomb calorimeter, (catalogue No CCBB: 33-0101). Total fatty acids (TFAs) in Na-SFA and Ca-SFA were determined according to AOCS (2000), while in feed and faeces as described by Sukhija and Palmquist (1988).

Blood Analyses

Blood plasma samples were withdrawn from three buffaloes at the end of the feeding period 4 h after feeding. Total protein, albumin, glucose, total lipids (TLs) and triglycerides (TGs) were determined calorimetrically using commercial kits (Bio Merieux 69280 Marcy-1, Etoile/France). Globulin was obtained by subtracting the albumin value from the total protein concentration and the albumin:globulin ratio was calculated by dividing albumin by corresponding globulin values. Total fatty acids (long-chain) in blood serum were determined according to Itaya and Ui (1965).

Rumen Degradability and Analysis of Rumen Fluids

Six male sheep equipped with a permanent rumen cannula (50 mm. inner diameter) were used for the *in-situ* trials and fed berseem hay (*Trifolim alexandrinum*) to cover their maintenance requirements. Nylon cloth (100% polyester) with a mean pore size of 120 µm was used for constructing the *in-situ* bags (8 × 10 cm) with nylon threads. Double bags containing approximately 4 g of dried experimental rations were incubated for 8, 16, 32, 48, 64 and 72 h to determine DM and OM degradability rates. A further 4 g were incubated for measuring protein degradability at the same times. Dry matter, OM and N were estimated according to the methods of AOAC (1990).

The data were fitted to the model of McDonald (1981) $Y = a + b\,(1–e^{-c\,(t-tl)})$ where:

Table 1. Chemical composition of Na-SFA and Ca-SFA (% DM basis).

Composition	DM	OM	AEE	TFAs[a]	OL[b]	Ash	CE Mcal/kg
Na-SFA[c]	61.73	92.60	81.00	68.95	12.05	7.40	6.562
Ca-SFA[d]	94.78	81.39	78.22	76.96	1.27	18.61	7.402

a = total fatty acids, b = other lipids, c = sodium salts of fatty acids, and d = calcium salts of fatty acids.

Table 2. Formulation and chemical composition of different rations and roughages (DM basis).

Ingredients	Control	Na-SFA	Ca-SFA	BH[d]	RS[e]
Content of ingredients (%)					
Con1[a]	42.86	—	—	—	—
Con2[b]	—	42.86	—-	—	—
Con3[c]	—	—	42.86	—	—
BH	14.29	14.29	14.29	—	—
RS	42.86	42.86	42.86	—	—
Chemical composition (%)					
DM	89.98	87.85	90.20	88.00	90.00
OM	87.67	86.93	86.20	86.00	83.50
CP	10.09	9.77	9.91	13.70	3.51
AEE	2.32	7.78	6.96	1.80	1.30
TFA	1.63	6.26	6.22	1.01	0.63
OL	0.69	1.52	0.74	0.79	0.67
Ash	12.33	13.07	13.80	14.00	16.50
Cell wall constituents (%)					
NDF	44.04	43.67	43.82	51.24	68.43
ADF	34.55	34.20	34.32	39.87	54.39
Cellulose	30.96	30.62	30.72	36.75	48.32
Hemicellulose	9.49	9.47	9.51	11.37	14.04
GE (Mcal)	3.884	4.028	4.060	3.856	3.647

a = concentrate without fat, b = concentrate containing Na-SFA, c = concentrate containing Ca-SFA, d = berseem hay and e = rice straw.

Y = degradability at time (t), a = the zero time intercept, b = potentially degradable fraction, c = rate of degradation of b and tl = lag time.

Samples of rumen fluids were withdrawn individually before feeding then after 4 h and 8 h after feeding from cannulated rams that were fed the experimental rations (3 for each group) for two weeks as an adaptation period at the end of the feeding trials. Rumen pH was immediately determined by the HANNA-Ph meter, model HI8424, total VFA concentration as described by Eadie et al. (1967) and VFA fractions (C2, C3 and C4) analysed according to Erwin et al. (1961). Free fatty acids (long-chain) in rumen liquor were determined using the method of Itaya and Ui (1965), and ammonia concentrations using the Conway method (1978).

Statistical Analyses

Results obtained were subjected to analysis of variance according to SAS (2000), and treatment means were ranked using Duncan's multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

Composition of Soapstocks, Rations and Roughages

The data in **Tables 1** and **2** show that the AEE content in Ca-SFA was lower than that in Na-SFA, while the reverse was the case for TFA's. The lower content of AEE in Ca-SFA was due to its higher ash content, while the lower content of TFA's in Na-SFA was due to the higher content of other lipids such as pigments, wax, etc, which are concentrated in Na-SFA during refining oils. As a result of the loss of large amounts of other lipids with washing water while converting Na-SFA to Ca-SFA, the levels were 1.27% and 12.05% for Ca-SFA and Na-SFA, respectively. The higher ash content in the ration containing Ca-SFA was related to higher ash content in Ca-SFA (18.61%). The content of NDF and ADF in experimental rations was lowered by adding either Na-SFA or Ca-SFA.

Rumen Breakdown of Rations

Rates of DM, OM and CP disappearance (**Table 3**) at different times after incubation in the rumen for rations contained Na-SFA were lower ($P<0.05$) than the corresponding values for Ca-SFA or control. However, no differences ($P>0.05$) were found between Ca-SFA and the control. Reduced DM, OM and CP breakdown in the rumen with added Na-SFA may be due to fatty acids interfering with the digestibility of feed components by inhibiting the activity of rumen microorganisms concerned with cellulose digestion or by reducing the retention of calcium due to excessive excretion of soap in the faeces (Roberts and McKirdy, 1965). Foaming and physical coating of dietary fibre with added Na-SFA has been proposed as a possible mechanism for the sometimes depressed DM, OM and CP disappearances observed under these conditions (Devendra and Lewis, 1974).

Degradation Kinetics

The rapidly degradable fraction (a) and slowly degradable fraction (b) of DM, OM and CP were similar with the Ca-SFA and control rations, and were reduced ($P<0.05$) with the ration containing Na-SFA (**Table 4**). These results might be due to Na-SFA having a higher inhibitory effect on rumen microbes than the triglyceride form, as suggested by Wu et al. (1993). Perry and Weatherly (1976) reported that either 2.5 or 5% dietary soapstock (DM basis) tended to decrease ruminal digestibilities of CP and CF when fed to beef cattle. Effective degradability (ED) and potential degradability (PD) of DM, OM and CP were significantly lowered ($P<0.05$) with adding Na-SFA compared with Ca-SFA rations. Undegradable DM, OM and CP were

Table 3. Mean values of fitted DM, OM, CP, NDF and ADF % disappearance for experimental rations at different intervals.

Disappearance	8h	16h	32h	48h	64h	72h
Dry matter (%)						
Control	23.97 ^{a}	37.73 ^{a}	56.93 ^{a}	68.73 ^{a}	75.93 ^{a}	78.43 ^{a}
Na-SFA	20.37 ^{b}	32.07 ^{b}	48.37 ^{b}	58.40 ^{b}	64.53 ^{b}	66.63 ^{b}
Ca-SFA	23.90 ^{a}	37.60 ^{a}	56.73 ^{a}	68.47 ^{a}	75.67 ^{a}	78.17 ^{a}
± SE	± 0.76	± 1.20	± 1.82	± 2.20	± 2.43	± 2.52
P<	*	*	*	*	*	*
Organic matter (%)						
Control	26.77 ^{a}	42.90 ^{a}	64.37 ^{a}	76.63 ^{a}	83.67 ^{a}	85.97 ^{a}
Na-SFA	21.47 ^{b}	34.40 ^{b}	51.60 ^{b}	61.43 ^{b}	67.07 ^{b}	68.93 ^{b}
Ca-SFA	26.00 ^{a}	41.70 ^{a}	62.60 ^{a}	74.50 ^{a}	81.37 ^{a}	83.57 ^{a}
± SE	± 0.44	0.71 ±	± 1.04	± 1.26	± 1.35	± 1.40
P<	*	*	*	*	*	*
Crude protein (%)						
Control	19.77 ^{a}	31.07 ^{a}	46.87 ^{a}	56.60 ^{a}	62.53 ^{a}	64.57 ^{a}
Na-SFA	17.40 ^{b}	27.40 ^{b}	41.37 ^{b}	49.87 ^{b}	55.13 ^{b}	56.97 ^{b}
Ca-SFA	19.73 ^{a}	31.00 ^{a}	46.83 ^{a}	56.53 ^{a}	62.50 ^{a}	64.50 ^{a}
± SE	± 0.31	± 0.49	± 0.74	± 0.90	± 0.99	± 1.04
P<	*	*	*	*	*	*

^{a}, ^{b} means in the same column for each category with different superscript are significantly different ($P<0.05$).

Table 4. Rumen degradation kinetics (%) of tested rations incubated *in sacco.*

Degradation	WL (%)	a (%)	b (%)	c (%/h)	PD (%)	ED (3%h-1)	B (%)	UND (%)
Dry matter (%)								
Control	13.96[a]	6.43 [a]	81.00 [a]	0.031	87.43 [a]	47.27 [a]	73.47 [a]	26.53 [b]
Na-SFA	11.90[b]	5.47 [b]	68.83 [b]	0.031	74.30 [b]	40.17 [b]	62.40 [b]	37.60 [a]
Ca-SFA	13.97[a]	6.40 [a]	80.70 [a]	0.031	87.17 [a]	47.13 [a]	73.20 [a]	26.80[b]
± SE	± 0.45	± 0.22	± 2.58	± 0.00	± 2.80	± 1.51	± 2.36	± 2.36
P<	*	*	*	*	*	*	*	*
Organic matter (%)								
Control	15.74[a]	5.43 [a]	87.67 [a]	0.035	93.07 [a]	52.53 [a]	77.33 [a]	22.67 [b]
Na-SFA	12.63[b]	4.37 [b]	70.27 [b]	0.035	74.63 [b]	42.13 [b]	62.00 [b]	38.00 [a]
Ca-SFA	15.30[a]	5.30 [a]	85.20 [a]	0.035	90.50 [a]	51.07 [a]	75.20 [a]	24.80 [b]
± SE	± 0.25	± 0.10	± 1.42	± 0.00	± 1.53	± 0.87	± 1.26	± 1.26
P<	*	*	*	*	*	*	*	*
Crude protein (%)								
Control	11.50[a]	5.30 [a]	66.70 [a]	0.031	72.00 [a]	38.90 [a]	60.50 [a]	39.50 [b]
Na-SFA	10.17[b]	4.67 [b]	58.80 [b]	0.031	63.50 [b]	34.33 [b]	53.33 [b]	46.67 [a]
Ca-SFA	11.50[a]	5.27 [a]	66.60 [a]	0.031	71.90 [a]	38.90 [a]	60.40 [a]	39.60 [b]
± SE	± 0.18	± 0.09	± 1.06	± 0.00	± 1.15	± 0.62	± 0.96	± 0.96
P<	*	*	*	*	*	*	*	*

[a], [b] means in the same column for each category with different superscript are significantly different (P<0.05).

a = the zero time intercept, b = potentially degradable fraction, c = rate of degradation of b, B = degradation of water insoluble fraction, UND = undegradable components, ED = effective degradability and PD = potential degradability.

Table 5. Nutrient digestibility, cell wall constituent and nutritive value for tested rations.

Item	Control	Na-SFA	Ca-SFA	± SE	P<
Nutrient digestibility, (%)					
DM	69.64[a]	64.89[b]	68.92[a]	± 0.73	*
OM	72.11[a]	67.67[b]	70.92[a]	± 0.86	*
CP	61.25[a]	56.83[b]	60.90[a]	± 0.71	*
AEE	69.26[b]	75.21[a]	77.39[a]	± 1.92	*
Energy	68.66[a]	64.67 [b]	67.33[a]	± 0.74	*
Cell wall constituent (%)					
NDF	68.62[a]	62.26[b]	67.96[a]	± 1.35	*
ADF	65.45[a]	60.46[b]	64.92[a]	± 1.10	*
Cellulose	71.71[a]	66.30[b]	71.20[a]	± 1.21	*
Hemicellulose	79.76 [a]	68.56[b]	78.61[ab]	± 3.03	*
Nutritive value, (%)					
TDN	67.97 [a]	64.03[b]	66.66[a]	± 0.74	*
DCP	7.27 [a]	6.54[b]	7.09[a]	± 0.08	*

[a], [b] means in the same row with different superscript are significantly different (P<0.05).

higher (P<0.05) with the ration containing Na-SFA compared with both the ration containing Ca-SFA and the control ration, but similar (P > 0.05) between the control and Ca-SFA rations. These results indicate that rumen degradabilities of DM, OM and CP with the Na-SFA ration were less compared with the Ca-SFA ration.

The data in **Table 7** show increased FFAs in the rumen with feeding a diet containing Na-SFA which in turn led to a decreased concentration of NH_3-N, a result reflecting the impact of added Na-SFA on protein degradability in the rumen. At the same time, the values for TVFAs show the impact of feeding Na-SFA on fibre degradability

Table 6. Feed intake and change in BWt for pregnant females fed experimental rations.

Item	Control	Na-SFA	Ca-SFA	± SE	P<
Feed intake on basis DM (kg)					
Concentrate, CFM	3.00	3.00	3.00	—	—
Berseem hay, BH	1.00	1.00	1.00	—	—
Rice straw, RS	3.00	3.00	3.00	—	—
TDMI	7.00	7.00	7.00	—	—
Concentrate: roughages	0.75	0.75	0.75	—	—
Changes in BWt (kg)					
BwtBP	565.40	567.70	566.10	± 7.79	ns
BwtPP	522.80	523.30	518.70	± 7.75	ns
Changing	42.60^{c}	44.40^{b}	47.40^{a}	± 0.41	*
Av. BWt	544.10	545.50	542.40	± 7.77	ns
Av. BWt$^{0.75}$	122.70	112.90	112.40	± 1.24	ns
Birth weight (kg)	34.30^{c}	36.00^{b}	38.80^{a}	± 0.38	*
Duration (day)	67.20^{a}	63.40^{ab}	61.80^{b}	± 1.61	*

^{a}, ^{b}, ^{c} means in the same row with different superscript are significantly different (P<0.05).

BWtBP = BWt before parturition at beginning of experiment; BWtPP = BWt postpartum.

resulting from the breakdown of fibre in the rumen. Eastridge (2002) reported that both chemical and physical forms of fat sources can affect digestion and reduce fibre digestibility in the rumen by inhibiting cellulolytic microorganisms.

Table 5 shows that the digestion coefficients of DM, OM, CP and energy were lower (P<0.05) with feeding the ration containing Na-SFA compared with that containing Ca-SFA, while no significant differences were found between the ration containing Ca-SFA and the control. These results are in good agreement with the data obtained from the *in sacco* study, where DM, OM and CP disappearances were significantly decreased with added Na-SFA compared with Ca-SFA. By contrast, undegradable DM, OM and CP were significantly higher as shown in **Table 4**. Palmquist (1994) and Kattab et al. (2001) found that the effect of added fat on digestibility depended on the type and level of fat supplementation. Jenkins (1994) suggested that when fat supplementation decreased protein digestibility in the whole tract, less nitrogen is absorbed across the rumen as ammonia and therefore available for use by body tissues for production. The reduced digestion coefficient of protein is attributed to depression of degradation in the rumen (Boggs et al., 1987).

Feeding rations containing Na-SFA or Ca-SFA significantly increased the digestion coefficient of AEE. The higher (P<0.05) digestibility of lipids associated with fat supplementation in Na-SFA and Ca-SFA rations might be related to the higher digestibility of the supplemented fat (Devendra and Lewis, 1974; El-Bedawy et al., 2005).

Digestibilities of NDF, ADF and cellulose were significantly lower with the ration containing Na-SFA compared with the Ca-SFA and control rations; however, no significant difference was found between the Ca-SFA-containing and control rations. These results might be due to the effect of long chain fatty acids (LCFA) on microbial growth in the NA-SFA ration, and in turn on rumen fermentation which affects fibre digestibility (El-Hag and Miller, 1972). Growth of the cellulolytic species *Butyrivibrio fibrisolvens, Ruminococcus albus* and *Ruminococcus flavefaciens* was inhibited by oleic acid in the presence of the soluble substrate cellobiose (Palmquist, 1988).

Nutritive values expressed as TDN and DCP were significantly lower with the ration containing Na-SFA compared with that containing Ca-SFA, while no significant difference was found between the control group and that containing Ca-SFA. Jenkins (1993) found that if the ability of microorganisms to ferment fibre is inhibited by fat, the fibre energy is lost in faeces.

Feed Intake and Bodyweight Changes

The concentrate: roughage ratio was fixed in the experimental rations to avoid changes in feed intake and to study the effect of rations containing Na-SFA or Ca-SFA on intake. No significant differences were found for bodyweight among tested groups at the beginning or the end of the experimental period (**Table 6**). Bodyweight increased to a significantly greater extent with feeding a ration containing fat than with the control diet, while animals fed the ration containing Ca-SFA had higher rates of gain (P<0.05) than those fed a ration containing Na-SFA. These results suggest that fat is generally favourable for foetal development in late pregnant buffaloes, especially Ca-SFA which contains calcium for developing the foetal skeleton. The data on birth weights supports this suggestion.

Changes in the live weight of dams during gestation are often assumed to be indicative of pre-natal foetal development (Amoah et al., 1996). Akingbade et al. (2001) reported that during late pregnancy there is preferential nutrient utilisation for foetal growth at the cost of mobilising maternal body tissue, which results in weight loss of does if the dietary supply of nutrients is inadequate (Al-Totanji and Lubbadeh, 2000). The pattern of foetal growth rate, calculated as the weight difference of dams before and after parturition during the experiment, corroborates these observations and also indicates that the last month of gestation is the period of most rapid foetal growth.

Rumen Parameters

The data in **Table 7** show increased FFAs in the rumen with feeding a diet containing Na-SFA which in turn led to a decreased concentration of NH_3-N, a result reflecting the impact of added Na-SFA on protein degradability in the rumen. At the same time, the values for

Table 7. Rumen parameters of sheep fed the experimental rations.

Item	Time (h)	Control	Na-SFA	Ca-SFA	± SE	P<
pH	0	6.50^{a}	6.97	6.57	± 0.168	ns
	4	5.84^{b}	6.79	6.03	± 0.207	ns
	8	6.13^{Bab}	6.82^{A}	6.60^{A}	± 0.072	*
	P<	*	ns	ns	—	—
TVFAs (meq/dL)	0	12.22^{A}	8.99^{Bb}	10.72^{Ab}	± 0.302	*
	4	14.43^{A}	11.06^{Ba}	12.51^{ABa}	± 0.505	*
	8	11.89	9.88^{ab}	11.81^{ab}	± 0.384	ns
	P<	ns	*	*	—	
Acetic (%)	0	59.32^{A}	52.51^{B}	57.24^{ABb}	± 1.180	*
	4	62.05	53.84	62.11^{a}	± 2.320	ns
	8	60.22^{A}	51.40^{B}	60.81^{Aa}	± 0.442	*
	P<	ns	ns	*	—	—
Propionic (%)	0	25.05^{b}	25.41	25.40^{b}	± 0.342	ns
	4	25.95^{a}	29.03	25.99^{a}	± 0.833	ns
	8	25.95^{a}	28.72	25.83^{ab}	± 0.587	ns
	P<	*	ns	*	—	—
Ac:Pr	0	2.37	2.07	2.25^{b}	± 0.006	ns
	4	2.39	1.87	2.39^{a}	± 0.117	ns
	8	2.32^{A}	1.79^{B}	2.35^{Aa}	± 0.038	*
	P<	ns	ns	*	—	—
Butyric (%)	0	10.63^{b}	10.58^{b}	10.94	± 0.367	ns
	4	12.17^{a}	13.79^{a}	12.32	± 0.538	ns
	8	11.16^{Bab}	12.48^{Aab}	12.28^{A}	± 0.180	*
	P<	*	*	ns	—	—
NH_3-N (mg/dL)	0	10.36^{b}	9.26^{Bb}	11.08^{Ab}	± 0.267	*
	4	13.38^{a}	10.67^{Ba}	12.59^{Aa}	± 0.204	*
	8	12.33^{a}	9.55^{Bb}	12.40^{Aa}	± 0.108	*
	P<	*	*	*	—	—
FFAs µmol/L	0	2.41^{B}	7.14^{A}	3.13^{B}	± 0.313	*
	4	2.86^{B}	7.34^{A}	4.45^{B}	± 0.333	*
	8	2.54^{B}	6.86^{A}	3.51^{B}	± 0.247	*
	P<	ns	ns	ns	—	—

^{A}, ^{B}, ^{C} means in the same row with different superscript are significantly different (P<0.05); ^{a}, ^{b} means in the same column within each category with different superscript are significantly different (P<0.05).

TVFAs show the impact of feeding Na-SFA on fibre degradability resulting from the breakdown of fibre in the rumen. Eastridge (2002) reported that both chemical and physical forms of fat sources can affect digestion and reduce fibre digestibility in the rumen by inhibiting cellulolytic microorganisms.

Mean pH values, propionic acid and FFAs in the rumen increased significantly while TVFAs, acetic, Ac:Pr ratio and NH_3-N significantly decreased when feeding a ration containing Na-SFA compared with that containing Ca-SFA or the control (**Table 7**). Butyric acid was not affected by added fat compared with the control diet. Increased release of FFAs in the rumen when feeding Na-SFA decreased both NH_3-N and TVFAs. Fatty acids, especially unsaturated fatty acids are antimicrobial and interfere with the normal function of rumen microbes (Palmquist, 1988). Devendra and Lewis (1974) reported that rumen fermentation is negatively affected as fatty acids become more unsaturated and/or are released faster from feedstuffs. The effects of adding fat on rumen fermentation depend on the source and content of fibre in the ration (Jenkins, 1994), and the type and level of fat (Abo-Donia et al., 2003).

Changes in Blood Parameters

Including Na-SFA in the ration decreased total protein concentrations in the blood of late pregnant buffaloes compared with Ca-SFA or the control rations (**Table 8**). This result is consistent with the data

Table 8. Blood parameters of buffaloes fed rations containing Na-SFA or Ca-SFA during the late pregnant period.

Parameter	Control	Na-SFA	Ca-SFA	± SE	P<
Total protein (g/dL)	6.51[a]	6.21[b]	6.49[a]	± 0.06	*
Albumin (g/dL)	2.61	2.59	2.67	± 0.05	ns
Globulin (g/dL)	3.88	3.80	3.83	± 0.04	ns
Albumin / Globulin	0.69	0.69	0.71	± 0.02	ns
Total lipids (g/dL)	5.00[b]	6.14[a]	6.31[a]	± 0.15	*
Triglyceride (mg/dL)	66.60[b]	72.97[a]	75.43[a]	± 0.89	*
FFA's (µmol/L)	19.35[c]	26.14[b]	30.47[a]	± 044	*
Glucose (mg/dL)	58.74[a]	52.74[b]	52.55[b]	± 0.72	*

[a], [b], [c] and means in the same row with different superscript are significantly different (P<0.05).

in Tables 3, 5 and 6 where protein degradability and digestibility decreased with the ration containing Na-SFA compared with that containing Ca-SFA or the control ration. Concentrations of albumin, globulin and their ratio were not affected by feeding rations containing either Na-SFA or Ca-SFA. The effect of Na-SFA on blood protein concentration might be dependent on the kind of fatty acids in Na-SFA (Aiad et al., 2005). Concentrations of total lipids, triglycerides and free fatty acids were significantly increased with feeding a ration containing fat compared with the control. No significant difference was found between Na-SFA and Ca-SFA except with respect to FFAs which were significantly higher with the Ca-SFA compared with the Na-SFA ration. The higher blood lipids might be due to inhibited lipogenic enzyme activities in the liver and adipose tissues of animals fed fat-containing rations (Storry, 1981). These results are related to the high content of fatty acids in Na-SFA and Ca-SFA. The present results are supported by those reported by Aiad et al. (2005). Palmquist and Conrad (1978) attributed the high blood plasma lipids of fat-supplemented cows to the greater quantity of fatty acids absorbed. All serum parameters were within the normal range as reported by William (1997). Glucose concentrations were significantly decreased with feeding a ration containing Na-SFA compared with feeding Ca-SFA or control rations.

CONCLUSIONS

Feeding diets supplemented with fat is suitable for foetal development, mammary adipose tissue and subsequent milk yield in late pregnant buffaloes. Soapstock as Na-SFA is a potential dietary fat source. Converting it to Ca-SFA reduces its negative effects and could be used as a source of energy in the rations of buffaloes especially during the late period of pregnancy.

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Effects of Nutritional Supplementation and Genotype on Milk Production and Fertility of Lactating Dairy Cattle under Tropical Conditions

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ABSTRACT

The objective of this study was to determine the effects of nutrition on milk production and fertility in lactating multiparous Friesian and Sahiwal cows. Forty in-calf cows comprising 20 Friesians and Sahiwals were selected and upon calving were randomly assigned to five dietary groups consisting of concentrate supplementation at the rates of zero to four kg fed twice daily after grazing on pasture for 24 weeks postpartum. Each group consisted of four Friesians and four Sahiwals. Pastures and concentrates were analysed using proximate analysis, milk samples were collected weekly to determine composition using infrared spectroscopy while blood was collected bi-weekly to determine progesterone levels using radioimmunoassay. Parameters recorded included milk yield and composition (percentages of fat, protein, solids not fat (SNF) and density. Reproductive data included d to beginning of luteal activity (progesterone >3nm/L), d to first insemination and conception to first service. Data were analysed by GLM of SAS. Level of supplementation, breed, parity and BWt of cow significantly affected milk yield. Heavier cows produced more milk with a mean increase of 0.2 kg for each kg increase in weight. Animals receiving four kg supplements twice daily had the highest mean milk yield per week ($P < 0.05$) in both breeds averaging 72.2 ± 4.4 and 43.1 ± 1.7 L for Friesians and Sahiwals respectively. Breeds differed also in terms of d to reach peak milk production and peak milk yield with Friesians and Sahiwal cows averaging 31.6 ± 6.0 and 42.2 ± 3.8 d to reach peak milk yield, while peak milk yields were 79.5 ± 5.9 and 58.4 ± 2.7 L respectively. Significant breed differences were also observed for percent fat, protein, SNF and density of milk. Sahiwal cows exhibited better reproductive performance than Friesians. It was observed that 18% of in-calf cows lost their foetus before term and 25% of them never showed heat by 120 d postpartum. Of these, 15% never showed any luteal activity, while 10% had silent heat. Sahiwals came into heat and started cycling earlier ($P < 0.05$) than Friesians but more Friesian cows ($P < 0.05$) conceived at first insemination and showed luteal activity later than the Sahiwals. There were within-breed differences between supplementation regimes ($P < 0.05$) for d to first heat and to start of luteal activity. However, the outcomes were quite variable and there were no clear patterns for effects of supplementation in both breeds. It is concluded that breed effects were more important than nutritional effects in determining milk production, composition and reproductive performance.

Key words: *Friesian and Sahiwal cows, nutritional supplementation, milk yield and characteristics, reproductive performance, breed effects, nutritional effects.*

INTRODUCTION

The lifetime productivity of a dairy cow depends on the number of calves born and the amount of milk produced during its active reproductive phase (De Vries, 2006). Efficient reproduction requires that calving intervals are optimised and there is adequate nutrition to support milk production and calf growth to reach puberty early in life (Gong et al., 2002).

Under tropical conditions, Friesian cattle have been reported to produce 72.5 L milk/week with a butterfat content of 3.7% and to have a lactation length averaging 290 d (Irungu and Mbugua, 1998) compared with Sahiwal cattle which produce 67.2 L/week and have a lactation length of 280 d (Muhuyi and Lokwaleput, 1998). Walshe et al. (1991) reported a range of 5–15 kg milk/cow/d in sub-Saharan Africa, and an average of 8.7 kg/cow/d from 69 herds in Kenya. The low milk production was attributed to various factors including the genetic potential of the animal, the nutritional inadequacy of the diet, parasitism and disease and late lactation.

Nutrition and other environmental factors have a profound influence on the production and reproductive performance of ruminants, but little is known about the complex relationship between nutrition and reproduction which is variable (Gong, 2002). Energy, protein, minerals and vitamins can all affect reproduction and insufficient intake of these nutrients is associated with suboptimal reproductive performance (Beam and Butler, 1998). Inadequate energy intake results in delayed puberty, prolonged postpartum intervals to first ovulation, increased incidences of silent heat, reduced conception rates and birth weights (Lopez et al., 2004).

Lactating cows are in a state of negative energy balance postpartum, because the energy required for milk production and to maintain body functions exceeds energy ingested and metabolic and endocrine changes lead to enhanced mobilisation of depot fat and breakdown of skeletal muscle to provide substrates for milk synthesis (Bauman, et al., 1988; Santos, et al., 2009). The concentrations of fat and protein increase during the advanced stages of lactation in pasture-fed dairy cows (Auldist et al., 1998). In exotic cattle peak milk yield occurs 50–70 d postpartum (Roche et al., 2006).

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The composition of milk varies considerably with breed, stage of lactation, feed, season of the year, and many other factors (Charles, 1998). Since the cow's diet is the ultimate source of most of the material used in milk synthesis, the conditions of feeding and the rations fed to the cow influence milk yield and composition (Robinson, 1997). To optimise milk composition, the nutritional status of cows can be adjusted through proper grazing and feeding management (Robinson 1997).

Low milk yields and poor fertility in tropical cattle are a result of nutritional factors due to seasonal variation of forage quantity and quality (Mukasa-Mugerwa et al., 1997). Topps (1994) showed a deficit of up to 15 MJ/d of metabolisable energy and effective rumen degradable protein of 235 g/day to support desired levels of milk output. The shortage of protein was considered to be more critical than energy. However, it has been shown that under good nutrition, average milk yields of 12–15 kg/d can be achieved, representing increases of 140–300% over the median daily milk yield (van der Valk, 1992).

This study was designed to determine the influence of nutrition on lactation and reproductive performance of lactating Friesian and Sahiwal cows in Kenya.

MATERIALS AND METHODS

Animals and Diets

Forty multiparous cows from two research station herds comprising 20 Friesians and 20 Sahiwal cows were selected based on relative weight within breed, parity (between three and five) and pregnancy status. The cows were two months to parturition at the beginning of the experiment and were randomly assigned to five dietary groups upon calving comprising eight cows per group i.e. four Friesians and four Sahiwals. They were grazed together on pasture leys of predominantly Rhodes grass and upon calving they were individualy fed a concentrate supplement, the amounts ranging from zero to four kg twice daily. The cows were adapted to their diets for 14 d and the study conducted from the time of calving up to 24 weeks (6 months) postpartum.

Analysis of Diets

The pastures were sampled monthly using a quadrant and analysed using the Van Soest method to determine their dry matter (DM), fibre and protein content (AOAC, 1995). The concentrate component of the diet was also analysed to determine DM, neutral detergent fibre (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL) and crude protein (CP).

Observations, Sampling and Measurements

After parturition, body condition was determined, the cow and calf were weighed using a weighing scale and randomly assigned to a dietary group. The calf was allowed to suckle for one week after which it was withdrawn; hand milking was done twice daily. Cow parameters recorded included date of calving, age at first calving, age, parity, breed, and daily milk yield (summed for the week).

The cows were then observed for behavioural signs of oestrus and served using artificial insemination (AI) by two AI practitioners using locally processed Friesian or Sahiwal semen. Cows observed on heat in the morning were served in the afternoon while those exhibiting heat in the later part of the afternoon were served the following morning. The cows were inseminated on up to four occasions, after which they were considered open.

Reproductive data included: d from calving to beginning of luteal activity as determined by progesterone profiles (≥ 3ng/mL) and d to first service and conception to first service. After insemination, the cows were monitored for return to heat. Pregnancy diagnosis was done by rectal palpation 90 d after insemination and confirmed by progesterone profiles. Conception rates were determined as the proportion of cows bred that became pregnant after the first AI. Other reproductive parameters recorded included embryonic or foetal loss as determined by progesterone profiles and rectal palpation. Blood samples were collected bi-weekly (20 mL) from each cow for 6 months after calving via jugular venipuncture using tubes containing ethylenediaminetetra-acetate (EDTA) at the rate of 1.8 mg/mL as an anticoagulant for plasma collection. Samples were centrifuged for 15 min at 1 600 rev/min to separate the plasma from the solid blood components. The plasma was then pippetted into 2 mL plastic vials and stored at –20 °C after which progesterone was determined using radioimmunoassay with ^{125}Iodine as the tracer (FAO/IAEA, 1999).

Homogenised milk samples (20 ml) were collected weekly (morning and afternoon milk) from each cow into plastic tubes for a period of 6 months after parturition. The milk samples were analysed immediately to determine milk components and characteristics which included percent butter fat (BF), protein (P), SNF, density and freezing point using infrared spectroscopy (Tietz, 1986). The percentage milk fat:protein and protein:SNF ratios were calculated to determine their association with reproductive and hormone parameters.

Daily milk yields were recorded for the morning and evening and eventually summed for the week for each cow postpartum. Lactation curves were then derived over the lactation period using plynomial regression equations.

Statistical Analyses

The data were analysed using the SAS program package release 8.2 (SAS, 2001). Analysis of variance was performed using the GLM procedure of SAS. Differences were considered to be significant if $P \leq 0.05$. Data are presented as means ± SEM. The model used was as follows:

$$Y_{ijkl} = \mu + b_i + t_j + (bt)_{ij} + p_k + d_m + \varepsilon_{ijklm}$$

where: Y_{ijkl} are the dependent observations of percentages of fat, protein and SNF, density (g/L), freezing point ˚C, milk yield (L/week), *d* to peak milk, peak milk and fat:protein and SNF ratios; μ, *b, t, p* and *d* are the overall mean and the fixed effects of breed, treatment, parity and body condition scores of each experimental animal while ε is the residual error.

Weight at calving, current age, age at first calving and d in milk were included in the model as covariates. Correlations between the various variables of the model were also calculated to determine degree of association.

RESULTS AND DISCUSSION

Feed Analyses

Results from the forage and concentrate analyses are shown in **Table 1**. These values are similar to those obtained in the East African region as reported by Smith et al. (2006) in Ethiopia and by Irungu and Mbugua (1998) in Kenya.

Milk Yields and Lactation Curves

Lactation curves of third degree polynomials were fitted and are represented in **Figures 1** and **2** for the two breeds. Most of the lactation curves are characterised by an increase in milk production up to peak milk output followed by a steady decline. These polynomials show fits for the Sahiwals whose r^2 is 0.91 while those of the Friesians had

Table 1. Mean ± SD of nutritional values (%) of feed components.

Nutritional Component	Forage	Concentrate
Dry Matter	86.8 ± 2.5	89.4 ± 3.7
Crude Protein	4.7 ± 2.2	16.8 ± 1.1
Neutral Detergent Fibre	41.7 ± 6.7	45.7 ± 9.5
Acid Detergent Fibre	42.6 ± 2.5	22.4 ± 4.9
Acid Detergent Lignin	4.7 ± 1.6	3.1 ± 0.6
Ash	8.9 ± 1.3	9.42 ± 2.1

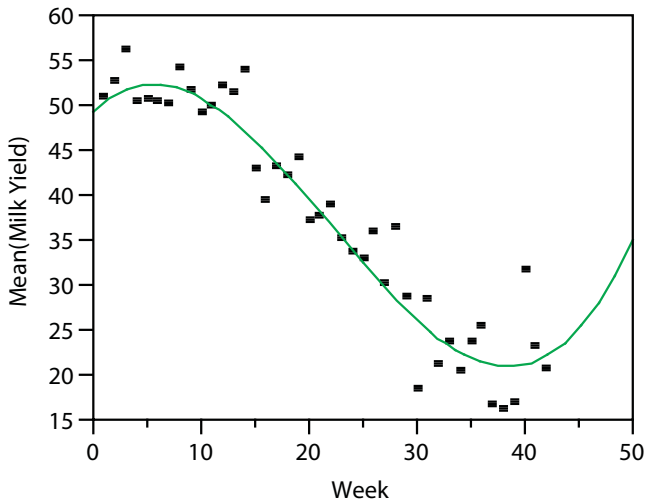

Figure 1. Sahiwal lactation curves.

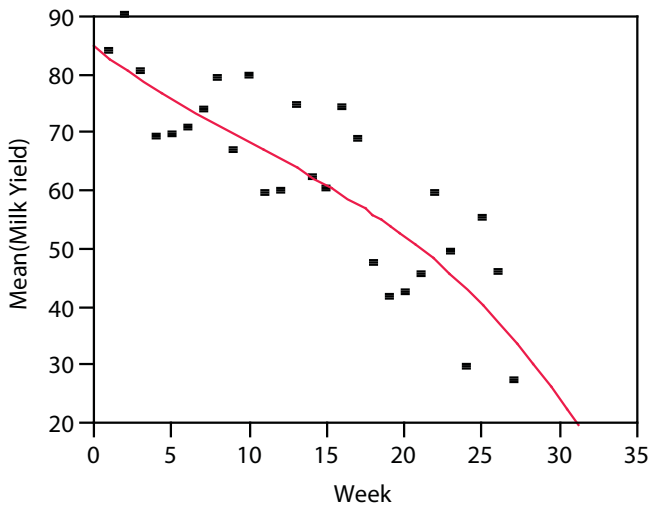

Figure 2. Friesian lactation curves.

a r^2 of 0.71; this could be an indication of inherent breed differences. These fits are similar to those found by Garcia and Holmes (2001). Friesians showed a shorter persistence with milk yield reaching 27% of peak milk yield at week 30 while that of the Sahiwals reached this level at week 40 postpartum; this is similar to the findings of Ilatsia et al. (2007) who found an average lactation length of 40.6 weeks for Sahiwals in Kenya, and to the 30% that was reported by Mech et al. (2008) as an indicator of the drying-off phase.

As per the model, breed, diet, breed by diet, parity, body condition and weight of cow had significant effects on milk yield. When milk yield is regressed on BWt of cows, it was found that heavier cows produced significantly more milk with a mean increase of 0.2 kg of milk for each increase in kg live weight. This was expected as older cows were heavier and had more developed udders. It has also been suggested that large cows have a larger rumen volume relative to their metabolic needs and are likely to channel more nutrients towards production rather than for maintenance (Preston, 1989).

There were breed and treatment differences in the lactation curves as depicted by milk yields and d to reach the peak (**Table 2**). The weekly mean milk yields irrespective of treatment were not significantly different ($P > 0.05$), being 51.9 ± 5.3 and 49.8 ± 2.5 L/ week for Friesian and Sahiwal cows respectively (**Table 2**).

Cows in Group 4 had the highest mean milk yield/week ($P < 0.05$) in both breeds which averaged 72.2 ± 4.4 and 43.1 ± 1.7 L for Friesians and Sahiwals respectively and could be assumed to be the optimal supplimentation for dairy cows not selected for high milk production. These findings contrast with those of Irungu and Mbugua (1998) who reported yields of 92.4 kg/week for Friesians with similar supplementation while Ilatsia et al. (2007) found lower values averaging 33.9 L/week for Sahiwals. Supplementation with three kg concentrate was able to provide adequate nutrients to tap the individual cow's potential which eventually fell to 58.4 L/ week and 42.8 L/week for Friesians and Sahiwals respectively upon provision of four kg of concentrate to cows in Group 5 (**Table 2**). This reduction could be due to the cows becoming overweight thus impacting negatively on milk production. On average, both breeds produced similar amounts of milk/week (mean 50.4 L., **Table 2**), but breed peaks differed significantly ($P < 0.05$), with Friesians attaining 79.5 L/week after 32 d postpartum while Sahiwals attained a pesk of 58.4 L/week at 42 d postpartum (**Table 2**). These peaks are earlier than the 50–70 d postpartum reported by Roche et al. (2006). This could be due to the differences in energy balance for the two breeds after parturition (Buttler et al., 2002).

Diet, breed by diet, parity, body condition and BWt of cow had significant effects on milk yield. There were significant ($P < 0.05$) breed differences for peak milk production and d to peak milk production in both breeds as indicated in **Table 2**. Friesian and Sahiwal cows averaged 31.6 ± 6.0 and 42.2. ± 3.8 d to reach peak milk yield respectively when diet was not considered, while the mean peak milk yields were 79.5 ± 5.9 and 58.4 ± 2.7 L (**Table 2**). These findings agree with Nebel and McGilliard (1993) who found that most cows reach peak yields between four and eight weeks postpartum and cows with high milk yield peaks also took longer to reach peak milk yield and vice versa.

There were significant interactions between breed and diet for d to reach peak yields and peak milk production but no consistent pattern emerged between these parameters as levels of supplementation increased.

Milk Composition and Characteristics

There were significant differences for the percentage of fat, protein, SNF, density and freezing point of milk between the two breeds, with Sahiwals having higher ($P < 0.05$) values than those of the Friesians (**Table 3**). These findings agree with those of Mwenya (1993), who indicated that local breeds of cattle produce relatively less milk but have higher values for milk components than exotics. Chenoset and Sansoucy (1998) found that these differences in composition could be due to differences in feed conversion and rumen function, being dependent on the quantities of volatile fatty acids and microbial proteins produced.

Table 2. Milk production by diet and breed.

Dietary Group	Breed	Milk Yield (L/week)	Peak Milk Yield (L/week)	Days to Peak Yield
1	Friesian	70.7 ± 4.7[a]	103.6 ± 3.4[a]	31.5 ± 3.6[a]
	Sahiwal	33.9 ± 1.3[b]	47.8 ± 0.8[b]	44.7 ± 2.5[b]
2	Friesian	57.9 ± 5.2[a]	59.2 ± 5.8[a]	25.6 ± 1.7[a]
	Sahiwal	47.1 ± 2,3[b]	65.7 ± 1.9[a]	31.1 ± 0.5[b]
3	Friesian	62.6 ± 4.3[a]	82.7 ± 2.3[a]	66.4 ± 3.8[a]
	Sahiwal	44.6 ± 1.9[b]	61.3 ± 0.1[b]	25.9 ± 0.5[b]
4	Friesian	72.2 ± 4.4[a]	92.6 ± 3.1[a]	62.9 ± 7.3[a]
	Sahiwal	43.1 ± 1.7[b]	61.1 ± 1.2[b]	51.5 ± 1.1[b]
5	Friesian	58.9 ± 4.3[a]	88.1 ± 0.9[a]	20.7 ± 0.2[a]
	Sahiwal	44.0 ± 2.1[b]	59.2 ± 1.4[b]	46.3 ± 0.9[b]
Overall	Friesian	51.9 ± 5.3[a]	79.5 ± 5.9[a]	31.6 ± 6.0[a]
	Sahiwal	49.8 ± 2.5[a]	58.4 ± 2.7[b]	42.2 ± 3.8[b]

Group 1 — pasture only; Group 2 — pasture +1 kg supplement twice daily; Group 3 — pasture +2 kg supplement twice daily; Group 4: pasture +3 kg supplement twice daily; Group 5 — pasture +4 kg supplement twice daily.

[a,b] Values with different superscripts within rows differ significantly ($P < 0.05$).

Table 3. Least square means ± SEM of milk components for the two breeds.

Breed	% Fat	% Protein	% SNF	Density (kg/L)	Freezing Point (°C)	Fat:Protein
Friesian	3.55 ± 0.22[a]	3.07 ± 0.03[a]	8.12 ± 0.08[a]	1.026 ± 0.04[a]	−0.53 ± 0.05[a]	1.17 ± 0.33[a]
Sahiwal	4.52 ± 0.14[b]	3.22 ± 0.02[b]	8.50 ± 0.05[b]	1.027 ± 0.26[b]	−0.55 ± 0.03[b]	1.40 ± 0.21[b]

[a,b] Values with different superscripts within columns differ significantly ($P < 0.05$).

It is apparent that the metabolism of these two breeds allows for different nutrient partitioning and that their nutrient requirements are also different which could be an inherent attribute of the Sahiwal considering its Zebu type (Charles, 1998). Fat, protein and fat:protein are important parameters of nutrient balance (Heuer et al., 1999). Milk fat percentage tends to increase and milk protein percentage tends to decrease in association with a negative energy balance post-partum due to mobilisation of adipose tissues. The fat:protein ratio has been suggested as a potential indicator of lack of dietary energy supply, and critical ratios between 1.35 and 1.5 have been suggested (Grieve et al., 1986). The ratios in **Table 3** show that Sahiwals were in greater energy deficit than Friesians which was suprising since Friesians might have been expected to be in a greater negative energy balance due to their higher mainatenance requirements. However, their relatively low milk yield might suggest that the nutrients saved for milk production by this lower yield produced the favourable energy balance recorded while the Sahiwals on average exceeded their milk yield potential (Ilatsia et al., 2007).

The freezing point of Friesian milk was similar to the –0.53 °C reported by Henno et al. (2008) in Estonian Friesians, but lower for the Sahiwal cows (**Table 3**), and could be due to the inherent breed differences which showed Sahiwals with consistently higher solid components in their milk than the Friesians. A decrease of 0.1% in milk protein results in an increase of 0.0024 °C in freezing point. The freezing point depression and density of milk depends upon its concentration of water-soluble components (Sherbon, 1988). The density of Sahiwal milk was higher ($P < 0.05$) than that of Friesians and so was the depression of freezing point (**Table 3**). This could be explained by the significantly higher total solids (% BF plus SNF) in Sahiwal milk causing the bigger depression of freezing point and a larger specific gravity as indicated by the measurements of density (**Table 3**). For all treatments it can be argued that breed effects far outweighed the dietary effects for milk components because within-breed variations did not show clear patterns as concentrate intakes increased; this agrees with the findings of Gonthier et al. (2005).

Freezing point depression and density are useful indicators of the solids in milk and animals in Group 3 had significantly higher values, particularly the Friesians as indicated by **Figures 3** and **4**. This is when the milk solids are at their highest and indicates that the optimal milk quality for the two breeds would be at this treatment. However, there were no clear patterns with respect to diet influencing freezing point and density in either breed.

There was a strong negative correlation between percent fat and density for both the breeds (**Table 4**). This can be problematic for marketing because as fat levels increase, the milk density tends to decrease and *vice versa*, although regulatory standards require that both fat and density should be high for good quality milk.

However, the high association between protein and both density and freezing point suggests that the level of protein is the main factor influencing density and freezing point depression due to its contribution to SNF because of its higher molecular weight (**Table 4**). Solids not fat exhibited strong positive associations with density, freezing point depression and protein in both breeds (**Table 4**). This is expected because the higher the solid component of milk the greater

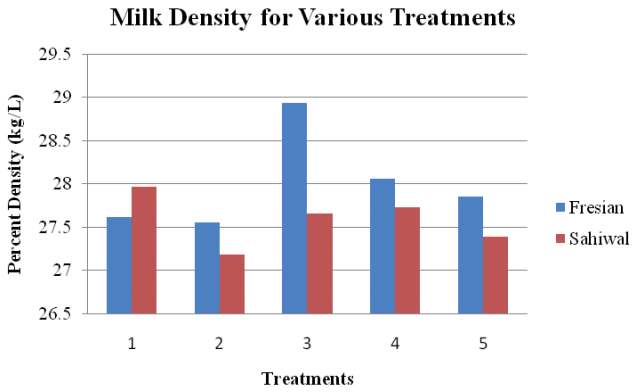

Figure 3. Milk density.

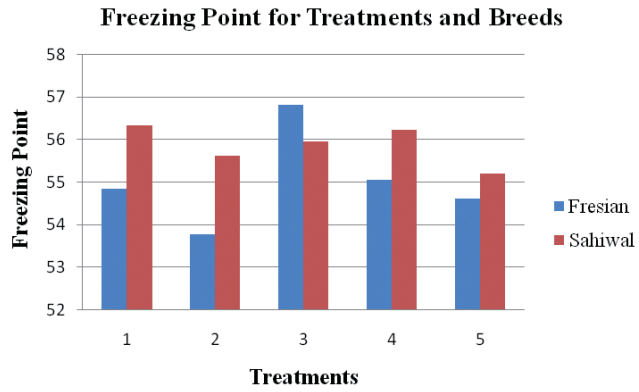

Figure 4. Milk freezing point (°C ×10–2).

Table 4. Correlations between milk constituents for cattle.

Friesian	Fat	SNF	Density	Freezing point	Protein
Fat	1	0.07	–0.45	0.03	0.15
SNF		1	0.86	0.99	0.99
Density			1	0.88	0.82
Freezing Point				1	0.99
Protein					1
Sahiwal					
Fat	1	0.15	–0.39	–0.01	0.21
SNF		1	0.85	0.97	0.99
Density			1	0.91	0.81
Freezing Point				1	0.97
Protein					1

the freezing point depression and the higher the density. Fat and protein were moderately positively associated in both breeds.

Parity affected mean milk yield ($P < 0.05$) which increased to 68.8 ± 1.9 L/week for parity five and declined in subsequent parities for both breeds; similar trends were observed for peak milk yield where parity six peaked at 92.1 ± 1.1 L/week. This was expected because the alveolar tissues in the mammary glands develop with increasing parity resulting in increased milk yield which takes longer to reach a peak. Similar findings were reported by Chase (1993). Parity also affected milk composition and its physical characteristics. Parity four milk had a significantly high mean protein content of 3.3 ± 0.02%, density of 1.028, SNF of 8.59 ± 0.05% and the lowest freezing point of –0.056 °C. This could be explained by the udder being adequately developed to take full advantage of the nutrients supplied for improved milk quality and production.

Body condition scores had significant effects on lactation with body condition five being associated with the highest mean milk yield of 75.5 ± 2.01 L which peaked at a mean of 103.2 ± 1.27 L and took 48.8 ± 1.9 d to reach peak milk yield. Milk composition and physical characteristics varied ($P < 0.05$) with body condition scores. However, animals with body condition four showed higher mean levels for fat, protein and SNF, an average density of 1.027 and a freezing point depression of –0.056 ^{0}C; this was the body condition under which both milk production and quality were optimal.

Reproductive Performance

Sahiwals had a better reproductive performance than Friesians. It was observed that 18% of in-calf cows lost their foetus before term and 25% of them never showed heat by 120 d postpartum (**Table 5**). Of the cows that calved, 15% did not show any luteal activity, while 10% had silent heat postpartum. Sahiwals came into heat and started cycling earlier ($P < 0.05$) than Friesians (**Table 5**) although, as indicated earlier, they were deemed to be in greater negative energy balance than Friesians. This finding could be due to adaptation i.e. being able to reproduce although the situation was less favourable given the fat:protein ratios. Alternatively, the Sahiwals could have a higher threshold for energy balance relative to the Friesians under tropical environments. Friesians had more ($P < 0.05$) cows conceiving at first insemination and showed luteal activity later than the Sahiwals (**Table 5**); this may have arisen because of the demand for nutrients for milk production which peaked earlier in Friesians than in the Sahiwals. The relatively less favourable reproductive attributes of the Sahiwals could also be a reflection of the influence of metabolic size on reproductive performance.

The beginning of luteal activity and expression of heat are important factors that influence when insemination should be done for conception to occur. The earlier it ocurs the better for reproductive effeciency. There were breed differences ($P < 0.05$) for d to first heat and start of luteal activity for most of the dietary treatments (**Table 6**). However, these outcomes were quite varied and did not show spe-

Table 5. Reproductive parameters of the two breeds.

	Breed		
Reproductive Characteristics	Sahiwal	Friesian	Overall
Cows Calving (%)	85	80	82.5
Foetal Loss (%)	15	20	17.5
Non-return to heat six months postpartum (%)	18[a]	31[b]	24.5
Mean ± SEM d to first heat	72.6 ± 1.7[a]	96.9 ± 5.4[b]	84.8
Mean ± SEM d to start of luteal activity	55.5 ± 1.2[a]	74.6 ± 1.9[b]	65.1
Cows showing heat after parturition (%)	82[a]	69[b]	75.5
Cows conceiving at first insemination (%)	21[a]	54[b]	37.5
Cows showing luteal activity after 120 d (%)	88	81	84.5

[a,b] Values with different superscripts within rows differ significantly ($P < 0.05$).

Table 6. Mean ± SEM d to first heat and start of luteal activity for the dietary groups.

	Mean days to first heat		Days to start of luteal activity (> 3ng/L)	
Dietary Group	Sahiwals	Friesians	Sahiwal	Friesians
1	61.5 ± 0.1	53.3 ± 3.9	53.0 ± 1.6	49.3 ± 3.1
2	83.9 ± 0.8[a]	51.5 ± 2.5[b]	29.3 ± 2.0[a]	63.4 ± 3.4[b]
3	35.7 ± 0.3[a]	116.1 ± 9.1[b]	60.7 ± 3.2[a]	72.2 ± 2.9[b]
4	109.1 ± 3.9[a]	42.4 ± 2.8[b]	66.8 ± 2.3[a]	58.7 ± 0.8[b]
5	60.4 ± 2.4[a]	243.3 ± 4.4[b]	58.5 ± 2.1[a]	101.6 ± 3.3[b]

[a,b] Values with different superscripts within row differ significantly ($P < 0.05$).

cific patterns in either breed, although on average the Friesians had more d to luteal activity and heat than the Sahiwals.

Overall, breed effects influenced milk production, composition and reproductive performance more than the nutritional effects. This can be attributed to the fact that the cows used in this study were not highly selected for increased milk production which could have skewed nutrient partitioning for milk production and reproduction. This reflects the adaptation of the two breeds to this production system.

CONCLUSIONS

There are interactions between nutrition and breed which affect milk production, composition and reproductive performance but the effects of breed far outweighed the nutritional effects due to the lack of clear response of these parameters when concentrate intake levels were gradually increased from zero to twice daily supplementation with four kg concerntrates. These findings may be attributed to the fact that the cows used in this study were not highly selected for milk production which would result in higher responses to supplementation as more nutrients are supplied to enhance milk production and reproduction.

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Milk Production and Reproductive Performances of Murrah Buffaloes in Tamil Nadu, India

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ABSTRACT

Data on the production and reproductive traits of Murrah buffaloes (1980 lactation records of 698 animals) were collected from the Central Cattle Breeding Farm, Alamadhi, Tamil Nadu, India. The overall least-squares means (±SE) for peak milk yield, days to attain peak yield, 305-d yield, lactation length and milk yield, service period, calving interval and dry period were 8.87±0.05 kg, 53.4±0.8 d., 1804.9±14.7 kg., 297.8±1.9 d., 1 855.6±16.1 kg., 225.0±5.5, 532.8±5.5 and 230.2±4.9 d respectively. Period of calving had a highly significant ($P < 0.01$) effect on all the traits studied except days to attain peak yield, where it had only significant ($P < 0.05$) effect. Season of calving had a significant ($P < 0.05$) effect on peak yield and lactation milk yield and a highly significant ($P < 0.01$) effect on days to attain peak yield, 305 d milk yield, milk yield/day of lactation and all the reproductive traits studied. The lowest calving interval was observed in southwest monsoon calvers and they differed significantly ($P < 0.05$) with winter and summer calvers. Parity had a highly significant effect ($P < 0.01$) on all the traits studied. Pair-wise comparison revealed that the lactation milk yield was lowest in first parity and differed significantly ($P < 0.05$) from other parities. In general, reproductive traits such as service period, calving interval and dry period were slightly higher than those observed elsewhere and hence better breeding management and introduction of genetic evaluation programmes are needed for genetic improvement of these traits.

Key words: *Murrah, buffaloes, production, reproduction, performance, non-genetic factors.*

INTRODUCTION

According to the 2003 livestock census, India possesses 185.2 million cattle and 97.9 million buffaloes, which is about 13.7% of the total cattle and 57.5% of the total buffalo population of the world. The dairy industry in India has made significant progress in the last few decades. Today, India is the largest producer of milk in the world. Milk production rose to about 88.1 million tonnes in 2003–2004 from 17.0 million tonnes in 1950–1951. At present, India's contribution to total world milk production is about 14.3% and the national per capita milk availability is 231 g/d. In India, although the proportion of buffaloes to cattle is 1:2, buffaloes contribute around 57% of the total milk obtained from cattle and buffaloes. Tamil Nadu with 9.14 million cattle and 1.66 million buffaloes produces an estimated 4.75 million tonnes of milk (Report, 2006). The Murrah breed is the finest genetic material of milk-producing buffalo not only in India but also probably in the world (Taneja, 1998). This breed has been used extensively throughout the country to upgrade the nondescript buffalo stock to improve the milk production. The breeding policy of Tamil Nadu State is to use Murrah or Surti as the breeds of choice to improve the nondescript buffaloes. As a result of these measures, the Murrah and graded Murrah populations have increased over the years. Although the water buffalo in the tropics out-produces other domestic animals, commercial milk production is adversely affected by a large number of factors such as late age at first calving, seasonality of oestrus, long calving interval and dry period. Therefore, it is necessary to evaluate the relative importance of various fixed environmental and physiological effects in influencing the milk production and reproductive traits for devising appropriate feeding and other managemental practices.

From the literature reviewed, it was found that the bulk of scientific information on buffaloes has come from the analysis of records made available from institutional and government farms in north India (Sethi and Khatkar, 1997; Dass and Sadana, 2000; Gogoi et al., 2002; Kundu et al., 2003a and b; Yadav et al., 2007). Information from the southern peninsular region, especially under the hot and humid coastal regions of Tamil Nadu is scanty. The home tract of Murrah buffaloes is a hot and dry climatic region in the north-western part of India. Breeding these buffaloes in the southern peninsular region of India, which is hot and humid, may affect their performance and adaptability. Hence the present study to both understand the performance and the influence of various non-genetic factors affecting economic traits of Murrah buffaloes in the coastal regions of Tamil Nadu and to suggest suitable managemental practices, selection and breeding strategies for genetic improvement of Murrah buffaloes under hot and humid climatic conditions of India.

MATERIALS AND METHODS

The study was based on data pertaining to Murrah buffaloes born and bred at the Central Cattle Breeding Farm, Alamadhi, Chennai, Tamil Nadu, India from 1979 to 2006 (28 years). This farm is located approximately at 13°N latitude and 80°E longitude at an altitude of about 20 m above mean sea level. The climate is generally hot, humid and tropical in nature. The mean annual maximum and minimum temperatures were 33°C and 24.7°C respectively and the mean relative humidity ranged between 69.2% and 76.2%. The buffaloes were housed in permanent sheds with open-type ventilation and maintained under stall-fed conditions. Roughage was provided in the form of green fodder and paddy straw. In addition, concentrate mixture was provided to all age groups as per the standard require-

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ments. Cows were hand-milked twice daily in the morning and evening.

Data on production and reproductive performance of Murrah buffaloes (1980 lactation records from 698 Murrah cows) were extracted from history and pedigree sheets. The traits studied were peak yield, days to attain peak yield, 305 d milk yield, lactation length and milk yield, milk yield/d of lactation, service period, calving interval, dry period and number of services/conception. Period and season were the fixed environmental effects considered for all the traits studied. As the calvings were less in a year, year-season analysis was not done. To utilise all available data the entire duration was divided into seven periods each with an interval of five years assuming that there would not be any major management changes within a period. Further, each calendar year was sub-divided into four seasons, viz. winter (January and February), summer (March to May), southwest monsoon (June to September) and northeast monsoon (October to December). The LSMLMW and MIXMDL PC-2 version computer program of Harvey (1990) was used to study the effect of various non-genetic factors and the means were compared using Duncan's multiple range test.

The model used for analysis was

$$Y_{ijkl} = m + P_i + S_j + O_k + e_{ijkl}$$

where

Y_{ijkl} is the l^{th} observation in i^{th} period, j^{th} season and k^{th} parity,
m is overall mean when equal sub-class frequencies exist,
P_i is effect of i^{th} period ($_i$ =1 to 7),
S_j is effect of j^{th} season ($_j$ =1 to 4),
O_k is effect of k^{th} parity ($_k$=1 to 6) and
e_{ijkl} is random errors NID (0, s^2_e).

RESULTS AND DISCUSSION

Production Traits

Least-squares means (±SE) of different production traits are presented in **Table 1**. Period of calving played a highly significant (P < 0.01) effect on the variation of peak yield, 305 d milk yield, lactation length, lactation milk yield and milk yield/d of lactation and it had only significant (P < 0.05) effect on days to attain peak yield. The highest peak yield, 305 d milk yield and lactation milk yield were observed in period six (1999–2002) and they differed significantly with the rest of the periods, except with periods five and seven, where differences were not significant. The lowest lactation length was observed in period three (1987–1990) and differed significantly (P < 0.05) from other periods except period seven (2003–2006), where it was not significant. There was a steep increase in peak yield, 305 d milk yield and lactation milk yield from the second-sixth periods and declined slightly in period 7 (2003–2006) and the values observed in period two differed significantly (P < 0.05) with the rest of the periods.

Season of calving had no effect on lactation length. However, it had significant (P < 0.05) effect on peak yield and lactation milk yield and a highly significant (P < 0.01) effect on days to attain peak yield, 305 d milk yield and milk yield/d of lactation. The peak yield of Murrah buffaloes that calved in the southwest monsoon season was the highest and it differed significantly (P < 0.05) with northeast monsoon calvers. However, there was no significant difference among other seasons. On the other hand, the lowest 305 d milk yield, lactation milk yield and milk yield/d of lactation were observed in cows calving in the northeast monsoon season. Winter and summer calvers had higher 305 d milk yields and lactation milk yield than the monsoon calvers. The yields observed among winter, summer and southwest monsoon calvers were not significantly different but they differed significantly (P < 0.05) with northeast monsoon calvers.

Parity had a highly significant (P < 0.01) effect on all the traits studied. First lactation peak yield was significantly (P < 0.05) lower than the rest. The yield increased from first to fourth parity and thereafter it started declining. On the other hand, the d to attain peak yield decreased from the first to fifth parity followed by a moderate increase in the sixth parity. Days to attain peak yield in first lactation were significantly (P < 0.05) higher (60.9 d) than the rest. The 305-d milk yield and lactation milk yield increased up to the third parity and they were maintained at the fourth parity and declined thereafter. Pair-wise comparison revealed that the 305-d and lactation milk yields observed in the first parity differed significantly (P < 0.05) from other parities. Similar to days to attain peak yield, the lactation length decreased with the advancement of parity. There was an initial sharp reduction (3.09%) in lactation length in the second parity followed by gradual decline later. The reduction in lactation length between first and second parities was statistically significant (P < 0.05). The milk yield/d of lactation increased linearly with the advancement of parity up to the fourth parity and then declined, and the values observed in the first parity differed significantly (P < 0.05) with the rest of the parities.

Reproductive Traits

Least-squares means (±SE) of different reproductive traits are set out in **Table 2**. Analyses of variance revealed that the period of calving influenced service period, calving interval, dry period and number of services per conception in a highly significant (P < 0.01) manner although there was no consistent trend over the periods. Lowest service periods, calving intervals and number of services per conception were found in period three (1987–90) and they differed significantly (P < 0.05) with other periods except the first period (1979–1982). On the other hand, the lowest and the highest dry periods were observed in periods one and five respectively, and the average dry period observed in period five was significantly (P < 0.05) different from the first, third and fourth periods.

Season of calving also influenced all the reproductive traits in a highly significant manner (P < 0.01). Murrah buffalo heifers freshening in the southwest monsoon had significantly (P < 0.05) shorter service periods, calving intervals, dry periods and number of services/conception than those calving in other seasons. Highest service periods, calving intervals and number of services per conception were observed in winter season calvers. The service periods and calving intervals observed in southwest monsoon calvers differed significantly (P < 0.05) with other seasons except with northeast monsoon calvers. On the other hand, the dry periods and number of services per conception recorded for southwest monsoon season calvers differed significantly (P < 0.05) with winter, summer and northeast monsoon season calvers.

The differences between service periods among parities were highly significant (P < 0.01). Service periods decreased with the order of lactation up to the fifth parity and the lowest values of 187.4 ± 13.7 d were observed at this parity. The reductions in service periods in the second and third parities were rather sharp and further decline was gradual. In general, pair-wise comparison revealed that the mean service periods of first and second parities differed significantly (P < 0.05) from other parities as well as between them. Similarly, Murrah buffaloes calving for the first time had the longest calving intervals and dry periods, which declined thereafter. The calving intervals and dry periods recorded in the first parity were significantly (P < 0.05) different from second and later parities. In addition, the mean calving intervals and dry periods observed between the sec-

Table 1. Least-squares means (± SE) for different milk production traits of Murrah buffaloes.

Effect	n	Peak yield (kg)	Days to attain peak yield	305-day milk yield (kg)	Lactation length (d)	Lactation milk yield (kg)	Milk yield/d of lactation (kg)
Overall mean (μ)	1980	8.87 ± 0.05	53.4 ± 0.8	1 804.9 ± 14.7	297.8 ± 1.9	1 855.6 ± 16.1	6.16 ± 0.04
Period of calving		**	*	**	**	**	**
P_1 (1979–1982)	268	8.47 ± 0.12^{b}	50.7 ± 1.7^{ad}	$1\,670.2 \pm 32.6^{b}$	295.6 ± 4.2^{b}	$1\,706.2 \pm 35.8^{a}$	5.74 ± 0.08^{b}
P_2 (1983–1986)	491	7.96 ± 0.09^{a}	54.9 ± 1.3^{bc}	$1\,584.2 \pm 23.6^{a}$	300.2 ± 3.1^{b}	$1\,629.9 \pm 25.8^{a}$	5.38 ± 0.06^{a}
P_3 (1987–1990)	430	8.30 ± 0.08^{b}	52.0 ± 1.3^{ab}	$1\,632.0 \pm 23.4^{ab}$	285.1 ± 3.0^{a}	$1\,658.5 \pm 25.6^{a}$	5.72 ± 0.06^{b}
P_4 (1991–1994)	265	9.16 ± 0.11^{c}	52.2 ± 1.6^{abc}	$1\,872.9 \pm 29.8^{c}$	298.3 ± 3.9^{b}	$1\,925.2 \pm 32.7^{b}$	6.43 ± 0.07^{c}
P_5 (1995–1998)	171	9.47 ± 0.13^{ce}	57.2 ± 1.9^{c}	$1\,947.2 \pm 36.1^{cd}$	307.2 ± 4.7^{b}	$2\,030.3 \pm 39.6^{c}$	6.55 ± 0.09^{c}
P_6 (1999–2002)	236	9.52 ± 0.11^{e}	51.4 ± 1.7^{ab}	$1\,974.1 \pm 31.1^{d}$	305.8 ± 4.1^{b}	$2\,055.6 \pm 34.1^{c}$	6.66 ± 0.08^{c}
P_7 (2003–2006)	119	9.18 ± 0.16^{ce}	55.0 ± 2.3^{bcd}	$1\,953.7 \pm 43.2^{cd}$	292.5 ± 5.6^{ab}	$1\,983.2 \pm 47.4^{bc}$	6.66 ± 0.11^{c}
Season of calving		*	**	**		*	**
Winter (Jan–Feb)	276	8.81 ± 0.10^{ab}	53.0 ± 1.5^{b}	$1\,839.0 \pm 28.6^{b}$	301.8 ± 3.7	$1\,888.6 \pm 31.4^{b}$	6.19 ± 0.07^{b}
Summer (Mar.-May)	150	8.84 ± 0.14^{ab}	57.9 ± 2.0^{b}	$1\,853.8 \pm 38.1^{b}$	293.3 ± 5.0	$1\,882.4 \pm 41.8^{b}$	6.34 ± 0.09^{b}
South-west monsoon (Jun–Sep)	724	9.03 ± 0.07^{b}	54.1 ± 1.1^{b}	$1\,793.1 \pm 19.8^{b}$	298.8 ± 2.6	$1\,849.6 \pm 21.8^{b}$	6.14 ± 0.05^{b}
North-east monsoon (Oct–Dec)	830	8.79 ± 0.06^{a}	48.3 ± 0.9^{a}	$1\,733.8 \pm 17.7^{a}$	297.4 ± 2.3	$1\,801.6 \pm 19.4^{a}$	5.99 ± 0.04^{a}
Parity		**	**	**	**	**	**
First	645	7.73 ± 0.08^{a}	60.9 ± 1.2^{b}	$1\,619.7 \pm 22.6^{a}$	310.4 ± 2.9^{c}	$1\,687.6 \pm 24.8^{a}$	5.38 ± 0.05^{a}
Second	457	8.85 ± 0.09^{b}	54.0 ± 1.3^{a}	$1\,832.8 \pm 23.5^{c}$	301.1 ± 3.1^{b}	$1\,894.5 \pm 25.7^{cd}$	6.22 ± 0.06^{b}
Third	311	9.37 ± 0.10^{cd}	51.1 ± 1.5^{a}	$1\,913.9 \pm 27.7^{d}$	302.3 ± 3.6^{bc}	$1\,967.3 \pm 30.4^{e}$	6.45 ± 0.07^{c}
Fourth	224	9.54 ± 0.12^{d}	50.6 ± 1.7^{a}	$1\,910.4 \pm 32.3^{d}$	299.0 ± 4.2^{bd}	$1\,966.5 \pm 35.5^{de}$	6.50 ± 0.08^{c}
Fifth	150	8.98 ± 0.14^{b}	50.0 ± 2.1^{a}	$1\,823.4 \pm 38.7^{c}$	289.8 ± 5.0^{abd}	$1\,853.7 \pm 42.4^{bc}$	6.33 ± 0.09^{bc}
Sixth and above	193	8.74 ± 0.13^{b}	53.5 ± 1.9^{a}	$1\,729.1 \pm 35.1^{b}$	284.3 ± 4.6^{a}	$1\,763.8 \pm 38.5^{ab}$	6.09 ± 0.09^{b}

n — number of observations.

* $P < 0.05$; ** $P < 0.01$.

Means bearing same superscript within classes do not differ significantly ($P > 0.05$).

Table 2. Least-squares means (± SE) for different reproductive traits of Murrah buffaloes.

Effect	N	Service period (d)	Calving interval (d)	Dry period (d)	Number of services per conception
Overall mean (μ)	1 550	225.0 ± 5.5	532.8 ± 5.5	230.2 ± 4.9	2.31 ± 0.05
Period of calving		**	**	**	**
P_1 (1979–1982)	220	182.8 ± 11.4^{af}	488.8 ± 11.4^{a}	181.6 ± 10.2^{a}	1.92 ± 0.11^{ad}
P_2 (1983–1986)	423	241.3 ± 8.1^{be}	548.2 ± 8.2^{be}	242.6 ± 7.3^{bc}	2.45 ± 0.08^{b}
P_3 (1987–1990)	316	176.1 ± 8.5^{a}	481.9 ± 8.5^{a}	191.5 ± 7.6^{a}	1.87 ± 0.08^{ad}
P_4 (1991–1994)	210	218.9 ± 10.4^{b}	527.7 ± 10.4^{b}	228.6 ± 9.3^{b}	2.29 ± 0.10^{b}
P_5 (1995–1998)	114	272.8 ± 14.0^{cd}	580.8 ± 14.0^{cd}	268.7 ± 12.5^{c}	2.82 ± 0.13^{c}
P_6 (1999–2002)	199	252.9 ± 10.7^{cde}	559.7 ± 10.7^{cde}	253.3 ± 9.6^{bc}	2.55 ± 0.10^{bc}
P_7 (2003–2006)	68	230.5 ± 17.6^{bdef}	542.4 ± 17.7^{bd}	245.1 ± 15.8^{bc}	2.30 ± 0.16^{bcd}
Season of calving		**	**	**	**
Winter (Jan–Feb)	211	246.8 ± 10.3^{b}	554.4 ± 10.3^{b}	248.0 ± 9.2^{c}	2.56 ± 0.10^{c}
Summer (Mar–May)	109	245.2 ± 14.0^{b}	553.4 ± 14.0^{b}	254.1 ± 12.5^{c}	2.44 ± 0.13^{bc}
South-west monsoon (Jun–Sep)	587	197.5 ± 7.2^{a}	505.5 ± 7.2^{a}	201.5 ± 6.4^{a}	2.02 ± 0.07^{a}
North-east monsoon (Oct–Dec)	643	210.6 ± 6.4^{a}	517.9 ± 6.5^{a}	217.1 ± 5.8^{b}	2.24 ± 0.06^{b}
Parity		**	**	**	**
First	513	280.1 ± 8.0^{c}	586.6 ± 8.1^{c}	274.1 ± 7.2^{c}	2.82 ± 0.08^{c}
Second	367	237.1 ± 8.3^{b}	544.9 ± 8.3^{b}	240.3 ± 7.4^{b}	2.40 ± 0.08^{b}
Third	259	218.4 ± 9.6^{ab}	526.4 ± 9.6^{ab}	220.9 ± 8.6^{ab}	2.24 ± 0.09^{ab}
Fourth	175	205.9 ± 11.6^{a}	512.7 ± 11.6^{a}	207.9 ± 10.4^{a}	2.19 ± 0.11^{ab}
Fifth	118	187.4 ± 13.7^{a}	495.5 ± 13.7^{a}	201.2 ± 12.2^{a}	2.00 ± 0.13^{a}
Sixth and above	118	221.2 ± 14.0^{ab}	530.8 ± 14.1^{ab}	236.8 ± 12.6^{ab}	2.24 ± 0.13^{ab}

n — number of observations.

* $P < 0.05$; ** $P < 0.01$.

Means bearing same superscript within classes do not differ significantly ($P > 0.05$).

ond and the fourth and fifth parities differed significantly ($P < 0.05$). The decline between the third and fifth parities was gradual and the differences between means were not significant.

DISCUSSION

The average peak yield obtained in the present investigation was higher than the value reported by some earlier workers (Rao and Rao, 1994; Kundu et al., 2003b), although Chhikara et al. (1998) and Suresh et al. (2004) reported higher values for Murrah buffaloes than those observed in the present study. The time to reach peak yield in the present study was substantially higher than that reported for Murrah buffaloes at different places in India (Kundu et al., 2003b; Suresh et al., 2004). Lactation milk yield of cattle and buffaloes up to 305-d of lactation is the criterion most commonly used for the selection of dairy animals and a study of the performance of this trait is of paramount importance for carrying out selection. The overall 305-d milk yield obtained for buffaloes in this investigation was comparable with the value reported by Ulaganathan et al. (1983) and higher than the values reported by other researchers (Kandasamy, 1987; Suresh et al., 2004). The overall least-squares means of lactation milk yield obtained were higher than those observed by Ulaganathan et al. (1984) and Patnaik (1988) in the same herd. Differences in the estimates might be due to sampling errors, genetic constitution of the herds, agroclimatic variations and management conditions. In general, the performance in terms of the first lactation milk yield of Murrah buffaloes at the Central Cattle Breeding Farm, Alamadhi is quite comparable (Sethi and Khatkar, 1997; Kumar et al., 2002) with those herds in Haryana indicating that there might not be any appreciable genotype x environment interaction.

The mean service period, calving interval and dry period recorded here were in agreement with other research reports on Murrah buffaloes (Kandasamy, 1987; Patnaik, 1988; Kundu et al., 2003b). However, much lower than the present estimates for the above reproductive traits were also reported by several researchers (Chhikara et al., 1995a; Dass and Sadana, 2000; Banik and Tomer, 2003). The mean number of services/conception recorded (2.31 ± 0.05) is also much higher than those reported by Kumar et al. (2003), but Dutt and Yadav (1988) and Chhikara et al. (1995b) found comparable estimates for Murrah buffaloes maintained at the National Dairy Research Institute, Karnal and the Buffalo Research Centre, Hisar in India.

The main factor controlling variations in the calving interval is the service period, which in turn depends on postpartum oestrus d and number of services/conception. In addition, many other factors have been implicated in lengthened calving intervals such as embryonic mortality, high milk production, seasonal and environmental fac-

tors, age of cow and sire used for service. The coefficient of variation obtained for the service period in the present study (67.3%) indicates that the herd was more heterogeneous for this trait. This strongly suggests better opportunities for improvement through good breeding practices. Hence, every effort should be made to reduce the service period sufficiently to reduce the calving interval.

The highly significant influence of period of calving observed in the present study on different production and reproductive traits was supported by similar findings on Murrah buffaloes maintained at different places in India (Kandasamy, 1987; Sethi and Khatkar, 1997; Suresh et al., 2004; Yadav et al., 2007). The difference in performance of the animals among different periods might be attributed to differences in management practices, sires used for breeding, environmental conditions such as ambient temperature, humidity, rainfall etc., and variations in feed and fodder availability.

The significant to highly significant effects of season of calving on different production traits corroborates the findings of earlier workers (Chhikara et al., 1998; Dass and Sadana, 2000) and indicates that there was a pronounced seasonal influence on the traits under study. Buffaloes calving in the winter season had longer lactation lengths and higher lactation milk yields than those calving in rainy seasons. This confirms the findings of earlier reports on Murrah buffaloes (Rao and Rao, 1994; Dass and Sadana, 2000; Gogoi et al., 2002). The higher lactation milk yields in winter and summer seasons might be due to the buffaloes calving in those periods having less gestational stress as a result of the longer service period and delayed conception; also, during their descending stage of lactation, there was an abundant availability of fodders coinciding with the monsoon seasons. The lowest milk yield in monsoon calvers might be because they suffered from heat and humidity stress and the non-availability of quality fodder during a major part of the lactation period.

The significant effect of season of calving on service period and calving interval is in agreement with earlier findings reported in literature for Murrah buffaloes (Chhikara et al., 1995b; Kumar et al., 2003; Suresh et al., 2004). It is generally observed that buffalo cows are seasonally polyoestrus between October and February and they breed regularly during this period. This could explain the shorter service period during monsoon seasons.

The higher number of services per conception in winter calvers recorded here might be due to the fact that animals calving in winter exhibit postpartum heat in the summer months; hence there would be reduction in conception rate. Conception rate is related to oestrous behaviour, time of oestrus detection and insemination and site of semen deposition. Among the different factors, accurate detection of oestrus is of paramount importance in any reproductive management programme, which is difficult in buffaloes during the summer season since most of them exhibit silent oestrus. Thus accurate detection of oestrus and management interventions to ameliorate the effects of heat load on conception rate should be implemented to reduce the number of services per conception. This in turn will have a positive effect on service period and calving interval.

The significant influence of parity on different production and reproductive traits is in accordance with the results obtained by other researchers (Dass and Sadana, 2000; Kundu et al., 2003b) on Murrah buffaloes. The highest 305-d and lactation milk yields obtained in the third parity indicate that lactational maturity is attained in the third lactation and is similar to the reports of Ulaganathan et al. (1983) and Kandasamy (1987). The significant influence of calving sequence on service period and calving interval and the longer first calving interval than the rest found in the present study concurs with other reports on Murrah buffaloes (Kandasamy, 1987; Dass and Sadana, 2000; Kundu et al., 2003a; Suresh et al., 2004). The reduction in service period and calving interval over parities might be due to differences in age of the animals and periodic culling of buffalo cows with longer calving intervals. The other plausible reason is that following the first two calvings, the physiological rhythm may be maintained (i.e. reduced postpartum oestrus days and better conception) which results in shorter inter-calving periods in pluriparous buffalo cows. Similarly, the longest dry periods in the first parity and significant reductions in later parities might be due to the reduced calving intervals, while the slight increase in reproductive traits from the fifth to sixth and later parities might be due to the lumping of all later records with the sixth parity.

CONCLUSIONS

This study revealed that non-genetic factors such as period and season of calving had highly significant effects on all the traits studied. In general, milk production of the farm-bred Murrah buffaloes at the Central Cattle Breeding Farm, Alamadhi was comparable with that of animals maintained under other government and institutional herds in India. However, the comparatively lower performance of Murrah buffaloes with respect to fitness traits indicates a lower adaptability of the breed to the hot and humid coastal region. Since temporary environmental factors play a major role on these fitness traits, better breeding management is needed for improvement. In addition, multi-trait evaluation using a combination of production and reproductive traits is needed for simultaneous improvement of production and reproductive performances of Murrah buffaloes. It is therefore imperative to emphasise improvements in husbandry practices and introduction of genetic evaluation programmes at the same time.

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Effects of Evaporative Cooling System on Productive and Reproductive Performance and some Physiological Parameters of Crossbred Holstein Friesian Cattle in Tropical Conditions

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ABSTRACT

The productive and reproductive performances and physiological changes occurring in crossbred primiparous cows raised in an evaporative cooling housing system (EVAP) and an open housing system (NEVAP) were compared. In the first experiment, 20 primiparous cows were randomly allocated to two groups of 10 animals. Each group was kept individually in a two-tie stall housing system with all animals being fed with the same ration after parturition through 10 weeks of lactation. Average ambient temperature, relative humidity and temperature-humidity index in EVAP and NEVAP systems were 25.4 °C and 28.5 °C; 86 and 70; and 77 and 81 respectively. Respiration rate and rectal temperature of cows in EVAP were significantly ($P < 0.01$) lower than cows in NEVAP, while dry matter intake (DMI) and DMI (% body weight [BWt]) were significantly higher ($P < 0.05$ and $P < 0.01$ respectively). Cows in the EVAP system had significantly ($P < 0.01$) higher milk yields but significantly lower water intakes than cows in the NEVAP system; however, differences in milk composition were not recorded. Cows with EVAP housing spent more time ($P < 0.05$) chewing than cows in the NEVAP system. In Experiment 2, 34 milking crossbred primiparous cows were randomly allocated into two groups of 17 animals kept under the same housing and feeding conditions as in Experiment 1. There was no significant difference in the interval to first postpartum ovulation between cows in EVAP and NEVAP or in follicular development although there was a tendency towards increased synchronisation and conception rates in the EVAP system. The results suggest that EVAP could improve the productive performance and to some extent also the reproductive performance of crossbred Holstein Friesian cows under tropical conditions.

Key words: *evaporative cooling system, heat stress, productive performance, reproductive performance, crossbred Holstein Friesian cows.*

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INTRODUCTION

Heat stress has a significant impact on dairy production. Heat stress occurs when the sum of the cow's physical heat production increases and the environmental heat becomes greater than cow's ability to dissipate heat. The principal climatic factors causing heat stress are temperature, humidity, solar radiation and wind speed (Armstrong, 1994). The most noticeable responses to heat stress are reduced feed intake, milk yield and impaired reproductive performance. Dry matter intake starts to decline and maintenance expenditure increases when environmental temperatures exceed 25 °C (NRC, 1981). However, the temperature-humidity index (THI) may describe more precisely the effects of the environment upon the cow's ability to dissipates heat. Milk yield and total digestible nutrient (TDN) intake decline slightly when the THI exceeds 72 and decline sharply when an index of 76 is exceeded (Johnson et al., 1963). During hot periods, dairy cows show signs of disrupted behaviour and impaired physiological function (Hahn and Mader, 1997). A coping strategy of cattle during heat stress is to decrease metabolic heat production by lowering feed intake, which adversely affects productivity. The major changes involved in this acclimatisation are in respiration rate (RR) and rectal temperature (RT), both as well as pulse rate being increased in cattle under heat stress (Marai et al., 1997; Bernabucci et al., 1999). For example, significant increases of RT from 38.7 °C to 40.6 °C and respiration from 42.5 to 85.3 breaths/min were found when ambient temperatures increased from 18 °C to 28 °C (Itoh et al., 1998).

It has been shown that a rise of 1 °C or less in RT is enough to reduce feed intake and milk production in dairy cows (Johnson et al., 1963). High environmental temperatures also increase water intake, which consequently reduce DMI due to gut fill (Bernabucci et al., 1999; Mallonee et al., 1985). Also, Ominski et al. (2002) found significant differences in DMI and water intake between themoneutral and heat stress phases, with heat exposure resulting in a 6.5% decrease in DMI and milk production decreasing by 4.8% when animals were exposed to heat stress compared with that produced during the thermoneutral phase. The amount of milk produced depended on the amount of feed ingested and the status of the hormonal system involved in milk production. Heat stress reduced daily milk yield by 21% as THI values increased from 68 in the spring to 78 in the summer (Bouraoui et al., 2002). Detrimental effects of heat stress on the reproductive performance of dairy cows have also been reported including suppressed intensity of oestrus, reduced

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preovulatory LH surge (Howell et al., 1994), altered ovarian follicle development (Wilson et al., 1998) and decreased embryo development (Hansen and Arechiga, 1999).

The objectives of this study were to characterise the effects of modifying the housing conditions by using an evaporative cooling system in which air is forced through a cooling pad on productive and reproductive performances of crossbred Holstein Freisian cows raised under tropical conditions.

MATERIALS AND METHODS

Experiment 1: Productive Performance

Animals and Management

Twenty primiparous 87.5% crossbred Friesian cattle were used; their average age at calving was 31.5 months. After calving they were divided into two groups of ten animals each. They were kept in a 20 x 10 m housing unit, divided into two parts. One group was kept in an evaporative cooling housing system (EVAP), 20 x 5 m and a height of 2.5 m, in which the temperature was reduced by forcing air through a cooling pad which was soaked by water for the whole day. Air movement was moved through the cooling pad by two fans each of 150 cm in diameter which automatically started to work when the ambient temperature in the barn increased to over 28 °C. Another group was kept in the open conventional housing system (NEVAP), 20 x 5 m and a height of 4 m. Both types of housing were individual tie-stall barns with individual feed and water troughs.

Ambient temperature and relative humidity of both housing systems were recorded between 0700 to 1900 h by using a dry-wet bulb thermometer for calculating the temperature humidity index (THI). THI was calculated according to NOAA (1976) in which:

$$THI = [1.8(temp) + 32] - [0.55 - 0.0055(rh)][1.8(temp) - 26]$$

and where *rh* is relative humidity (dry-wet bulb thermometer).

Maximum and minimum temperature in both housing systems were recorded daily using a digital thermometer.

Diets, Body Weight, Feed and Water Intake Measurements

Diets were formulated to meet NRC requirements (NRC, 1989). All animals received feed in the form of a total mixed ration (TMR). The roughage:concentrate ratio was 45:55 (DM basis). The roughage used in this experiment was silage composed of corn stalk and pineapple waste at a ratio of 1:1. From the beginning to the end of the experiment, which covered the period from parturition to 10 weeks postpartum, animals of both groups were fed the same ration. Food and water were available *ad libitum*.

Body weight (BWt) of individual animals was measured weekly throughout the experiment, while DMI was measured daily from parturition until week ten postpartum. The amount of feed offered and orts were weighed daily. Orts were removed in the morning before the next feeding. Samples of feed were collected and dried at 105 °C immediately to determine DM. Water consumption of each cow was measured daily at 0600 h for three consecutive days using individual water meters and an average calculated.

Rectal Temperatures and Respiration Rates

From 8–10 weeks postpartum, rectal temperature (RT) and respiration rate (RR) were measured in all animals every 2 h between 0700 to 1900 h for three consecutive days. RT was recorded with a digital electronic thermometer, while RR was measured by observing movement of the flank for 1 min three times from which an average was calculated.

Ruminal Fluid Collection and Analysis

At the end of experimental period, oro-ruminal intubation was used to collect ruminal fluid 2.5 h after feeding in the morning (Whitelaw et al., 1970). Ruminal content was obtained by sucking with an air pump, strained immediately using two layers of cheesecloth and a 60 mL aliquot of the filtered fluid preserved by adding 3 mL *6 N* hydrochloric acid and kept at -20 °C. Ruminal fluid was analysed for volatile fatty acids (VFA) by the method modified from Erwin (1961).

Eating Behaviour

Between weeks 8–10 of the experimental period, the behaviour of all animals was observed continuously for two consecutive d using video recorders. Tape records were used for assessing times spent eating, ruminating and chewing.

Milk Production and Composition

Milk production was recorded daily from parturition until 10 weeks postpartum. Milk samples were collected weekly on two occasions (a.m. and p.m.) and kept at –20 °C for analysis. They were analysed for lactose, fat and protein using MilkoScan 133B (Foss Electric, Hillerød, Denmark).

Experiment 2: Reproductice Performance

Animals and Management

The effect of EVAP and NEVAP on follicular development, the time of ovulation and conception rate were investigated in 34 primiparous 87.5% crossbred Friesian cows. They were separated into two groups of 17 animals and kept individually in the same type of housing and on the same feeds as in Experiment 1.

Starting at between 3 and 7 weeks postpartum the reproductive tract of all animals was monitored weekly by rectal palpation and ultrasonography using B-mode real time ultrasound (Aloka SSD500) and a 5 MHz linear array rectal transducer to measure follicular size and determine the presence of a corpus luteum. From 1–12 weeks postpartum blood samples (20 mL) were collected twice weekly at intervals of 3–4 d from the jugular vein. Plasma was separated and kept at -20 °C for progesterone analysis by RIA (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA). Calving to first ovulation interval was defined by the first increase in plasma progesterone (≥ 1.0 ng/mL), ovulation being assumed to have occurred 7 d before elevation of progesterone concentration. Between 85–90 d postpartum oestrus synchronisation was performed by giving all animals an initial intramuscular injection of 100 µg of gonadorelin (gonadotropin-releasing hormone [GnRH]; Fertagyl®) followed 7 d later by 500 µg of cloprostenol ([prostaglandin] PGF2α agonist Estrumate®) intramuscularly. Ultrasonography of the ovaries was performed once daily from the time of initial treatment with GnRH to the time of PGF2α treatment and then every 4 h until 120 h post-treatment. Follicular size was measured and ovulation confirmed by disappearance of the follicle. Blood samples used to determine plasma progesterone were collected daily for 3 d after the PGF2α injection. Corpus luteum regression was defined as a cow having plasma progesterone concentration > 1.0 ng/mL and then declining to a level < 1.0 ng/mL. A cow was considered synchronised only if she met the criteria stated above for both ovulation and CL regression. Synchronisation rate was defined as the percentage of cows which showed regression of the corpus luteum in addition to ovulation of a dominant follicle.

At approximately 110 d postpartum, cows received 100 µg of gonadorelin followed seven d later by an intramuscular injection of 500 µg of cloprosternol; a second injection of 100 µg gonadorelin

was performed on d 9. Sixteen to 24 h after the second injection of gonadorelin the cows were inseminated (Ovisynch). Ultrasonography was used to identify the embryonic vesicle and conceptus at 22, 28, 35 and 42 and 60 d after insemination. The presence of embryonic fluid and a foetus in the uterine horn, a palpable amniotic vesicle and foetal membrane slip were considered positive indicators of pregnancy. The conception rate (CR) was defined as the proportion of cows where early establishment of pregnancy occurred. Blood was collected at d 22 after insemination for progesterone analysis. Early embryonic loss at d 18 after insemination was declared if progesterone was below 1.0 ng/mL at d 22. To declare early embryonic loss at d 28, both plasma progesterone at d 22 and ultrasonography of the uterus at d 28 were used.

Statistical Analyses

All data were reported as the mean value ± SE. The unpaired t-test was used to estimate the statistical significance of differences in values between groups. The proportional data of cows were analysed using Chi-square. Significant differences were declared at $P < 0.05$ and $P < 0.01$.

RESULTS

Temperature, RH, THI and Physiological Changes

Mean environmental RH, THI and physiological changes during the experimental periods are presented in **Table 1**. The temperature under EVAP housing was lower than the temperature in the NEVAP system, the average difference being 3.1 °C. During the day, ambient temperature was higher than 24 °C in both type of housing, the level which has been suggested to be critical for dairy cattle. There was a large difference in mean RH between EVAP (86%) and NEVAP (70%), but when average THI was calculated, THI in the EVAP system tended to be higher (77 vs 81). Respiration rate and rectal temperature of cows raised in the EVAP system were significantly lower ($P < 0.01$) than those of cows kept in the EVAP system.

Dry Matter Intakes, Weight Gains, Milk Yields and Composition

The dry matter intake of animals in the EVAP system (13.3 kg/d) was significantly higher ($P < 0.05$) than in the NEVAP system, more so ($P < 0.01$) when expressed as a percentage of BWt. (**Table 2**) There

Table 1. Mean environmental temperatures, relative humidity, temperature-humidity index, respiration rate and rectal temperatures under EVAP and NEVAP cooling systems (mean ± SE, n = 20).

Parameter	EVAP	NEVAP
Minimum temperature (°C)	22.2 ± 0.7	23.6 ± 0.6
Maximum temperature (°C)	29.1 ± 0.3	35.8 ± 0.3
Average temperatue (°C)	25.4 ± 0.4	28.5 ± 0.3
Mean RH (%)	86 ± 0.7	70 ± 3.2
Mean THI	77 ± 0.5	81 ± 1.4
RR (breaths/min)	53 ± 0.7^{a}	67 ± 2.4^{b}
Rectal temparature (°C)	38.7 ± 0.03^{a}	39.4 ± 0.09^{b}

Different superscripts in the same row are significantly different ^{ab} ($P < 0.01$).

Table 2. Body weights (BWt), dry matter intakes, milk yields and water intakes of crossbred Friesian heifers maintained in EVAP and NEVAP cooling systems (mean ± SE, n = 20).

Parameters	EVAP	NEVAP
Initital BWt (kg)	374.3 ± 15.3	358.4 ± 10.9
Final BWt (kg)	392.5 ± 10.8	378.7 ± 15.4
Average BWt (kg)	384.1 ± 12.6	364.6 ± 13.9
DMI (kg)	13.3 ± 0.4^{a}	11.1 ± 0.5^{b}
DMI	15.4 ± 0.3^{a}	12.9 ± 0.4^{b}
DMI/%BWt	3.47 ± 0.22^{c}	3.03 ± 0.06^{d}
Milk yield (kg/d)	16.9 ± 1.9^{a}	12.6 ± 0.6^{b}
4%FCM (kg/d)	14.6 ± 2.5^{c}	11.1 ± 0.5^{d}
DMI/4%FCM	0.92 ± 0.13	1.01 ± 0.04
Water intake (L/d)	54.4 ± 3.5^{c}	93.6 ± 8.0^{d}
Water intake/DMI (L/kg)	4.5 ± 0.3^{c}	10.6 ± 0.8^{d}

Different superscripts in the same row ares significantly different ^{ab} ($P < 0.05$), ^{cd} ($P < 0.01$).

was, however, no difference between the groups in terms of weight gain over the experimental period.

Cows in EVAP produced significantly more milk than cows in NEVAP ($P < 0.05$), but differences in milk composition were not significant except that cows in the EVAP system produced a higher amount of 4% fat corrected milk (FCM) than cows in the NEVAP system ($P < 0.01$).

It was found that animals in EVAP drank 54.4 L water/d compared with 93.6 L/d in the NEVAP system ($P < 0.01$). Expressed as L/kg DMI, water intake under the NEVAP system was also significantly higher ($P < 0.01$).

There were no differences in eating and ruminating times under the two systems of housing (**Table 3**) but cows in the EVAP system tended to spent more time eating and ruminating and significantly more time chewing ($P < 0.05$) than cows in NEVAP, and as a result spent less time resting ($P < 0.05$). Production of VFAs was essentially the same under both systems (**Table 3**).

Reproductive Performance

One cow in the control group was excluded from the study due to a foot problem. The reproductive performance of the cows under the two systems of housing are shown in **Table 4**. The success of oestrus synchronisation in cows under EVAP housing was higher than cows raising in NEVAP, but not significantly so. Follicular size and d to first ovulation were similar in both groups, but conception rates by 22 d and 60 d after insemination were higher in cows housed under EVAP conditions, but again the differences were not statistically significant. Embryonic loss was somewhat lower on d 18 in cows housed under EVAP conditions but by d 28 the rate of embryonic loss in both groups was similar.

DISCUSSION

THI is widely used in hot areas all over the world to assess the impact of heat stress on dairy cows. If the heat stress level classified by Hahn and Mader (1997) is used, it can be concluded that cows in the EVAP group were under mild stress while those kept under NEVAP conditions were under medium stress. However, cows kept under the former conditions suffered mainly from high RH while those in the NEVAP group experienced heat stress through the high ambient temperature. All animals in this experiment were therefore heat stressed although at different levels of severity. Nevertheless, environmental temperatures and THI were higher for the NEVAP group, especially during the day although it is likely that the THI of the EVAP group fell below 74 during the night and as a result cows in this group might compensate for their lower day-time consumption by eating more during the night. On the other hand, the THI under NEVAP conditions may have exceeded 74 due to increased RH at night. Cows in NEVAP would therefore have less chance to compensate their consumption during the cooling period. This would explain why animals kept under EVAP housing consumed 22.7% more feed

Table 3. Chewing behaviour and volatile fatty acids of cows maintained in EVAP and NEVAP cooling systems (mean ± SE, n = 20).

Parameter	EVAP	NEVAP
Total eating time (min/d)	227.1 ± 6.9	192.4 ± 6.2
Total ruminating time (min/d)	349.5 ± 5.2	255.9 ± 13.7
Total chewing time (min/d)	576.7 ± 4.3[a]	448.3 ± 12.8[b]
Resting time (min/d)	863.3 ± 4.7[a]	981.7 ± 14.7[b]
Volatile fatty acids (VFA, mmol/mL)		
Acetate	119.2 ± 6.4	107.7 ± 8.9
Propionate	42.7 ± 3.4	43.8 ± 3.1
Butyrate	19.0 ± 1.0	19.8 ± 1.4

Different superscripts in the same row are significantly different [ab] ($P < 0.05$).

Table 4. Reproductive performance of cows maintained under EVAP and NEVAP housing systems (mean ± SE, n = 17).

Parameters	EVAP	NEVAP
Synchronisation rate (%)	82.4 (14/17)	52.9 (9/17)
Size of the largest ovulatory follicle at PGF2α injection (mm)	11.5 ± 0.6	10.2 ± 0.5
Maximal size of the largest ovulatory follicle (mm)	14.6 ± 0.5	14.2 ± 0.4
Days to postpartum ovulation [a]	31.4 ± 4.3	26.1 ± 3.6
Conception rate (%) within 22 d after insemination[b]	43.8 (7/16)	23.5 (4/17)
Conception rate (%) within 60 d after insemination[b]	25.0 (4/16)	7.6 (3/17)
Embryonic loss (%) within 18 d[c]	56.2 (9/16)	76.5 (13/17)
Embryonic loss (%) within 28 d[c]	75.0 (12/16)	82.4 (14/17)

[a] one cow from each treatment did not show ovulation; b number of pregnant cows/number of inseminated cows; c number of nonpregnant cows/number of inseminated cows.

than animals under NEVAP conditions. The higher feed intake of cows in the EVAP group had a direct effect on milk production. These animals produced 4.3 kg/d more milk and 3.5 kg/d more of 4% FCM than cows kept under the NEVAP system. Cows in the EVAP group were therefore more productive.

These findings are consistent with the results of Bouraoui et al. (2002) who found negative correlations between daily THI and both milk yield (r = –0.76) and feed intake (r = –0.24) and that milk yield decreased by 0.41 kg/cow/d for each point increase of THI above 69. Also, Johnson (1985) and Du Preez et al. (1990) showed that milk production was not affected by heat stress when THI values were between 35 and 72 and that both milk production and feed intake began to decline only when THI reached 72 and continued to decline sharply at THI values of 76 or greater. Shearer and Beede (1990) reported that heat stress influenced milk composition through its effect on feed intake, which was the main response of dairy cattle to high environmental temperatures (Collier et al., 1981). No significant differences were recorded in milk composition in this study, but cows in the EVAP group tended to produce milk of superior composition. A similar response was reported by Strickland et al. (1989) and Abelardo et al. (2002) who although not finding an effect of cooling on milk fat percentage, recorded an increase in protein levels in animals kept under a cooling regime.

Indicators of heat stress in cattle include increases in RT, RR and pulse rate (Lemerle and Goddard, 1986; Itoh et al., 1998 and Marai et al., 1997). RT is a sensitive indicator of thermal balance and may be used to assess the negative effects of hot environments on growth, lactation and reproduction in cows (West, 1999). It has been shown that a rise of 1 °C or less in RT is enough to reduce intake and production in dairy cows. A cow normally has 15–30 breaths/min and RRs of 80–90 breaths/min are considered a clear indication of heat stress (Stowell, 2000). Eigenberg et al. (1999) reported a positive correlation between RR and ambient temperature and at these temperatures thermoregulation by increasing evaporative heat loss from the upper respiratory passages would be apparent (Thatcher and Collier, 1986). In the present study, there were significant differences between RT and RR values for cows in the EVAP and NEVAP groups, the significantly greater increases recorded for the latter being characteristic of heat stressed animals. Several studies have shown that evaporative cooled cows had lower values for RT and RR than those that were not cooled (Abelardo et al., 2002; Chen et al., 1993; Huber et al., 1994).

Increased water intake is a further major physiological reaction to heat stress. Bernabucci et al. (1999) reported that exposure to high temperatures was responsible for both increased water intake and reduced DMI. In this study, dairy cows maintained under NEVAP housing consumed more water than those exposed to evaporative cooling. A finding explained by established physiological knowledge that water consumption increases with increasing environmental temperatures because of the greater water losses incurred from sweating and water vaporisation arising from more rapid respiratory rates (panting), both effects aimed at increasing evaporative cooling for the cow (NRC, 1981).

Climatic conditions also influence the behaviour of dairy cows. Cows try to avoid activity during the hotter day, concentrating their grazing/eating during the relatively cooler early morning and late afternoon periods extending into the cool of the evening. Under the conditions of this study, there were no statistical differences between the groups in times spent eating and ruminating, although due to their lower level of feed consumption cows in the NEVAP group tended to spend less time eating and ruminating. Also, when total chewing time (eating time + ruminating time) was considered, NEVAP animals spent significantly less time chewing. This finding is in agreement with the study of Prasanpanich et al. (2002) who reported that rising temperature and humidity contributed to declining eating activity, and with the finding of Cowan et al. (1993) that increasing temperature during the day forced the early cessation of grazing in lactating cows.

Heat stress can depress the reproductive performance of dairy cows including by decreasing intensity of oestrus, reducing the preovulatory LH surge and decreasing secretion of luteal progesterone (Howell et al., 1994). Also, estradiol is necessary during the preovulatory period to produce an LH surge and ovulation. Cows in EVAP which were in a better status of heat stress may have produced enough estradiol to initiate oestrus and ovulation. The time from giving PGF2α to oestrus or ovulation is influenced by the oestrus cycle and the follicular development stage in the follicular wave at the time of PGF2α treatment (Stevenson et al., 1998). In this study, follicular development at the time of PGF2α treatment was expected to be similar in both groups because the dominant follicle was synchronised by the GnRH injection. However, the response rate to synchronisation of ovulation in cows kept in EVAP housing tended to be greater than in cows kept under NEVAP conditions. One of the deleterious effects on lactating cows which experience heat stress is a reduction in follicular growth (Wilson et al., 1998), but in this study no relationship was found between follicular size and type of housing. However, the size of largest ovulatory follicle at the time of PGF2α treatment in cows maintained under the EVAP system tended to be greater than cows kept in the NEVAP system. These findings suggest that EVAP housing may improve hormonal status by virtue of increased oestradiol secretion arising from the greater size of the ovulatory follicle and the higher ovulation rate.

High ambient temperatures significantly increase embryonic loss (Sugiyama et al., 2003) and Ryan et al. (1993) reported that a high percentage of embryonic loss occurred between d 7 and 14. In this study cows kept under EVAP housing suffered lower embryonic losses during the earliest stages of pregnancy, suggesting that the cooling system used could alleviate the effect of heat stress on embryonic loss at the early stage of pregnancy. However, the advantage noted at conception declined after 18 d. Indeed, the degree of embryonic loss after d 18 was higher in cows housed in the EVAP system indicating that the cooling system was not good enough to get rid of whole heat stress which can affect the loss of embryo at the later stage of pregnancy. However, the conception rate in cows housed in EVAP system was greater at 60 d after insemination suggesting that EVAP could nevertheless improve conception among crossbred cows at the earlier stage of pregnancy.

CONCLUSIONS

The EVAP housing system led to lower environmental temperatures and values for THI during the day time compared with the conventional housing system, leading to reduced RR and RT. In addition, DMI, milk yields and 4% FCM were higher in cows maintained under the EVAP system, and some reproductive indices were superior compared with those of cows kept under NEVAP housing. The results suggest that EVAP has the potential to alleviate the stress occurring from heat exposure, reducing thereby the deleterious effects of heat stress on the productive and reproductive performance of crossbred lactating cows. However, further study is needed to determine the effects of this cooling system on animal health, and more broadly its economic benefit under the conditions found in Thailand.

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Molecular Characterisation of Bulgarian Livestock Genetic Resources and their Optimal Utilisation for Animal Production

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ABSTRACT

This study was undertaken to determine the genetic structure and diversity among two local cattle breeds from Bulgaria, the Rhodope Shorthorn and Grey cattle. A panel of 11 microsatellites was used for the evaluation. For these loci, allele frequencies, heterozygosity, HWE, genetic disequilibrium were determined. Both populations displayed a relatively high level of genetic variation as estimated by allelic diversity and heterozygosity. Heterozygosities ranged from 0.5424 (SPS 115) to 0.8983 (TGLA 227) for the Rhodope population and from 0.6333 (TGLA 53) to 0.9333 (TGLA227) for Grey cattle, with similar average values for the two groups (0.7858 and 0.7757). These results clearly suggest that these breeds are suitable to preserve as genetic resources.

Key words: *microsatellites, genetic structure, genetic diversity, linkage disequilibrium, animal production.*

INTRODUCTION

One of the factors for improvement of livestock breeds in future is to develop new breeds with desirable traits based on cross-breeding between traditional livestock breeds resistant to important diseases and commercial breeds. If done effectively, this would yield animals with productive characteristics of the commercial breed and the disease resistance of the traditional breed. However, when livestock breeds become extinct, their unique genes are lost forever. Loss of local breed populations contradicts the principles for sustainable development of animal breeding and the correct management of animal genetic resources (AnGR). The old Bulgarian breeds, traditionally bred, have been adapted to local conditions and they are resistant to diseases. Due to this they are usually preferred for organic raising of animals (which excludes the use of veterinary medicines and preparations). Utilising local breeds is therefore an effective strategy for contributing to the achievement of local food security objectives.

The necessity to extend, maintain and conserve genetic diversity has been outlined in the First Report on the State of the World's Anmal Genetic Resources (FAO, 2007). In this context a number of molecular techniques have provided new DNA markers for the study of genetic variation (Nijman et al., 1999; Hansen et al., 2002). During recent years, different studies of cattle breeds based on microsatellite markers have aimed at characterising the genetic variation, genetic relationships between cattle breeds from Italy (Ciampolini et al., 1995, Spain (Martin-Burriel et al., 1999; Canon et al., 2001), Belgium (Mommens et al., 1999), Poland (Radko and Duniec, 2002), and the Czech Republic (Czernekova et al., 2006).

The use of microsatellites with high polymorphism information content for correctly identifying cattle, assists in better operation of breeding programmes and breed improvement.

Understanding the diversity, distribution, basic characteristics, comparative performance and the current status of Bulgaria's animal genetic resources is essential for their efficient and sustainable use, development and conservation. For sustainable management, diversity needs to be considered and understood at the species level, between and within breeds.

The aims of this study were therefore to describe the cattle production systems and assess the genetic diversity by analysing genetic variability of eleven microsatellite markers in two populations of Bulgarian local breeds with a view towards promoting their conservation.

Bulgarian Animal Genetic Resources and their Productivity

Bulgaria is rich in animal genetic resources, i.e. in cattle, horses, pigs, sheep, goats, dogs and poultry. Two cattle breeds (Grey and Rhodope Shorthorn); nineteen sheep breeds (Blackhead Pleven, Local Stara Zagora, Local Karnobat, Splotch-Faced Maritza, White Maritza, Karakachan, Cooper-Red Shoumen, Replyan, Duben, Middle Rhodopean, Kotel, Middle Stara and Planina, Sofia (Elin-Pelin), Strandza, Koprivshtitza, Sakar, Teteven, West Stara Planina and Breznik); one goat breed (Local Long-Haired [Kaloferska] goat); four horse breeds (East Bulgarian, Karakachan, Danubian, and Pleven); one dog breed (Karakachan); and one poultry breed (Black Shoumenska hen) have been reported so far (Gelev et al., 2008 and 2009).

The high value of Bulgarian local breeds reflects the genes they possess which provide their excellent adaptive capabilities, high resistance to diseases and ability to produce high quality meat, milk and eggs. However, intensified use of commercial breeds has had serious effects on local types of domestic animals, which as a rule are less productive. Undoubtedly, the increased import of highly-selected breeds and the complete ignorance and undeserved lack of interest in local genotypes have contributed to their decline. There is a real danger that more of them will disappear forever.

Within Bulgaria there are three recognised breeds of domestic cattle: the Local Grey and its offshoot the Iskar-Grey which

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are selected mainly for productivity, and the Rhodope Shorthorn. The Rhodope breed is a representative of the brachyceros type of cattle, and the Grey is a result of prolonged cross-breeding between the brachyceros type and a primigenius type. This cross-breeding has taken place with varying degrees of intensity and continuity in different places in Bulgaria, the result of which in the recent past has been a rich diversity of combinations. It has been established that in lower-lying regions where forage conditions are better, larger and more productive primigenius types are predominant. The opposite is also true: the influence of the smaller, less productive brachyceros type increases with altitude.

They are bred mainly in the region of the rivers Iskar, Vit, Osam, and Rositsa, from whence Iskar - Grey cattle breed received its name. The population of this breed has declined from 74 152 in 1957 to just 281 today. Put another way, Iskar cows in 1957 represented 7.1% of the cows in the country, and today they represent practically nil.

As suggested by its name, the Rhodope Shorthorn evolved in the Rhodope mountains. Together with the Albanian and southern Montenegran cattle, the Rhodope cattle is the last remnant of the prehistoric brachyceric cattle in Europe. It was domesticated over 8 000 years ago and is also referred to as the Thraco-Illyrian Brachyceros. Small in size, they are capable of using any type of mountain pasture. In the winter when food is scarce, they usually lose about 20% (50 kg) of their body weight, but regain it in less than one month in the spring by grazing.

In 1957, there were 429 975 Local Grey cattle (40–42% of the cattle in the country) and there were 52 956 Rhodope Shorthorn cattle in 1961 (Danchev 1994; Dimitrov and Dimitrova 1994; Dimitrov et al., 1998) but nowadays they are on the brink of extinction. According to the information in the EAAP-Animal Genbank of the European Association for Animal Production (/http://www.tiho-hannover.de/einricht/zucht/eaap/), both these breeds are classified as "critically endangered". The calculated number of females (NFN) is 280 for Grey cattle and for Rhodope Shorthorn it is less than 250. According to the most recent monitoring by the Executive Agency of Selection and Reproduction in Bulgaria (Gelev et al., 2008 and 2009), for the Grey cattle breed there are 1 506 cows with 729 females under selection while for the Rhodope Shorthorn breed, there are 604 cows with 97 females under selection control.

The average live weight of Grey Iskar cows is 390 kg. Average milk-yield is 2 500–2 600 L, with a maximum of 6 929 L. The fat content in the milk is on average 4.2%, with a maximum of 5.7%. The fat content of the milk is on average 4.5%, with a maximum of 5.9%. They are exceptionally hardy and strong. In addition to milk, these animals are used to provide tractive power (up to the age of 15 or 20 years).

The average live weight of Rhodope Shorthorn cattle is around 220 kg., their average milk yield is between 1 100–1 200 L but varies from 400 to 1 900 L. Maximum yields are obtained between the fifth and 11th lactations.

Comparisons among all the local and improved breeds of cows in Bulgaria show that the Rhodope Shorthorn breed is second only to the Bulgarian Red cows in production per 100 kg live weight. Moreover, they live 2–3 times longer than the highly-selected breeds and can produce offspring and milk into an advanced age.

In order to avoid extinction, the Bulgarian government has formed breeding populations of these autochthonous domestic animals. Also, in 2003 the Executive Agency for Selection and Reproduction in Animal Breeding in Bulgaria formulated a programme for conservation of AnGR and relevant guidelines for performing selection activities. In addition, a National Gene Bank was established recently for conservation of semen, as was patenting of the autochthonous

Table 1. Chromosome location and primer sequences of eleven microsatellite loci analysed.

Cattle chromosome	Locus	Size range (bp)	Primer sequence	Reference
18	TGLA 227	64–115	F:5`-cga att cca aat ctg tta att tgc t-3` R:5`-aca gac aga aac tca atg aaa gca-3`	Barendse et al., 1994
2	BM 2113	116–146	F`:5`-cgt gcc ttc tac caa ata ccc-3` R:5`-ctt cct gac aga agc aac acc- 3`	Bishop et al., 1994
7	TGLA 53	147–197	F:5`-gct ttc aga aat agt ttg cat tca-3` R:5`- atc ttc aca tga tat tac agc aga-3`	Barendse et al., 1994
5	ETH 10	198–234	F:5`-gtt cag gac tgg ccc tgc taa ca-3` R:5`-cct cca gcc cac ttt ctc ttc tc-3`	Toldo et al.,1993
20	TGLA 126	104–133	F:5`-cta att tag aat gag aga ggc ttc t-3` R:5`-ttg gtc tct att ctc tga ata ttc c-3`	Barendse et al., 1994
21	TGLA 122	130–193	F:5`- aat cac atg gca aat aag tac ata c – 3 R:5`- aat cac atg gca aat aag tac ata c-3`	Barendse et al., 1992
3	INRA 023	193–235	F:5`-gag tag agc tac aag ata aac ttc-3` R:5`- taa cta cag ggt gtt aga tga act c-3`	Vaiman et al., 1994
19	ETH 3	90–135	F:5`-gaa cct gcc tct cct gca ttg g-3` R:5`-act ctg cct gtg gcc aag tag g-3`	Toldo et al.,1993
9	ETH 225	135–165	F:5`- gat cac ctt gcc act att tcc t-3` R:5`- aca tga cag cca gct gct act-3`	Steffen et al., 1993
1	BM1824	170–218	F:5`-gag caa ggt gtt ttt cca atc-3` R:5`-cat tct cca act gct tcc ttg-3`	Bishop et al., 1994
15	SPS 115	234–258	F:5`- aaa gtg aca caa cag ctt ctc cag-3` R:5`-aac gag tgt cct agt ttg gct gtg -3`	Mommens et al., 1998

breeds as well as monitoring their populations (Gelev et al., 2008 and 2009). One of the requirements for strengthening protection of these AnGR is to apply DNA analysis for the purposes of identification and for paternity testing. With the growing availability of new techniques in molecular biology, it is nowadays possible to characterise genetic resources at the DNA level.

MATERIALS AND METHODS

Blood samples were collected from 89 animals belonging to the two breeds, namely 30 unrelated Grey cattle (G) and 59 Rhodope Shorthorn cattle (R) from the regions of Sredetz and Smoljan. Total genomic DNA was extracted using GFX Genomic Blood Purification Kit (Amersham Biosciences, UK).

As recommended by the International Society of Animal Genetics (ISAG), for diversity studies in cattle 11 microsatellites (**Table 1**) were selected, using the Stockmarks for Cattle Bovine Genotyping Kit (Applied Biosystems). Multiplex amplification was carried out in a final volume of 15 µL containing of 50 ng of template DNA, 0.5 units of AmpliTaqGold™ polymerase, 3.0 µL Stockmarks buffer, 400 µL of eash dNTP and 5.5 of primer mix. The reactions were carried out using a thermocycler (GeneAmp 9700, Applied Biosystems) in an initial denaturation phase of 15 min at 95 °C, followed by 31 cycles of 45 sec at 94 °C, 45 sec at 61 °C, and 1 min at 72°C. A final extension was carried out at 72°C for 1 h and then at 25 °C for 2 h. The amplified products were mixed with fluorescent dye group and analysed in a 5% (w/v) denaturing gel using an ABI PRISM^{TM} 377 DNA Sequencer. The fluorescence data were collected by GeneScan^{TM} Analysis 2.0 and analysed using Genotyper^{TM} 2.0 software.

The GENEPOP package (Raymond and Rousset, 1995) was used to perform an exact test for deviation from Hardy-Weinberg equilibrium (HWE), allele frequencies, the expected (He) and observed heterozygosity (Ho), and genotypic linkage disequilibrium. Fisher's method was used to combine the two populations and 11 microsatellite loci with 4n degrees of freedom, computed as in Weir and Cockerham (1984) and Robertson and Hill (1984).

RESULTS AND DISCUSSION

One hundred and seventy eight alleles were detected from the 11 loci surveyed — 118 alleles in Rhodope (R) and 60 in Grey (G) cattle yielding a mean value of 9.0 alleles and 7.5 per locus respectively. Since the evaluation of polymorphism is strictly dependent on the allele number and the frequency distribution of the alleles, estimates of allele frequencies are essential.The allele frequencies of 11 microsatellites are listed in **Tables 2** and **3**. These frequencies revealed that not all markers were equally informative.

All loci were polymorphic in both breeds. The alleles detected at different loci varied with disparate frequency. At the most polymorphic locus TGLA 53–13 alleles were in the range of 154 to 186 bp. Alleles of 160 bp and 170 bp were the most frequent in both breeds at this locus. The allele 166 bp was present in Rhodope but absent in Grey cattle and allele 186 bp was present in Grey and absent in Rhodope Shorthorn cows. The data for another high polymorphic locus - TGLA 122 showed the absence of alleles 138bp, 140 bp and

Table 2. Allele frequencies of the 11 microsatellites loci analysed in Bulgarian Grey cattle.

TGL227		TGL53		TGL122		ETH3		BM2113	
Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq
78	0.014	154	0.086	142	0.100	103	0.071	125	0.157
80	0.043	160	0.329	144	0.343	109	0.029	127	0.029
82	0.114	162	0.043	148	0.029	115	0.071	133	0.300
84	0.200	164	0.043	152	0.028	117	0.229	135	0.200
86	0.114	168	0.171	154	0.371	119	0.143	137	0.114
90	0.072	170	0.029	156	0.029	121	0.029	139	0.129
92	0.172	172	0.029	164	0.043	123	0.100	141	0.071
94	0.071	174	0.029	172	0.028	125	0.286		
96	0.057	176	0.057	174	0.029	127	0.042		
98	0.014	180	0.086						
100	0.014	182	0.057						
104	0.015	184	0.041						

SPS115		TGLA126		INRA023		ETH 225		ETH10		BM1824	
Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq
248	0.571	109	0.014	199	0.186	140	0.371	210	0.071	180	0.157
250	0.014	117	0.529	207	0.115	144	0.143	216	0.057	182	0.200
252	0.029	119	0.300	209	0.129	146	0.129	218	0.286	184	0.386
254	0.157	121	0.014	211	0.057	148	0.157	220	0.329	190	0.2571
256	0.086	123	0.043	215	0.471	150	0.157	222	0.257		
258	0.029	125	0.100	217	0.042	158	0.043				
260	0.114										

Table 3. Allele frequencies of the 11 microsatellites loci analysed in Rhodope Shorthorn cattle.

TGL53		TGL122		TGL227		INRA023		ETH3	
Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq
154	0.014	138	0.008	80	0.100	175	0.017	109	0.017
160	0.043	140	0.017	82	0.343	197	0.008	113	0.008
162	0.114	142	0.195	84	0.029	199	0.144	115	0.119
164	0.200	144	0.288	86	0.028	205	0.068	117	0.331
166	0.114	146	0.008	90	0.371	207	0.161	119	0.127
168	0.072	152	0.034	92	0.029	209	0.102	121	0.017
170	0.172	154	0.144	94	0.043	211	0.119	123	0.034
172	0.071	156	0.051	96	0.028	213	0.017	125	0.305
176	0.057	164	0.051	98	0.029	215	0.237	127	0.034
178	0.014	172	0.017	100		217	0.102	131	0.008
180	0.014	176	0.136	102		219	0.025		
182	0.015	178	0.051						
184	0.015								

ETH225		BM2113		ETH10		SPS115		TGL126		BM1824	
Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq
138	0.008	125	0.008	212	0.017	248	0.686	117	0.051	180	0.051
140	0.288	127	0.144	214	0.017	250	0.034	119	0.525	182	0.331
144	0.229	129	0.008	218	0.517	252	0.068	121	0.153	184	0.432
146	0.025	131	0.186	220	0.195	254	0.076	123	0.008	190	0.178
148	0.186	133	0.153	222	0.220	256	0.042	125	0.042	192	0.008
150	0.085	135	0.288	224	0.017	258	0.025	127	0.220		
156	0.008	139	0.169	226	0.017	260	0.068				
158	0.153	143	0.042	226	0.017						
160	0.017			226	0.017						

178 bp in the Grey cattle population. The most frequent allele in the studied populations was the 144 bp allele, while the rarest alleles were 138bp and 152bp (0.008) for the Rhodope population and 156 bp (0.017) for the Greys. ETH 3 amplified ten and nine alleles respectively for Rhodope and Grey cattle with the most common alleles being 117 bp and 125 bp. In both breeds the 121bp allele had the lowest frequency. Four to five alleles were detected in locus BM1824. The most common allele was 184 bp. Allele 192 bp was present only in the Rhodope Shorthorn population.

The results obtained in the present study concerning allele frequencies in Bulgarian local cattle are in agreement with the data obtained by Radko and Duniec (2002). However, Czernekova et al. (2006) reported higher numbers of alleles in the same loci for Czech cattle — from seven at BM1824 to 14 at TGLA 227. According to Peelman et al. (1998) and Cervini et al. (2006) who have analysed European cattle and *Bos indicus* Nellore cattle, the number of TGLA53 locus alleles in Holstein Friesian (13 alleles), Belgian Red Pied (12 alleles), East Flemish (12 alleles) and Belgian Blue (10 alleles) cattle and Nellore cattle (13 alleles) were very similar to that found in Rhodope Shorthorn (13 alleles) and Grey cattle breeds (12 alleles). High polymorphism was noted also at loci TGLA 122, with 12 and 10 alleles respectively for R and G cattle, TGLA 227 (11 and 12 alleles) and ETH 3, with 10 and 9 alleles for R and G cattle. The lowest number of alleles in both breeds was observed at locus BM1824. The same variation of number of alleles were shown by Zhou et al. (2005) for five native Chinese cattle breeds, while the number of alleles at locus TGLA 227 in the present study is higher than observed by Martin-Burriel et al. (1998) and Armstrong et al. (2006) for Spanish native and Creole cattle breeds (seven alleles). The Rhodope Shorthorn population had a greater mean number of alleles (9.0) than the Grey cattle (7.5), although this may have been due, in part, to the much larger sample size.

Both populations were described according to the expected (He) and observed (Ho) heterozygosity (**Table 4**). The data in **Table 4** show a common trend in the two groups. Expected heterozygosity ranged from 0.5424 (SPS 115) to 0.8983 (TGLA 227) for the Rhodope population and from 0. 6333 (TGLA 53) to 0.9333 (TGLA 227) for Grey cattle. All the microsatellite loci showed an expected heterozygosity greater than 0.500. The overall mean heterozygosity across all populations and all markers had similar average values for both groups i.e. 0.7858 and 0.7757 respectively. In an analysis of six Spanish native breeds, Martín-Burriel et al. (1998) reported an average expected heterozygosity between 0.56 and 0.68. Additionally, Rendo et al. (2004) found an expected heterozygosity between 0.69 and 0.76 in four Western Pyrenees cattle breeds using 11 microsatellite markers, while in a study of 15 Portuguese cattle breeds Mateus et al. (2004) found an average expected heterozygosity between 0.63 and 0.74. Similar results were reported by Zhou et al. (2005) for five native Chinese populations which displayed a high heterozygosity, namely 0.51 to 0.86. The values obtained here for expected heterozygosity

Table 4. Observed and expected heterozygosity and number of alleles in Bulgarian cattle breeds studied.

Locus	Expected heterozygosity He	Observed heterozygosity Ho	Number of alleles N
TGLA 227			
R	0.8983	0.8536	11
G	0.9333	0.8885	12
BM 2113			
R	0.8475	0.8141	8
G	0.8000	0.8172	7
TGLA 53			
R	0.7966	0.8383	13
G	0.6333	0.8517	12
ETH 10			
R	0.6610	0.6505	7
G	0.8333	0.7425	5
SPS 115			
R	0.5424	0.5143	7
G	0.7000	0.6488	6
TGLA 126			
R	0.6610	0.6531	6
G	0.6667	0.6149	6
TGLA 122			
R	0.8814	0.8369	12
G	0.6667	0.7523	10
INRA 23			
R	0.8814	0.8635	11
G	0.8333	0.7351	6
ETH 3			
R	0.8644	0.7702	10
G	0.8000	0.8351	9
ETH 225			
R	0.8814	0.8045	9
G	0.8333	0.7787	6
BM 1824			
R	0.7288	0.6749	5
G	0.8333	0.7402	4
Mean			
R	0.7858	0.7513	9.0
G	0.7757	0.7641	7.5

were higher than those reported by Citek and Rehout (2001) and Czernekova et al. (2006) for endangered populations of Czech Red, Czech Pied, Polish Red, and German Red cattle, which were in the range of 0.396–0.495 and 0.650 to 0.764 respectively. Our study showed the highest level of heterozygosity at locus TGLA 227 in both populations. This means that this marker could be included in subsequent genetic diversity studies of cattle populations. Also, the heterozygosity found in our samples of Bulgarian local cattle breeds was considerably higher than that found in studies on commercial breeds that used similar microsatellites. For example, Hansen et al. (2002), and Maudet et al. (2002) showed that highly selected commercial breeds were much less diverse and more inbred than local breeds, which reinforces the importance of local breeds as reserves of genetic diversity for sustainable agriculture.

According to classical genetics, a population is in Hardy–Weinberg equilibrium (HWE) if the gene (p and q) and genotype frequencies, p2 (AA), 2pq(Aa), and q2 (aa) are not changing from one generation to another. Evolutionary factors such as genetic drift, selection, mutation, and migration are the forces which can modify HWE in the population. Genetic drift is due to a small population size, where mating of related individuals is conducive for enhanced inbreeding. The selection of non-neutral alleles and mutations contribute to genetic differentiation among populations. In this study, deviation from proportions of HWE were noted for all the loci except for TGLA 53 ($P < 0.05$) in Rhodope Shorthorn and for BM 2113, ETH 3, TGLA 122 and TGLA 53 ($P < 0.01$) in Grey cattle breeds. Ciampolini et al. (1995) reported that Hardy–Weinberg equilibrium was not maintained in a study of microsatellites for four Italian beef breeds. Migration (gene flow) from an external population is a possible factor contributing to the observed deviation from HWE in the present investigation.

Tests of genotypic disequilibrium across populations resulted in 110 comparisons. Significant linkage disequilibrium ($P < 0.05$) was found between locus pairs BM2113 and TGLA53 on the one hand and ETH 3 on the other in the Rhodope Shorthorn breed. In the second local Bulgarian breed a genetic disequilibrium was observed between the BM2113 and ETH3 and ETH 225, BM1824 and ETH10 loci. ($P < 0.05$). Gametic disequilibrium can arise from a variety of causes including epistatic selection, physical linkage, genetic hitchhiking. Because the markers studied were mapped to different chromosomes (respectively BM1824 on chromosome 1, BM2113 on chromosome 2, ETH 3 on chromosome 9, ETH 225 on chromosome 10 and TGLA 53 on chromosome 16), one can exclude physical linkage. The most probable reason may therefore be gametic selection.

CONCLUSIONS

This study extends knowledge of the genetic diversity, genetic structure and molecular characterisation of small populations of local cattle breeds in Bulgaria that are on the brink of extinction. Grey and Rhodope Shorthorn cattle breeds were characterised genetically using DNA markers. All loci were polymorphic indicating that the microsatellite markers used are suitable for studying genetic diversity. The comparison between the two local breeds shows that they displayed a remarkably high variability. This clearly suggests that these breeds have however, potential value to be preserved as genetic resources.

More work and analysis will be required in the future to increase the efficiency of studying a larger number of microsatellites. Additional information on productive, morphological, and fitness-related traits of these breeds is needed, however, as these factors should also be taken into account when ranking breeds for the purposes of conservation and sustainable use.

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Effect of Temperature and Humidity on Heat Stress Responses in Vietnamese Yellow Cattle

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ABSTRACT

Four female cattle (local Yellow breed), eight months of age, were fed a diet of 50% urea-treated and 50% untreated rice straw *ad libitum*, with free access to water. The levels of ambient temperature/relative humidity (RH) were random combinations of 25, 29, 35 and 39 °C and 70, 80 and 90% RH, achieved in an experimental chamber fitted with air conditioners, heaters and ultrasonic humidifiers. The treatments were based on the natural conditions which frequently occur in an indoor animal house in Central Vietnam in summer. Feed intake decreased linearly while water intake increased with increasing ambient temperature. Heart rate increased in direct proportion to the air temperature but was not affected by RH levels. Body temperature only increased when the chamber temperature reached 39 °C and RH was 90%, while respiration rate increased when the ambient temperate exceeded 35 °C. Measurements of HSP70 (heat shock protein, a biochemical stress indicator) from leukocytes using PCR showed that HSP70 was evident when RH reached 90% with an ambient temperature of 25 °C, or with an RH of 70% and an ambient temperature of 39 °C.

Key words: *Yellow cattle, heat stress, physiology indexes, HSP70.*

INTRODUCTION

In the life of animals the environment is important particularly ambient temperature and relative humidity (RH), which often vary. Hot weather and high humidity can reduce breeding efficiency, milk production, feed intake, weight gains, and sometimes cause death (Boyles, 2008). In addition to causing discomfort, high temperatures increase the maintenance energy required to keep the animal cool. An animal's ability to tolerate weather conditions depends on its type or breed and its body condition. The aim of this study was to obtain quantitative information on the changes in heat stress indicators in local Yellow cattle in response to changes in ambient temperature and relative humidity in Central Vietnam.

MATERIALS AND METHODS

Animals

Four female cattle (local Yellow breed) aged eight months were used; they had an average live weight of 80 kg at the beginning of the study. The animals were housed individually in metabolism pens in one chamber and de-wormed before the experiment.

Diets

The animals were given a diet consisting of 50% rice straw (90.3% DM and 6.6% CP (DM based) and 8 MJ/kgDM of ME) and 50% urea-treated rice straw (50.5% DM, 8.8% CP (DM based) and 10.3 MJ/KgDM of ME). All animals were fed a*d libitum* with free access to water.

Environmental Treatments

The environmental conditions were achieved in an experimental chamber fitted with air conditioners, heaters and ultrasonic humidifiers with ventilators (**Table 1**). All animals were kept under the same conditions at the same time then changed to the next treatment using the following design:

The treatments were based on the natural conditions which frequently occur in an indoor animal house in Central Vietnam in summer. Temperature and humidity were recorded using thermo-hygrometers. The experiment was carried out from March to May.

During the first 15 d the cattle were kept outside the chamber while they adapted to the diet; they then spent five d adapting to the chamber. Following this, each treatment was imposed for a period of four d, i.e. three d of heat and humidity treatment in which the treatments were imposed from 7.00 to 17.00 h followed by an ambient temperature of 25 °C and RH of 70% from 17.00 to 7.00 h, during each d; these latter conditions were also imposed on the first d which was free from treatment in each 4-d period.

Feeding

Fresh feed and water were supplied at 8.00 h and again at 18.00 h in the afternoon. Feed refusals and water intake were recorded at 17.00 and 06.00 h.

Table 1. Relation between temperature, relative humidity and their index.

Temp. °C/RH%	THI	Temp. °C/RH%	THI	Temp. °C/RH%	THI
(1)25/90	76	(5)29/70	80	(9)35/90	93
(2)39/80	97	(6)39/90	100	(10)25/80	75
(3)35/70	89	(7)35/80	91	(11)39/70	95
(4)29/90	83	(8)25/70	74	(12)29/80	81

Temp = temperature; RH = relative humidity; THI = temperature and humidity index.

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Physiological Measurements

Feed and water intakes were measured. The respiration rate was counted by observing the rate of movement of the diaphragm, heart rate by listening to a stethoscope between the 3rd and 4th ribs, and body temperature using a 42 °C thermometer placed in the animal's rectum.

Blood Sampling and RNA Isolation

Blood sampling was at 13.00 h on the last two d of heat treatment. Blood samples were collected from catheters fitted in the jugular vein into 10 mL heparinised tubes. Blood was kept on ice and immediately taken to the laboratory for isolation of total RNA from leukocytes. The procedure for isolating total RNA followed the Technical Manual provided by the manufacturer (Promega, 2007) using SV Total RNA Isolation Kits (50 prep). All RNA samples were frozen at –20 °C until the application of a real-time PCR (RT-PCR) technique to identify Hsp70 (heat shock protein, a biochemical stress indicator).

Real-time PCR Protocol for Heat Shock Protein (Hsp70) Assay

Access Quick RT-PCR System Kits (100 reactions) from Promega (USA) were used. The RT-PCR component consisted of 25 µL 2x Access-Quick Master Mix, 1 µL of each primer (10 µM), 2 µL RNA template (50 ng), nuclease-free water to a final volume of 50 µL; 1 µL 5u AMV Reverse Transcriptase was added as the final component and mixed by gently pipetting. Reverse transcription was carried out at 42 °C for 60 min and the initial denaturation at 94 °C for five min; this was followed by 30 cycles of 30 s denaturation (94 °C), 30 s annealing (55 °C) and 1.30 min extension (72 °C), and a final cycle of seven min and final extension at 72 °C carried out in a thermocycler (iCyler, Bio-rad).

The Hsp primer pairs used for analysis by RT-PCR were designed using the bovine primers for Hsc70 described by Kennie (2000), from sequence data for Hsc70, Hsp70-1 and Hsp70-2 (DeLuca-Flaherty and McKay, 1990; Grosz and Skow, 1994) as follows:

The primers (**Table 2**) were purchased from Integrated DNA Technologies (USA).

Data Processing

Excel software was used for data processing and a general linear model ANOVA followed by Minitab version 13.1 was used to assess significant differences ($P < 0.05$) among treatments for the feed and water intake, the respiration rate, heart rate and body temperature.

RESULTS AND DISCUSSION

The effects of changes in environmental temperature and relative humidity on the various parameters measured in the Yellow cattle are shown in **Table 3**.

Heart Rate

The heart rate increased in direct proportion to temperature increases but not significantly with increases in humidity (**Table 3**). The pulse rate is an indicator of the number f times the heart beats in a minute (Wiggins, 2007). Pulse rates can increase as a result of stimulation of the sympathetic nervous system from a number of stresses, including thermal stress.

Body Temperature

Body temperature did not vary with the levels of temperature in the chamber at humidity levels of 70% and 80%. However, at 90% humidity, the changes in body temperature following increases in chamber temperatures were significant ($P < 0.001$).

If body temperature remains steady in hot conditions, the temperature regulatory mechanism is clearly able to cope with temperature and relative humidity environments to a point beyond which heat exchange to the environment is compromised in cattle. When environmental temperature increases, feed intake will decrease to reduce heat production from digestion in general and from rumen microbial fermentation in particular. Cattle can markedly increase subcutaneous evaporative water loss by sweating. In Vietnamese Yellow cattle, the sweat glands are well developed, and in hot environments the evaporation of water from sweat to the air can significantly cool and affect thermal balance (Withers, 1992) However, in this study, evaporative cooling through the sweat became limiting at 90% RH, resulting in an increase in body temperature ($P < 0.001$).

Respiration Rate

In this study the respiration rate became higher than normal (10–30 respirations/min) when the temperature in the chamber reached 35 °C (Tinh et al., 1996; Tho and Tien, 1990). This observation agrees with a recent study on Yellow cattle in Vietnam (Thanh and Wang, 2007).

An increase in lung ventilation not only increases gas transfer but also results in more loss of heat and water (Eckert and Randall, 1988) and Withers (1992) concluded that respiratory evaporative water loss is a major avenue for evaporative water loss, especially for non-sweating mammals. This is why respiration rate is sensitive to the thermal environment. Yellow cattle have an extensive sweat

Table 2. Heat shock protein primers.

Hsp Gene (species)	Primer Pair	PCR Product Length (base pairs)
Hsc70 S	5′ AAGATGCTGGAACTATTGCTGG 3′	1474 bp
Hsc70AS	5′ ATCAACCTCTTCAATGGTGG 3′	1474 bp
Hsp70-1 S	5′ AGGACTTCGACAACAGGCTGGTGAA 3′	1098 bp
Hsp70-1 AS	5′ CTCTTGTGCTCAAACTCGTCCTTCT 3′	1098 bp
Hsp70-2 S	5′ TCATCAACGACGGAGACAAGCCTA 3′	1165 bp
Hsp70-2 AS	5′ ATCGATGTCGAAGGTCACCTCGATCT 3′	1165 bp

S = sense DNA strand primer (forward); AS = antisense DNA strand primer (reverse).

Table 3. Effects of ambient temperature and relative humidity on Yellow cattle.

Heart Rate (Beats/min)					
RH%	Temp. °C				
	25	29	35	39	SE
70	65.7^{ab}	71.1^{ab}	88.0^{cd}	91.0^{cd}	1.7***
80	73.2^{ab}	69.8^{ab}	83.5^{cd}	86.9^{cd}	1.7***
90	66.8^{ab}	70.9^{ab}	86.8^{c}	93.7^{d}	1.7***
Body Temperature (°C)					
70	38.7	38.7	38.8	39	0.1
80	38.6	38.7	38.8	38.9	0.1
90	38.6^{abc}	38.6^{abc}	38.7^{abc}	39.2^{d}	0.1***
Respiration Rate (Respirations/min)					
70	25.2^{ab}	26.0^{ab}	42.9^{cd}	48.9^{cd}	2.4***
80	24.2^{ab}	27.8^{ab}	42.1^{c}	51.4^{d}	2.1***
90	24.2^{ab}	25.4^{ab}	42.5^{c}	60.1^{d}	2.9***
Feed Intake (kg/d)					
70	4.02^{d}	3.54^{c}	2.87^{ab}	2.50^{ab}	0.1***
80	4.21^{d}	2.94^{abc}	2.75^{abc}	2.73^{abc}	0.1***
90	3.98^{d}	3.29^{c}	2.59^{ab}	2.47^{ab}	0.1***
Water Intake (L/d)					
70	6.31^{ab}	6.93^{ab}	8.74^{c}	13.02^{d}	0.26***
80	5.98^{ab}	6.35^{ab}	7.67^{c}	13.09^{d}	0.26***
90	6.08^{ac}	7.13^{bc}	6.43^{abc}	13.78^{d}	0.24***

^{abcd} Means with different superscripts within rows are different at $P < 0.05$.

gland system, and are adapted to the hot and humid environment in Vietnam. However, cattle sweat at only 10% of the rate of humans and are therefore more susceptible to heat stress (Keown and Grant, 1993)

Feed Intake

Feed intake fell by 12% after increasing the temperature from 25 °C to 29 °C ($P < 0.001$) and fell by 38% at 39 °C and 80–90% RH (**Table 3**).

At ambient temperatures above the thermoneutral zone, feed intake is reduced in ruminants (McDonald et al., 1995), and cattle will automatically reduce their feed intake during hot weather (Keown and Grant, 1993; Linn, 1997). McDonald et al. (1995) found that feed intake fell by two per cent for every 1 °C rise in average daily temperature above 25 °C. The high reduction recorded here could be due to the high fibre content of the diet (only rice straw) since fibre digestion results in a higher heat increment of feeding (sum of heat produced from rumen fermentation and nutrient metabolism (Linn, 1997).

Water Intake

Water intake increased with increases in chamber temperatures, being double at a chamber temperature of 39 °C compared with intakes at temperatures of 25 °C, even when RH levels reached 90%.

Because evaporation from the skin or respiratory epithelium is the most effective means of reducing heat stress, there is a close link between water balance and temperature control in hot environments (Eckert and Randall, 1988).

Heat Shock Protein (Hsp70) in Leukocytes

Heat shock proteins (HSPs), also called stress proteins, are a group of proteins that are present in all cells in all life forms. They are induced when a cell is subjected to various types of environmental stresses like heat, cold and oxygen deprivation.

The Hsp70 family is one of the most studied due to the fact that one of its members, Hsp70, is highly heat-inducible and all organisms examined to date produce Hsp70 family in response to elevated temperatures. Two important members of the mammalian Hsp70 family are the cytosolic proteins, Hsc70 (heat-shock cognate protein) and Hsp70. They are very similar in biochemical properties and have a high sequence homology (about 95%).

Reverse transcription-polymerase chain reaction (RT-PCR) has become the method of choice for quantifying mRNA expression in many laboratories for detecting gene(s), replacing Northern blots and *in situ* hybridisation. In RT-PCR, RNA is isolated from cells or tissues and then used as a template for reverse transcription of mRNA to complimentary DNA (cDNA). The cDNA then acts as the template for the PCR using primers designed to amplify a selected cDNA region, known as the target sequence.

In this study, the RT-PCR and primers were used to identify the Hsc70, Hsp 70-1, and Hsp 70-2 RNA from cattle leukocytes while the cattle were undergoing the heat treatments. At an environment of 29 °C/80% RH (THI = 81), Hsc, an important member of Hsp70 was expressed (**Figure1**). At the level of 29 °C/90% RH (at 29 °C/96% RH and 29 °C/97% RH samples) and 35 °C/70% RH (at 35 °C/72% RH sample) Hsp 70-1 and Hsp 70-2 were also present (**Figure 2**).

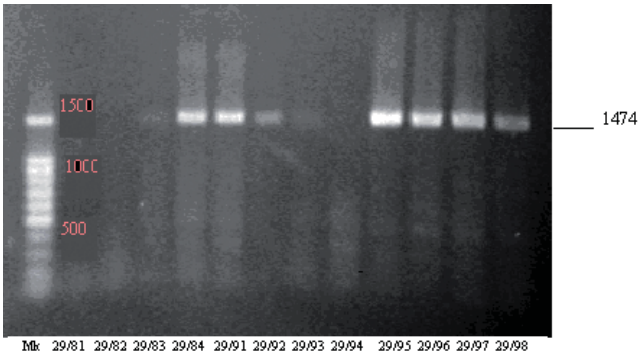

Figure 1. Identification of the Hsp70 (PCR product length of Hsc70: 1474) in leukocytes.

29/81–29/84: samples from four cattle kept at 29°C/80% RH d 1
29/91–29/94: samples from four cattle kept at 29°C/90% RH d 1
29/95–29/98: samples from four cattle kept at 29°C/90% RH d 2

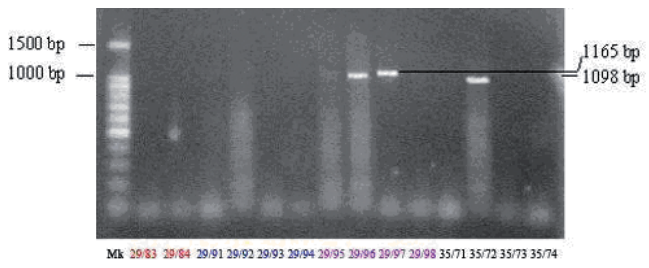

Figure 2. Identification of the HSP70–1 (1098 bp) and HSP70–2 (1165 bp) in leukocytes

29/83–29/84: samples from two cattle kept at 29°C/80%RH d 1
29/91–29/94: samples from four cattle kept at 29°C/90%RH d 1
29/95–29/98: samples from four cattle kept at 29°C/90%RH d 2
35/71–35/74: samples from four cattle kept at 35°C/70%RH d 1

CONCLUSIONS

Yellow cattle are well adapted to hot and humid tropical conditions, but sudden changes in weather can cause heat stress in these cattle. In this study, when the ambient temperature reached 29°C and RH 80%, heart and respiration rates increased, feed intakes declined and water intakes increased. Under these chamber conditions, heat shock protein, a chemical indicator of heat stress, were expressed in leukocytes of the cattle. This showed that Yellow cattle were stressed at a THI of 81 under the conditions in the chamber.

Farmers should be aware about this critical set point and pay attention to managing cattle to avoid heat stress and the resulting decrease in production. So what is needed is a house with shade, a well prepared diet, and access to water in the hot season. More studies are needed to identify diets that can be recommended to livestock keepers during hot periods and especially when rapid climate changes occur that may reduce production and fertility.

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Simple and Environmentally Friendly Options to Improve Livestock Performance under Smallholder Conditions

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ABSTRACT

This paper describes a set of technical possibilities that are simple, inexpensive and efficient and that may improve livestock performance and contribute to environment protection. Sufficient evidence exists in the literature highlighting the beneficial effects of plant secondary metabolites, in particular of tannins and saponins, with respect to increasing animal productivity, health and product quality. Moreover, feeding of low levels of tannins to ruminants might also reduce methane (CH_4) production, benefiting the environment. Many countries would benefit immensely from identifying plants and plant products having bioactive moieties that could be used as alternatives to antibiotics, growth promoters and antiparasitic drugs. The conservation of many agroindustrial by-products in the form of silage, feed blocks and pellets is another promising option to decrease feeding costs, increase animal productivity and mitigate environment pollution. Some shrub and tree species such as cactus (*Opuntia* spp.) and *Moringa oleifera*, and novel agroindustrial by-products such as *Jatropha* kernel meal from the non-toxic *Jatropha curcas*, the detoxified kernel meal from the toxic *J. curcas* and kernel meal from *M. oleifera* have considerable potential to increase livestock performance and improve farmers' incomes. Appropriate strategies for the transfer of various options and mechanisms to boost their adoption should be targeted. Participatory approaches based on the involvement of farmers, technicians, scientists, local institutions and policymakers are recommended to achieve this objective.

Key words: *Alternative feed resources, natural compounds, fodder shrubs and trees, feed blocks and pellets, smallholder farms, livestock performance.*

INTRODUCTION

Ruminant production is one of the main sources of income for rural populations living in arid and semi-arid zones. The lack of adequate year-round feed resources is probably the most important factor contributing to the low productive and reproductive performances of these animals (Ben Salem and Smith, 2008). In addition to rangeland degradation and global warming, the recent leap in the prices of concentrate feeds and the international economic crisis are seriously threatening the sustainability of livestock-based production systems. Until recently, antibiotics and other chemicals in feeds were used to improve livestock performance and health. However, the use of these additives has been banned in the EU since 2006 because of the risk to humans of chemical residues in food and of antibiotic resistance being passed on to human pathogens (Makkar et al., 2007).

A large number of recent research programmes focus on identifying alternative options that enhance animal performance and improve product quality without risk to human health (Makkar et al., 2007; Martin et al., 2010). Some promising cost-effective and environmentally friendly options which have recently proven to be efficient in improving ruminant performance and health include the use of plants (Wina et al., 2005; Makkar et al., 2007), plant extracts (Cheecke, 1999) or natural compounds (Makkar et al., 2007) as potential alternatives to growth promoters and antibiotics. Moreover, the development of simple and inexpensive techniques to improve the value of local feed resources (e.g. browses, cacti, agroindustrial by-products) could help smallholders to better manage livestock feeding throughout the year.

In this paper, we discuss some findings available in the literature on the benefits of alternative options based on locally available feed resources and how they can improve livestock performance and health while conserving the environment. Expected improvement of farmers' incomes and opportunities for their adoption by farmers are also highlighted.

TECHNICAL OPTIONS TO IMPROVE LIVESTOCK PERFORMANCE AND PROTECT THE ENVIRONMENT

Rumen Manipulation with Plant Secondary Compounds

Plant defensive compounds commonly but loosely known as plant secondary compounds include phenolics, saponins, alkaloids, non-protein amino acids, essential oils and glycosides. Tannins and saponins are the most widely occurring components from these groups. They have both beneficial and adverse effects depending upon their nature and the amount an animal consumes. In various studies, foliage as well as fruits and seeds of fodder shrubs and trees have been reported to suppress ruminal protozoal populations.

This natural defaunating (i.e. protozoa eliminating) activity of some multi purpose trees and shrub-derived feeds arises from their plant secondary metabolites (Leng et al., 1992). Digestion of feeds

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by ruminal microorganisms results in emission of CH_4 from livestock. The excretion of CH_4 from the rumen can represent a loss of up to 12% of the digestible energy, depending on the type of diet (Martin et al., 2010). Reduction of CH_4 emission (a greenhouse gas) from agricultural sources, representing 30–40% of total CH_4, is a priority for both developed and developing countries. Reducing CH_4 production from ruminants can be of direct economic benefit because it is accompanied by greater energy use efficiency of the feed by the animal. Inclusion of feed additives of plant origin in particular plant secondary metabolites and oils in diets (Ben Salem et al., 2005; Jouany et al., 2008), feeding diets rich in unsaturated fatty acids (Martin et al., 2010), and modifying feeding practices and supplementing roughage-based diets with deficient nutrients have been investigated in some laboratories with the aim of reducing CH_4 emissions and increasing productivity (Martin et al., 2010). Dietary manipulations result in CH_4 reduction by decreasing fermentation of organic matter in the rumen (Martin et al., 2010) and shifting the site of digestion from the rumen to the intestines, diverting hydrogen away from CH_4 production during ruminal fermentation (Benchaar et al., 2001), inhibiting methanogenesis by ruminal bacteria (Moss et al., 2000) or by optimising rumen fermentation, thereby decreasing CH_4 emission/unit of organic matter digested.

Tannins

The term 'tannin' refers to 'tanning' or preserving skins to create leather; tannins also contribute to the astringency of many popular drinks such as tea and wine. They are classified as:

- Hydrolysable tannins (HTs), which are potentially toxic and decrease the nutritive value of feedstuffs and thus have in general negative effects on livestock performance. In the Mediterranean area, these compounds can be found for example in *Quercus* spp. foliage. Although HTs have potential to bind feed proteins and increase the availability of proteins post-ruminally and thereby decrease CH_4 emission from ruminants, their *in vivo* effects have not been investigated;
- Condensed tannins (CTs), also known as proanthocyanidins, which are widespread in dicotyledonous species and occur infrequently in Graminacae. They are present mainly in the foliage of a wide range of shrubs, trees and herbaceous roughages like Sulla (*Hedysarum coronarium*) and sainfoin (*Onobrychis viciifolia*). CTs bind to proteins in the rumen, reduce protein degradation and when dietary crude protein (CP) concentrations exceed animal requirements for CP, these effects can improve performance (Min et al., 2003; Waghorn, 2008). However, when dietary CP levels are low and fibre concentrations are high, CTs are nearly always detrimental (Makkar, 2003).

In contrast to HTs, numerous studies have been carried out to identify and determine the bioactivity and study the effects of CTs (purified or CT-containing plants) on digestion (Min et al., 2003), productive and reproductive performances (Min et al., 2003; Waghorn, 2008) and product quality in ruminants (Vasta et al., 2008). It is now clear that depending mainly on their origin, level and structure, diet ingredients, animal species and physiological stage, CTs under specific conditions may have beneficial effects on ruminant performance (Ben Salem et al., 2005; Waghorn, 2008). Considering the objective of the current review, we report and discuss some examples illustrating the benefits from CTs in ruminant nutrition.

CTs to Promote Protein Value of Feeds

Although generally regarded as antinutritional, certain tannins at low concentrations are known to alter rumen fermentation of carbohydrates and proteins and microbial protein synthesis to the benefit of ruminants (Min et al., 2003; Makkar et al., 2007; Waghron, 2008). Since tannins are widely distributed in herbaceous and woody vegetation, identification of tanniniferous feedstuffs having beneficial effects on ruminant digestion would provide useful means to exploit the use of such feedstuffs to improve efficiency of ruminant digestion.

Feeding a small amount of foliage from a the tanniniferous legume shrub *Acacia cyanophylla* Lindl which is widespread in Tunisia with soyabean meal (SBM) resulted in a significant increase in the daily weight gain of Barbarine lambs (67 g/d vs 43 g/d) on oaten hay (Ben Salem et al., 2005). This positive effect was obtained at a total tannins:dietary protein ratio of 0.021 tannic acid equivalents (g/g protein) and when SBM (200 g/d) was given immediately after consumption of the entire amount of *Acacia* foliage (100 g/d) by animals. However, feeding *Acacia* and SBM at the same time or feeding SBM first and then *Acacia* foliage did not improve lamb growth when compared with animals on the control diet, i.e. hay + SBM. These findings were ascribed to decreased protein degradation and ammonia concentration in the rumen due to binding of tannins. Such a beneficial effect was also demonstrated on lambs and kids receiving oaten hay supplemented with fresh *Acacia* foliage and protein-rich concentrate (Ben Salem, unpublished). In a recent study (Ben Salem and Benyoussef, unpublished), lambs grazing on Sulla grew better than those on the same pasture but drenched with polyethylene glycol, MW 4 000 (PEG), a tannin deactivating reagent. Sulla contains moderate levels of CTs and at the same time is relatively high in CP. Therefore, the binding effect of Sulla-CT with proteins could explain the improved performance of lambs.

This strategy of feeding small amounts of CTs and dietary protein is simple and can promote the livestock sector under smallholder conditions by targeting the choice of plant species and management of grazing animals. For example, animals could be allowed to graze in an *Acacia* plantation (CT source) for a short time and then transferred to a grass dominated pasture (protein source, e.g. lucerne) for a longer period.

CTs to Control Gastrointestinal Parasites (GIP) in Ruminants

The presence of GIP disturbs mainly protein metabolism (Min et al., 2003), and along with reducing food intake, this may explain the decreased growth of ruminants harbouring high parasitic loads (Hoste et al., 2006). Treatment against GIP is necessary to improve the performance of such animals. Commercial anthelmintics are used mainly in the commercial sector but seldom by smallholders, particularly in developing countries; since the drugs are expensive and their indiscriminate use may lead to anthelmintic resistance in worm populations (Hoste et al., 2006) particularly in small ruminants. Currently, alternative solutions are being sought which address public concern for more sustainable production systems by relying less on the use of chemicals to improve feeding efficiency and livestock health. Nutritional manipulation of the host animal in order to improve host resistance and/or resilience to parasitic infections is a promising option.

Recent studies showed that the incorporation of CT-containing feedstuffs in the diet reduced GIP (e.g. Akkari et al., 2008). Nevertheless, the extent of the GIP decrease varied among these studies probably because different CT sources and levels, diet composition and animals were used. According to Hoste (2005), tannins might interfere directly with the biology of various nematode stages and could also indirectly improve host nutrition by protecting dietary proteins from ruminal degradation which in turn could modulate worm biology. Recent studies in France and Tunisia showed that repeated feeding of sainfoin hay (Hoste et al., 2006) and *Acacia cyanophylla*

foliage (Akkari et al., 2008) reduced faecal egg counts in kids and lambs, respectively. It seems that some other natural secondary compounds such as flavonol glycosides and sesquiterpene lactones could also have anthelmintic activity (Hoste et al., 2006), although to our knowledge this hypothesis remains to be investigated.

The feeding of tannins also changes the partitioning of nitrogen excretion in that less is excreted in the urine and more in faeces. This decreases the loss of nitrogen to the environment from manure. In addition, the rate of release of nitrogen from the manure of animals on tannin-containing diets is lower, which is also advantageous for crop production (Makkar et al., 2007). Lower CH_4 emission from ruminants on tannin-containing feed has also been demonstrated (Waghorn and Clark, 2006).

Saponins

Saponins are glycosides of aglycone linked to one or more sugar chains. These have a wide range of biological activities. For example they are antiprotozoal (see reviews by Makkar et al., 2007 and Jouany and Morgavi, 2007), and they interact with mucous membranes and influence nutrient transport (Cheeke, 1999) and absorption (Jouany and Morgavi, 2007). Also, the detergent action of saponins kills rumen protozoa which could lower ammonia levels in the rumen thereby improving the efficiency of microbial synthesis (Jouany and Morgavi, 2007; Wina et al., 2005). Defaunation has been shown to have several advantages for ruminants. Suppression or elimination of protozoa may enhance the flow of microbial protein from the rumen, increase the efficiency of feed utilisation and thereby improve animal nutrition. Saponins are also known to increase the permeability of the intestinal mucosal cells (Jouany and Morgavi, 2007). Reduced CH_4 production from rumen fermentation by saponins has also been demonstrated in various studies (Martin et al., 2010; Wina et al., 2005).

The possible use of natural plant products as growth promoters provides cheaper, safer and more consumer-acceptable alternatives to synthetic compounds. Lately inclusion of saponin- containing plants in the diets of ruminants has received wide interest due to the positive effects listed above (reviewed by Makkar et al., 2007; Wina et al., 2005). For example, preliminary results from a research programme on the effects of saponin-containing feedstuffs in ruminant feeding indicate that the administration of a small amount (30–40 g/d) of fenugreek (*Trigonella foenum-graecum* L.) seeds, which are relatively rich in saponins, increased lamb growth and milk production in dairy ewes (H. Ben Salem, unpublished). *Agavae americana* (Cactacae family) is also high in saponins (ca. 80 g/kg DM), and in a recent study in Tunisia, an *Agavae* extract (120 ppm saponins) increased the growth rate of Barbarine lambs (Nasri and Ben Salem, unpublished).

The overall positive effects of tanniniferous and/or saponin-containing forages on feed efficiency and for controlling GIP, and thereby improving the productive and reproductive performance of ruminants should encourage the uptake of practical options for using plants containing these natural plant secondary compounds in grazing systems. These options offer promising solutions to reduce the use of chemicals in livestock production systems, enhance livestock productivity and decrease both emission of CH_4 and discharge of nutrients to the environment.

Essential oils

Many plant extracts contain essential oils (EOs), which are naturally occurring volatile components responsible for their characteristic essence and colour. These secondary metabolites have antimicrobial properties that make them potential alternatives to antibiotics for manipulating microbial activity in the rumen. Therefore, plant-derived EOs could be used for improving the efficiency of nutrient utilisation in ruminants and reducing negative impacts on the environment. Compared with tannins and saponins, *in vivo* studies on the effect of EOs on rumen fermentation and performances of ruminants are scarce, although some Mediterranean institutes have recently initiated research on this topic. Available data suggest that EOs have potential to improve nitrogen and energy utilisation and inhibit ruminal methanogenesis (Benchaar et al., 2008).

Promising Fodder Shrubs and Trees

Cactus

The popularity of cactus (*Opuntia* spp.) as a feed in dry areas of some regions and countries (North Africa, Ethiopia and northern Brazil, among others) is increasing. Characterised by a remarkable tolerance to drought conditions, high water use efficiency, a rapid dissemination and growth, a high biomass yield and multipurpose uses, cactus is a promising range species that can promote the livestock sector in dry areas and improve farmers' incomes. Cactus cladodes are high in soluble carbohydrates, calcium and β-carotene (Ben Salem and Abidi, 2009), but they are low in fibre and CP (Stintzing and Carle, 2005). Therefore, provision of fibre and protein sources is recommended when feeding cactus to ruminants.

The following approaches have been tested for increasing the nitrogen content of cactus cladodes:

- Gonzalez (1989) noted that the CP content of fertilised cactus was almost double that of unfertilised cactus (99 g/kg vs 55 g/kg DM). However, the option of fertilising cactus fields has not been accepted by farmers in many countries especially when cactus is cultivated for forage rather than fruit production. Farmers would prefer to use manure or fertilisers for fruit trees and/or vegetable crops;
- Breeding is another way to select nitrogen rich clones of cactus. Some selected clones of cactus (e.g. clone TAMUK accession 1270) contained higher than normal CP contents of 110 g/kg DM (Felker and Inglese, 2003);
- Radiation induced mutation of cactus cladodes is being tested in Tunisia to increase their nitrogen content (INRAT-IAEA);
- Solid state fermentation seems a promising microbial process to produce protein from cactus. The microorganisms (algae, bacteria, fungi and yeasts) are considered a source of cell protein. They grow rapidly and could be cultivated on diverse substrates like cactus, rendering them rich in protein. The fermentation of cactus with *Aspergillus niger* resulted in a 12.8% increase in CP content (Oliveira, 2001). Also, Araújo et al. (2005) reported a remarkable increase (up to 400%) in the proportion of protein (260 g/kg DM) in cactus cladodes fermented with yeast (*Saccharomyces cerevisiae*). This procedure of protein enrichment of cactus is technically interesting, but its economic benefit should be evaluated before diffusion at the farm level.

Cactus cladodes are low in total extractable phenols and total tannins, condensed tannins and saponins (Ben Salem and Abidi, 2009). However, they are remarkably high in oxalates. Oxalates are present in a wide range of spiny and spineless cactus cultivars and clones at levels ranging from 70–150 g/kg DM (Ben Salem et al., 2002). It is well documented that oxalates form complexes with some minerals mainly calcium and magnesium and the ingestion of high amount of soluble oxalates is toxic to animals. However, since most cactus oxalates are present in insoluble form, cactus feeding has little adverse effects on the animal (Ben Salem and Abidi, 2009).

Replacing concentrate feeds (i.e. corn or barley) with cactus cladodes had no effect on digestion, lamb growth and cattle milk

production and quality, provided that energy from concentrate feeds was replaced by the equivalent energy from cactus cladodes (Ben Salem and Abidi, 2009). Total replacement of corn and barley with cactus could be achieved without any negative effects. However, with forages such as hay, straw and silage the replacement level should not exceed 50% otherwise digestion, daily gain and milk production is impaired (Ben Salem and Abidi, 2009).

Cactus could be used as fresh, dried or ensiled material:

- Cactus cladodes are fed mostly fresh to cows, sheep, goats and dromedaries. In order to avoid material loss, it is recommended to cut cladodes into small slices (using knives or electric choppers) before offering to animals. Tegegne et al. (2005) concluded that compared with a control diet (without cactus) sheep performed better when a proportion of grass hay offered was replaced by fresh cactus. Milk production of dairy cattle was not affected when fresh cactus replaced 12–36% of sorghum silage (Wanderley et al., 2002). Also, total replacement of barley (300 g) by fresh cactus (ca. 3.5 kg) had no effect on hay intake, *in vivo* organic matter digestibility and nitrogen balance in male lambs and kids (Abidi et al., 2009);
- Cactus cladodes could be dried and ground, and the meal obtained used as a supplement feed for animals. Veras et al. (2002) reported that lambs on elephant grass hay supplemented with corn or cactus meal exhibited similar organic matter (OM) intakes and OM and neutral detergent fibre (NDF) digestibilities. Although data on the replacement value of cactus meal for common feedstuffs (e.g. concentrate feeds) are limited, the benefit:cost ratio of this alternative strategy should be studied before diffusion to farmers;
- Cactus ensiling has been evaluated at the laboratory level and to our knowledge this technique is still not adopted at the farm level. Çürek and Özen (2004) evaluated the nutritive value of cactus cladodes which were chopped (1–2 cm), wilted (DM content 35%) and then ensiled. Based on pH and organic acids content, the quality of this silage was found acceptable. However, its nutritive value was low. It might be advantageous to ensile cactus mixed with other ingredients. Abidi et al. (unpublished data) ensiled fresh cactus cladodes with olive cake and wheat bran. Replacing oaten hay with this silage had no effect on digestible nutrient intakes but decreased the average daily gain of concentrate supplemented lambs from 50 g to 37 g. Cactus ensiling seems a simple technique, but its adoption would depend largely on the benefit:cost ratio which amongst others should include the costs of technology transfer efforts.

In addition to feed shortage, water scarcity compromises livestock performances in dry areas. Because of its succulence, cactus could overcome this constraint. Indeed, ruminants do not need to drink water when receiving cactus cladodes (ca. 35 g DM/kg metabolic weight) (Ben Salem and Abidi, 2009).

Moringa oleifera

Moringa oleifera Lam. (syn. *Moringa pterygosperma* Gaert.) is native to the western and sub-Himalayan parts of northwest India, Pakistan and Afghanistan and is now widely cultivated across Africa (e.g. Nigeria, Senegal, Tanzania), South America and Southeast Asia (e.g. Malaysia, Indonesia). This plant is grown intensively in plantations and produces over 100 tons of dry green foliage/ha with a protein content of 18–25% which has a biological value comparable with soyabean (Foidl et al., 2001). In addition, this foliage has a number of antioxidants (Makkar et al., 2007). Almost every part of this plant has value as food or feed. For example *Moringa* leaves are exceptionally rich in pro-vitamin A, vitamins B and C, Fe and several amino acids (Fuglie, 2001).

The beneficial effects of *Moringa* leaves on milk production and growth are well documented (Foidl et al., 2001). Also, proteins in the meal have an antibiotic effect (Makkar et al., 2007) and hence have the potential to modify rumen fermentation. These proteins have also been shown to decrease degradability of feed proteins in an *in vitro* rumen system (Hoffmann et al., 2003), and could enhance the post ruminal protein supply. In addition, defatted seed meal is free of most plant secondary metabolites such as tannins, saponins, alkaloids, inhibitors of trypsin and amylase, lectin and cyanogenic glucosides, but contains glucosinolates (Makkar and Becker, 1997). Use of the defatted kernel meal in the diet as an additive at a level of 4 g/d increased body weight of lambs (Ben Salem and Makkar, 2009), and kernel meal proteins also reduced CH_4 production from ruminants (Makkar et al., 2009; patent). These beneficial effects are attributable to the presence of cationic proteins in the meal (Makkar et al., 2007).

Jatropha curcas

Jatropha curcas L. belongs to the family Euphorbiaceae, and is native to tropical America and grown throughout the tropics. It is a drought-resistant shrub or small tree, commonly called Physic nut. The seeds contain 27–40% inedible oil which is easily converted into biodiesel and complies with USA and EU standards. The plant is easy to grow and widely used for many purposes, e.g. erosion control, fencing, firewood, green manure, various medicinal uses, feed, and soap production (Makkar and Becker, 2009). Because of the quality of its oil, *J. curcas* has created immense interest as a possible bio-fuel crop, and its plantations are expanding in many tropical countries including those in semi-arid areas. It even grows in upper Egypt in the hot desert sand when irrigated with sewage water from the city of Luxor (Becker and Makkar, 2008). There are two genotypes of *J. curcas*, a toxic and a non-toxic one. To the best of our knowledge, the non-toxic genotype is found only in Mexico. Today's global production of *J. curcas* from plantations is negligible. However, it is believed that approximately 25–30 million ha are currently being established, largely with the toxic genotype.

The kernel meal obtained from the non-toxic genotype has a CP content of approximately 60% with good amino acid composition (deficient only in lysine compared with soybean meal), but it contains heat labile antinutritional factors such as trypsin inhibitor and lectins (Makkar et al., 2007). The heat-treated kernel meal from the non-toxic genotype has been demonstrated to be an excellent fish feed, and it is also expected to be an excellent protein source for other high yielding farm animal species. Phorbol esters are absent in kernel meal from the non-toxic genotype but present in high concentrations in the kernel meal from the toxic genotype (Makkar et al., 2007). The toxicity of kernel meal from toxic *J. curcas* is therefore attributed to phorbol esters. Recently detoxification of kernel meal from the toxic genotype has been achieved at the University of Hohenheim, Germany (Makkar and Becker, 2010; patent). The detoxified kernel meal containing 62–64% CP was fed to common carp. The performance of the group in which 75% of fish meal protein was replaced by the detoxified *Jatropha* kernel meal was comparable with the group in which 75% of fish meal protein was replaced by soybean meal, but was lower than the group fed 100% fishmeal. On the other hand, the performance of the group in which 50% of fish meal protein was replaced by detoxified *Jatropha* kernel meal was better than that of groups in which 50% and 75% of fish meal protein was replaced by soybean meal and similar to that of the 100% fishmeal group. Histopathological studies showed no abnormalities

in liver, intestine and spleen and various haemotological and biochemical parameters in blood were in the normal range, suggesting that detoxified Jatropha kernel meal could replace 50% of fish meal protein in carp diet (Kumar et al., 2008). Similar results have been observed for trout and tilapia (Makkar, unpublished).

Better Use of Agro-industrial By-products (AGIBPs)

Huge amounts of AGIBPs are produced by the farming and wider food industries, but the use of these resources for livestock feeding is still limited because of their geographical separation from animals and associated high transport and storage costs, alternative uses and the relative opportunity costs, and the low managerial capabilities of the farmer. Some technologies have been developed to increase the use of these unconventional feed resources in ruminant feeding e.g. Ben Salem and Nefzaoui (2003) described a number of formulae for AGIBPs-based silages, feed blocks and pellets, and their positive effects on livestock performance and on decreasing feeding costs.

AGIBPs ensiling

For farmers with transportation facilities and flocks located in close proximity to food industries such as olive oil and fruit juice extraction, appropriate ensiling is a promising technique for efficient use of AGIBPs in livestock feeding. Micro silos (e.g. plastic bags) could be used to ensile these AGIBPs.

Feed blocks (FBs)

Feed blocks manufactured by the cold process are made from a mixture of one or more AGIBPs (e.g. olive cake, tomato pulp, etc.), binder (e.g. quicklime, cement or clay), water and common salt, and urea with or without molasses. This technique was used in the 1930s to overcome feed shortages and droughts in Tunisia. Nowadays, it is used in more than 60 countries. Makkar (2007) summarised the experiences of some countries in manufacturing and using FBs. Depending on their composition, FBs are used to partially or totally replace common concentrate feeds offered to ruminants receiving low quality diets or grazing in degraded natural rangelands. They have a role in stimulating the digestion of low quality feed resources and their use has resulted in economic benefits to farmers. Ben Salem and Znaidi (2008) noted that lambs supplemented with 500 g concentrate and those supplemented with 125 g of the same concentrate and olive cake-based FBs had similar growth rates. Recently, some FB variants have been developed and used which incorporate vitamin E (AD3E) to improve the fertility of rams or PEG as a tannin-inactivating agent to increase the utilisation of tannin-rich browses and trees (Ben Salem and Nefzaoui, 2003). Also, medicated FBs containing anthelmintic agents such as fenbendazole, pineapple leaves, nematophagous fungi and tannins to control internal parasites have been used in some countries, for example Bangladesh, Vietnam, Malaysia and Australia, and minerals such as phosphorus, copper, selenium etc. have also been incorporated in FBs to mitigate their deficiency in the diets of cattle, yak and sheep (Makkar, 2007). The success of FB technology depends mainly on proper formulations and their favourable benefit:cost ratio. In addition, this technology has been most successful in regions with strong extension services and where FBs were prepared and promoted by private industry.

AGIBPs-based pellets

Conserving AGIBPs in the form of pellets is another promising option. Nefzaoui and Ben Salem (unpublished data) have developed olive cake-based pellets and determined their nutritive value. The formulation of these pellets was inspired by the ingredients forming the FBs, being composed of olive cake, wheat bran, rapeseed meal, wheat flour residue, salt and minerals. The *ad libitum* intake of these pellets by sheep averaged 2.5 kg/d, and their cost was about half that of lucerne pellets which are imported by Tunisia and subsidised and which produced almost similar weight gains. Mechanisation, however, is necessary for making pellets.

CONCLUSIONS

Several simple approaches are available for improving livestock performance under smallholder conditions. Many of these also reduce livestock mediated environmental pollution, and some increase the environmental sustainability of livestock production systems. The possible use of natural plant products as growth promoters provides cheaper, safer and more acceptable alternatives to synthetic compounds. Successful transfer of these alternative options to farmers will require participatory approaches involving a wide variety of stakeholders.

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SESSION 2

EFFECTS OF NUTRITION, REPRODUCTION, GENETICS, AND ENVIRONMENTAL FACTORS ON ANIMAL PRODUCTIVITY

Ruminal Fungi for Increasing Forage Intake and Animal Productivity

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ABSTRACT

Increased fungal activity in the rumen has the potential to increase production from ruminants by increasing dietary intake. To obtain new information on ruminal fungi involved in the breakdown of fibrous plant tissues, *Neocallimastix frontalis* was incubated with fragments of wood. Examination by electron microscopy has provided evidence for extra-cellular structures released by the fungus during degradation. The branched-linear structures, about 0.2–0.4 µm in length, migrated to and appear to attach by one end before aligning to form a thick layer on plant cell walls. We suggest that the structures migrating from *N. frontalis* contain multi-enzyme assemblages of polysaccharidases and are the fungal equivalent of cellulosomes released by many fibrolytic bacteria. When *Caecomyces communis* was incubated with plant tissue, the tissues were macerated and fibrillated as the fungus grew along fibres. This physical disruption was caused by bulbous rhizoids expanding inside the fragments. The novel breakdown process involved both rhizoidal contact with cell walls and degradation which occurred when small extracellular particles attached to cell walls. At some degradation sites, extracellular vesicles were also observed. The structure of the assemblages exported by *C. communis* differed markedly from those of *N. frontalis* probably reflecting the different modes of action for the two fungi. To locate activity from *Caecomyces* spp. in the rumen, fragments of ruminal digesta were examined. *Caecomyces* spp. were observed but only on the very small fragments indicating that these fungi are involved in the final stage of particle breakdown and fibre clearance from the rumen. Because of their specific fibre degrading properties it is suggested that this fungal genus is a key target for increasing feed intake and productivity in ruminants.

Key words: *fungi, rumen, forage intake, fibre breakdown and clearance, enzymes, physical disruption.*

INTRODUCTION

In grazing ruminants productivity is usually limited by the amount of feed an animal is able to ingest (intake). Because forage is the primary source of energy, the greater the intake the greater the production. However, intake is often restricted by slow clearance of resistant plant material from the rumen. Recalcitrant tissues are digested only slowly and thus inhibit further intake until they are released from the rumen as particles 1 to 2 mm in length (Ulyatt et al., 1986).

In the rumen, fibre breakdown and fibre clearance arise from a combination of two separate processes: physical breakdown of plant tissues during chewing and rumination, and fibre degradation during digestion. The digestion process is carried out by fibrolytic bacteria such as *Ruminococcus albus*, *R. flavefaciens* and *Fibrobacter succinogenes*, fungi and some protozoa (Demeyer, 1981; Chesson and Forsberg, 1997). Of these microbes, the fungi are most promising targets for improving fibre breakdown (Orpin and Joblin, 1997). Ruminal fungi have been associated with increased feed intake in sheep (Gordon and Phillips, 1993) and with improved growth rates in buffalo calves (Tripathi et al., 2007).

This paper describes results from studies on two species of fungi; one which digests very recalcitrant lignocellulosic material, and one which has a novel capacity to physically disrupt and macerate fibrous plant tissues. Ruminal fungi are known to secrete a wide range of cell wall degrading enzymes (Joblin et al., 1990; Teunissen and Op den Camp, 1993) and the genes coding for some of these polysaccharidases have been cloned (Bassam et al., 1995 and refs therein). We present here results from a scanning electron microscopy (SEM) and transmission electron microscopy (TEM) examination of fungus/cell wall interactions inside plant fragments during degradation. The aim was to compare the modes of action of *Neocallimastix frontalis* and *Caecomyces communis*, species with different morphologies.

Neocallimastix frontalis has thin filamentous rhizoids (rhizomycelia) (Orpin, 1994), degrades cell walls in forage (Akin, 1994), and has been shown to digest resistant lignocellulosic tissue in some woods (Joblin and Naylor, 1989). In the present work, *Populus tremuloides* wood, readily degraded by *N. frontalis* (Joblin and Naylor, 1989) but not by the highly cellulolytic bacterium *R. albus* (Joblin and Naylor, unpublished), was selected as substrate because it provides thick secondary cell walls ideal for TEM examination.

In contrast to *N. frontalis*, fungi belonging to the *Caecomyces* genus, a reclassification of the *Sphaeromonas* genus (Gold et al., 1988), have non-filamentous rhizoids which are large and bulbous (Orpin, 1994). These appear ill-suited to penetrate plant cell walls but one study has shown that *C. communis* fibrillated plant tissue during fermentation (Joblin, 1989). In the present work, *C. communis* was incubated with sisal (*Agave sisalona* L) to examine interactions between the fungus and plant cell walls. After confirming the physical disruption of tissue, the study was extended to fragments from ruminal digesta. This showed that the mode of action of *Caecomyces* spp. in the rumen is similar to that observed *in vitro*.

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MATERIALS AND METHODS

Media and Cultures

The anaerobic culture techniques and methods for preparing media under O_2-free CO_2 were those used previously for culturing ruminal fungi on solid substates (Joblin, 1981; Joblin et al., 2002). All cultures were in screw-top Hungate tubes (Bellco Biotechnology, Vineland, NJ) and incubations were carried out at 39 °C. Medium B, a modification of the rumen fluid-containing medium of Joblin et al. (2002), consisted of per L: salts solution A (170 mL), salts solution B (170 mL), clarified ruminal fluid (300 mL), yeast extract (500 mg), tryptic case (1 g), $NaHCO_3$ (5 g), distilled water (360 mL), 0.01% resazurin (0.3 mL), and cysteine hydrochloride (500 mg). For sisal medium, pieces of sisal twine (5–8 mm long) were added to each tube before addition of pre-reduced medium (10 mL).

A similar medium was prepared for *Populus tremuloides* (PT). Pieces of PT (approximately 12 mm × 5 mm × 3 mm) were removed from a block of wood using a razor blade, dried *in vacuo* over P_2O_5 for 24 h and 20–30 mg added to each tube prior to addition of medium B (10 mL). Media containing solid substrates were autoclaved at 121 °C for 15 min. For agar roll tubes, melted agar (at 41 °C) containing sisal fibres (3–5 mm long) was inoculated with *C. communis* zoospores and the tubes rolled at 12 °C to solidify agar. To provide zoospores for inocula, *N. frontalis* PNK2 and *C. communis* CS123 obtained from the Rumen Culture Collection now at the Grasslands Research Institute were grown for 3 d in sisal medium.

Microscopy

After incubation, culture fluid was removed by aspiration and fixative solutions added directly to culture tubes. For histochemistry, sections (0.5 µm) were cut with a diamond knife, stained with (0.05%) toluidine blue in 0.1M sodium phosphate buffer (pH 7.2) and examined by bright-field microscopy. For SEM, solid substrates were fixed in 4% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.2 for 5 h at room temperature and then washed successively with the buffer for 5, 10 and 15 min. Substrate samples were post-fixed with 1% OsO_4 in the same buffer for 2 h at room temperature, washed twice with buffer, dehydrated and dried to critical point with liquid CO_2. For fixation of rumen contents, 8 mL of 25% glutaraldehyde was added to 200 g freshly collected digesta from a sheep fed fresh forage, mixed thoroughly and left for 24 h. For SEM of plant fragments from the rumen, samples were prepared as above. Specimens were mounted on Al stubs, sputter-coated with gold and examined using a Cambridge Model 250 Mk III Scanning Electron Microscope (Cambridge Instruments, UK). For TEM, samples fixed as before, were washed with 0.1 M potassium phosphate buffer (pH 7.2), post-fixed with OsO_4 at 4 °C, dehydrated and embedded in epoxy resin. Thin sections, cut with a diamond knife, were stained with uranyl acetate and co-stained with lead citrate before examination using a Philips 201C Transmission Electron Microscope.

RESULTS AND DISCUSSION

Neocallimastix frontalis

Chips of *P. tremuloides* wood were not degraded by *C. communis* but 31% (dry weight) was solubilised by *N. frontalis* after 6 d incubation (data not shown).

This result agrees with that of a previous study which showed that *P. tremuloides* was readily digested by *N. frontalis* and that cellulose, xylan and galactomannan were the major cell wall components degraded (Joblin and Naylor, 1989). *N. frontalis* releases highly active extracellular cell wall-degrading polysaccharidases (Wood et al., 1986; Joblin et al., 1990; Teunissen and Op den Camp, 1993). A light microscopy examination of chip sections (**Figure 1a**) revealed that the secondary cell walls were highly degraded whereas middle lamellae between cells were resistant to degradation. Cell wall degradation progressed successively from outer cells to inner cells with cell walls in the centre of chips showing little degradation (**Figure 1a**). The transfer of fungus between cells probably involved cell degradation of the type seen in forage cell walls (Akin, 1994) together with zoospore migration through pit apertures. Mature sporangia (fruiting bodies) containing zoospores were observed in inner cells (not shown). A fungal rhizoid penetrating a secondary cell wall is shown in **Figure 1d**.

In general, during degradation rhizoids grew in the lumen of cells rather than attached to cell walls (**Figures 1a–c**) and cell walls were degraded by erosion processes (**Figures 1b–c**) as would be expected for extracellular enzymes. This is in agreement with findings that the cell walls of forage are eroded by filamentous ruminal fungi during digestion (Akin, 1994).

Access to degraded *P. tremuloides* chips (**Figure 1a**) enabled us to select cell walls at differing stages of degradation for further examination. **Figure 2a** shows rhizoids together with an actively degrading secondary cell wall. The rhizoids have internal membraneous or hydrogenosome-like structures (Munn, 1994) and the degrading cell wall is covered with an electron-dense layer. At higher magnification (**Figure 2b**), numerous branched-linear structures (about 0.2–0.4 µm long) were observed between rhizoids and the degrading plant cell wall. These structures appear to be migrating towards the cell wall for attachment as expected for extracellular polysaccharidases (Chesson and Forsberg, 1997). The structures attach by one end and a layer is formed on the cell wall surface (**Figure 2b**). The appearance of the layer (**Figure 2b**) suggests that the structures are orientated parallel to rather than perpendicular to the cell wall during degradation.

Figure 2c shows a longitudinal section of a rhizoid in contact with an actively degrading cell wall. Examination at higher magnification (**Figure 2d**) revealed that the rhizoid is rich in polysomes, suggesting high polypeptide synthesis activity. The branched-linear structures present between rhizoid and plant cell wall and on the outer surface of the rhizoid (**Figure 2d**) have a similar appearance to the extracellular structures in **Figure 2b**.

To the best of our knowledge these extracellular structures have not been observed before during cell wall degradation by ruminal fungi. We suggest that they contain the multiple enzyme complexes necessary for cell wall degradation and are functionally equivalent to the cellulosomes released by many anaerobic bacteria during cell wall degradation (Bayer et al., 1998). This is supported by observations that one of the most active cellulases known is produced by *N. frontalis* (Wood et al., 1986) as part of a multi-enzyme complex similar to that found in cellulosomes (Wilson and Wood, 1992). The bacterial cellulosome contains at least 26 different polypeptides including structural proteins (Bayer et al., 1998). Genes encoding cellulosome-like components have been isolated from ruminal fungi (Nagy et al. 2007; Steenbakkers et al., 2008) and these include genes for both polysaccharidases and polypeptides with putative structural properties (e.g. dockerins) of the type found in cellulosomes (Raghothama et al., 2001; Steenbakkers et al., 2001 and refs therein).

Close examination of a region of cell wall which had been extensively degraded (**Figures 2e–f**) revealed that the electron-dense layer on the residual cell wall had a different appearance to that on actively-degrading cell walls (**Figures 2b, 2d**). In this case, the components of the layer appear to align perpendicular to the cell wall

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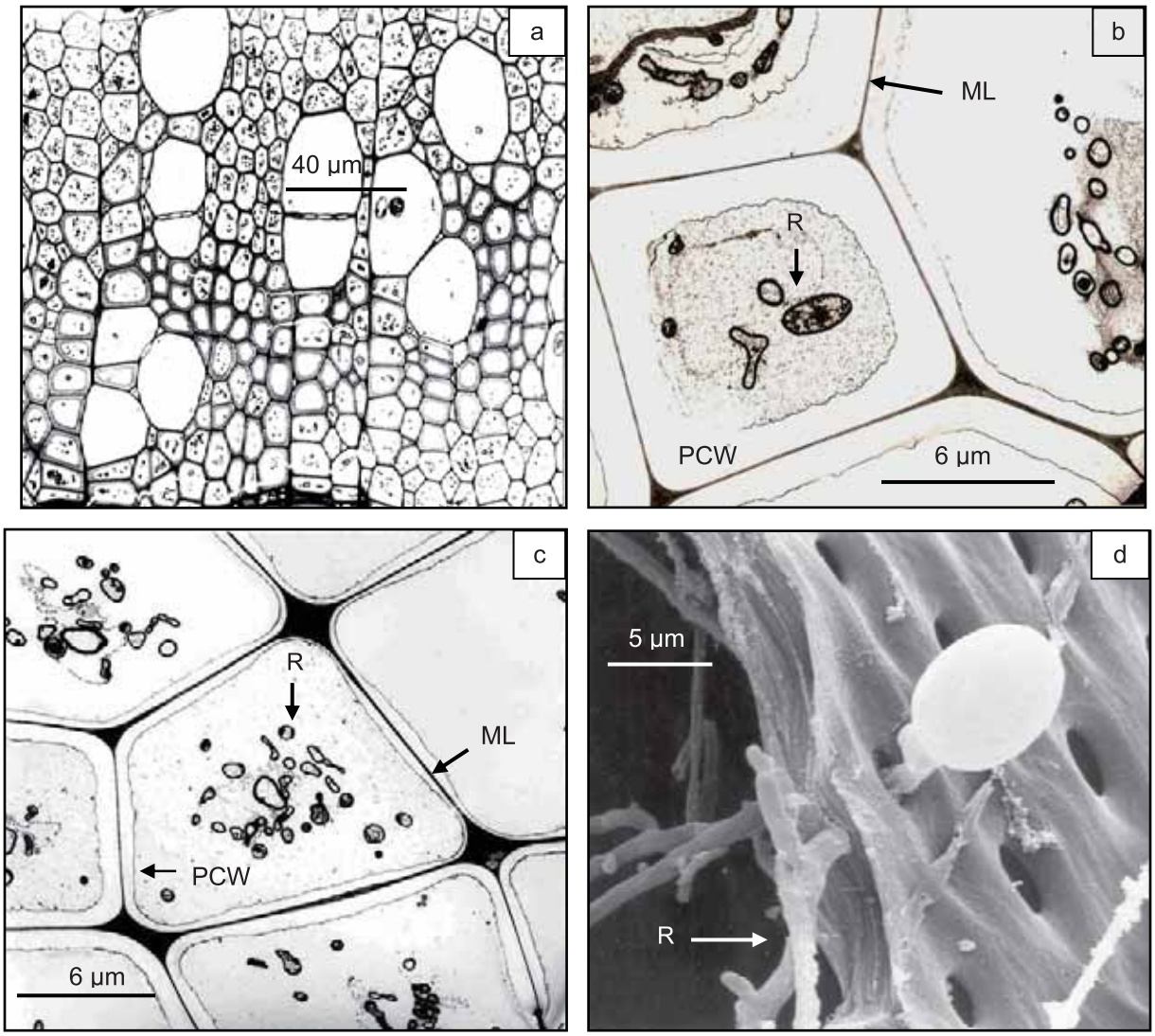

Figure 1. Degradation of *Populus tremuloides* wood by *Neocallimastix frontalis*. a — light micrograph of a chip section after 6 d incubation; b — TEM micrograph of actively degrading cell walls; c — TEM micrograph of extensively degraded cell walls; d — SEM micrograph of a rhizoid penetrating a pit-aperture cell-wall; R — rhizoids; ML — middle lamella; PCW — plant cell-wall.

(**Figure 2f**), perhaps as a consequence of interactions with lignin or polyphenolic components in the nearby middle lamella.

Caecomyces communis

Figure 3a shows sisal fibres in agar roll tubes after 6 d incubation. Fungal establishment began at the end of fibres (not shown). As the fungus grew and bulbous rhizoids expanded within fibres (**Figures 3c–d**) plant tissue became macerated and fibrillated (**Figures 3a–b**). Fracture planes developed between fibrils during fungal growth (arrows in **Figure 3c**) and at the completion of incubation tissues had a shredded appearance.

Our findings indicate that the disruption of plant tissues by *C. communis* arises predominantly from expansion of bulbous rhizoids within tissues and support previous observations (Joblin, 1989). Large bulbous rhizoids reached diameters of around 50 µm (**Figure 3b**) and contained little of the electron-dense material observed in small rhizoids (**Figures 3e–f**). The large bulbous rhizoids (**Figures 3c–3f**) probably contain mainly vacuoles (Wubah et al., 1991). Examination by TEM (**Figures 3e–f**) showed that the process involved both contact of fungus with plant cell walls as well as cell wall degradation. Contact points (or attachments) between fungus and plant cell-wall are arrowed in **Figures 3e–f** and **Figures 4a–b**.

There was no degradation of middle lamellae (**Figures 3e–f**) but at sites of sisal cell wall degradation (**Figure 4**) small electron-dense particles appear to be migrating from fungal rhizoids to the plant cell-walls (**Figures 4b–d**) as would be expected for degradation involving extra-cellular enzymes. Extra-cellular cellulases and xylanases are known to be released from *C. communis* (Hodrová et al., 1998; Matsui and Ban-Tokuda, 2008). The particles formed electron-dense layers on degrading cell walls. At some degradation sites, extracellular vesicles were observed together with particles (**Figure 4c**). We suggest that the particles are the *C. communis* equivalent of bacterial cellulosomes. It is noteworthy that these putative multi-enzyme assemblages differ markedly in structure from those released from *N. frontalis* (**Figures 2b, 2d**) suggesting that the macromolecular structure of the polysaccharidase-complexes released from *C. communis* is very different to those of *N. frontalis*.

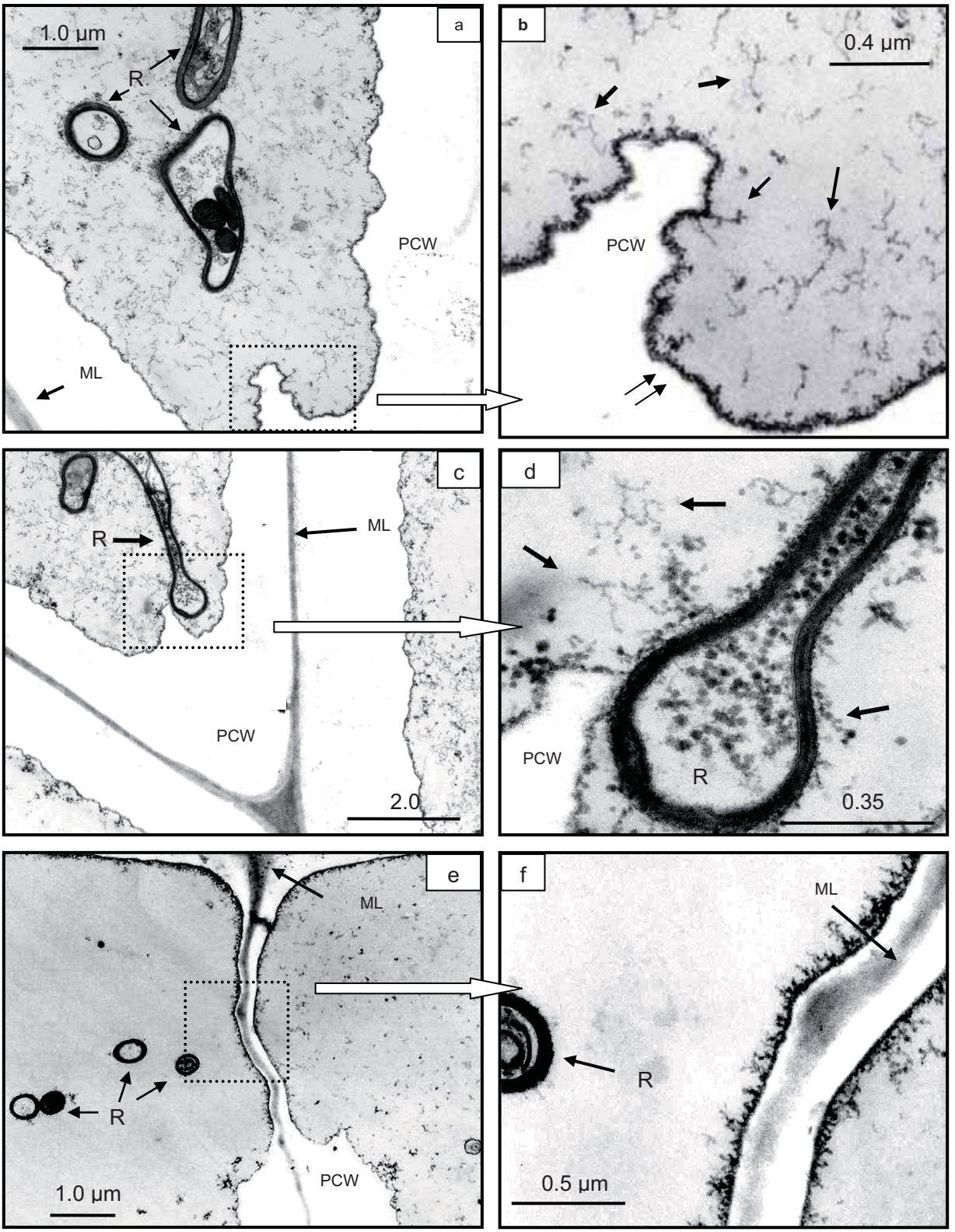

Figure 2. Degradation of *Populus tremuloides* cell-walls by *Neocallimastix frontalis.* TEM micrographs showing: a and b — rhizoids, extracellular structures (arrows) and the electron-dense layer (double arrows) on a degrading cell-wall; c and d — rhizoid in contact with degrading cell wall and extracellular structures (arrows); e and f — extensively degraded cell wall with attached layer components perpendicular to the cell wall. PCW — plant cell-wall; ML — middle lamella; R — rhizoid.

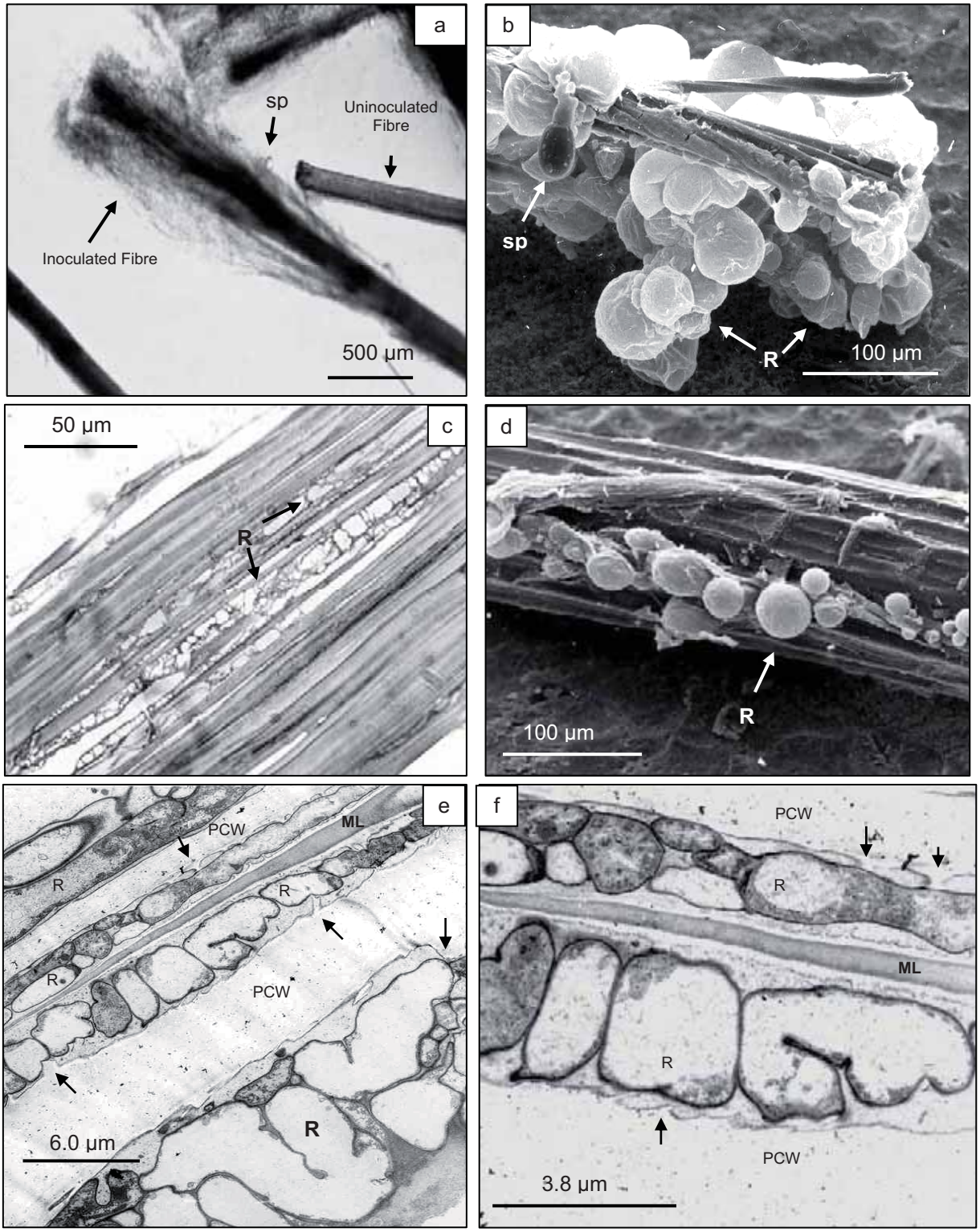

Figure 3. Breakdown of sisal fibre by *Caecomyces communis*. a — light micrograph showing fibre disruption; b — SEM micrograph of fibrillated tissue and rhizoids; c — light micrograph showing internal growth of rhizoids; d — SEM micrograph showing fibre fracture; e and f — TEM micrographs showing internal rhizoid growth and rhizoid/cell-wall contacts (arrows). SP — sporangium; PCW — plant cell wall; ML — middle lamella; R — rhizoids.

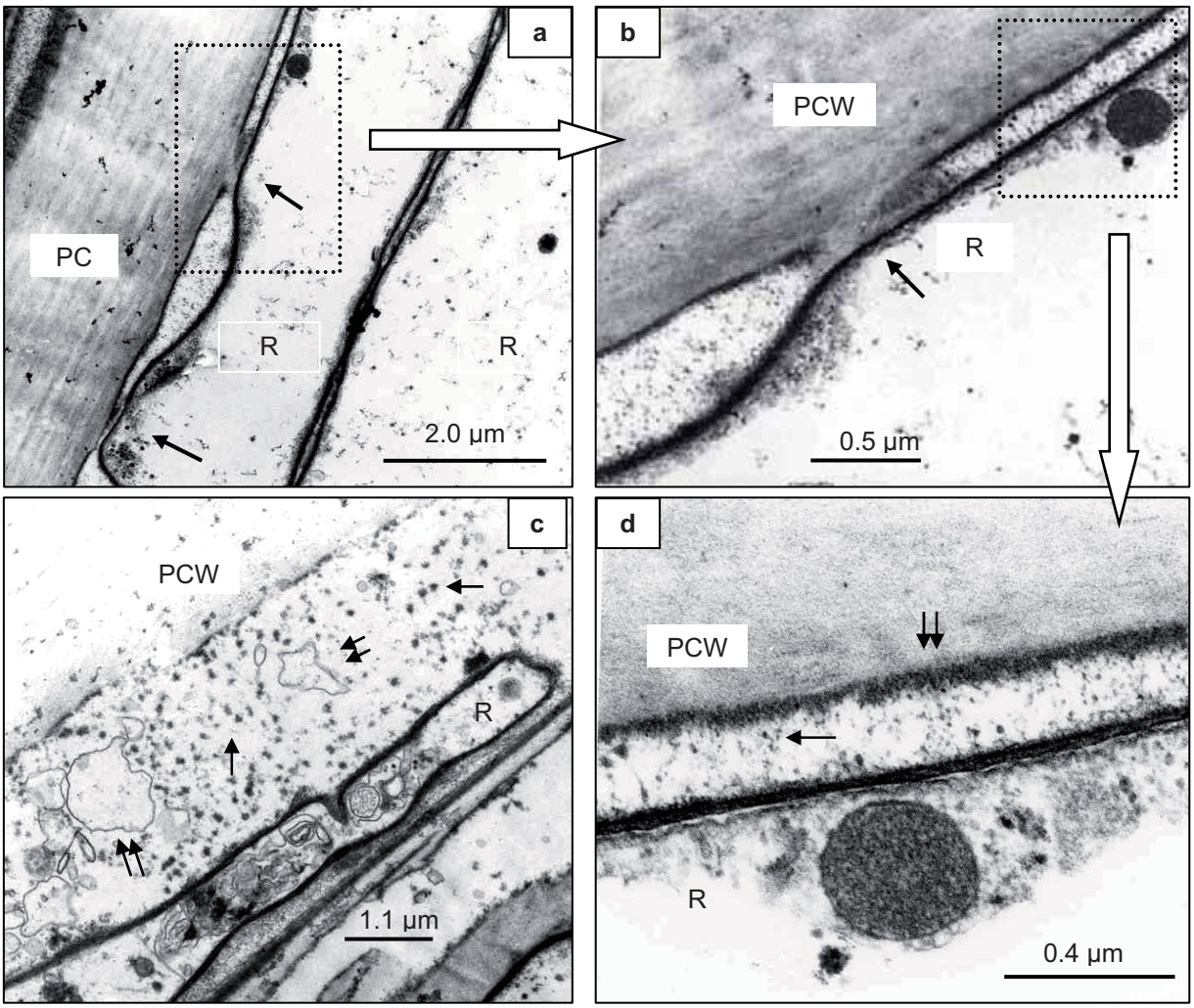

Figure 4. TEM micrographs of sisal cell wall degradation by *Caecomyces communis.* a and b — rhizoid contact with cell wall (arrows); c — extracellular particles (arrowed) and vesicles (double arrows) between rhizoid and degrading cell wall; d — particles (arrows) between rhizoid and cell wall and electron-dense layer (double arrows) on cell wall. PCW — plant cell wall; R — rhizoid.

To find evidence of *Caecomyces* spp. activity *in vivo*, plant fragments from a sheep rumen were investigated. An SEM examination found sporangia similar to those of *N. frontalis* (Orpin and Joblin, 1989) on fragments but, despite extensive efforts, failed to find caecomyces-like rhizoids. In a final study, small fibrillated fragments which had been discarded were examined. This revealed the presence of bulbous rhizoids (**Figure 5**) on many particles. The rhizoids had a similar appearance to those of *C. communis* growing *in vitro* (**Figures 3b, 3d**) and grew parallel to the axis of plant fibres (**Figures 5a–c**) as observed in sisal fragments incubated with *C. communis* (**Figure 3d**). These findings show that *Caecomyces* spp. are involved in the breakdown of small particles in the rumen. We suggest that this is their ecological role. It is likely to be a key process in fibre clearance because only the small particles are released from the rumen during digestion (Ulyatt et al., 1986).

CONCLUSIONS

The rumen is essentially a fermentation vat of microbes adapted to digesting lignocellulosic tissues in order for the host animal to thrive on a forage diet. Ruminant production would be revolutionised if fermentation in the rumen could be controlled in the manner of industrial fermentations which produce antibiotics, metabolites, enzymes, microbial cells etc. A major goal is to increase fibre clearance.

This study has shown that the fibre breakdown abilities of *Caecomyces* spp. are unusual. They fragmented and macerated plant tissues in a manner leading directly to particle size reduction — a property not found in other fungi. In the rumen they appear to have a specific role in fragmenting small particles such as those released during chewing and rumination — and which require further size reduction before release from the rumen. When large stem-cylinders from alfalfa hay were incubated with *C. communis* (data not shown), the waxy cuticles were removed intact. Removal of the protective cuticle would expose fragment surfaces to increased attack from ruminal bacteria. This 'cuticle peeling' property requires further investigation because it was not always reproducible in *in vitro* incubations. It is concluded that these fungi play a key role in fibre clearance from the rumen. Inoculation of animals with more effective and competitive *Caecomyces* strains, or methods which enhance

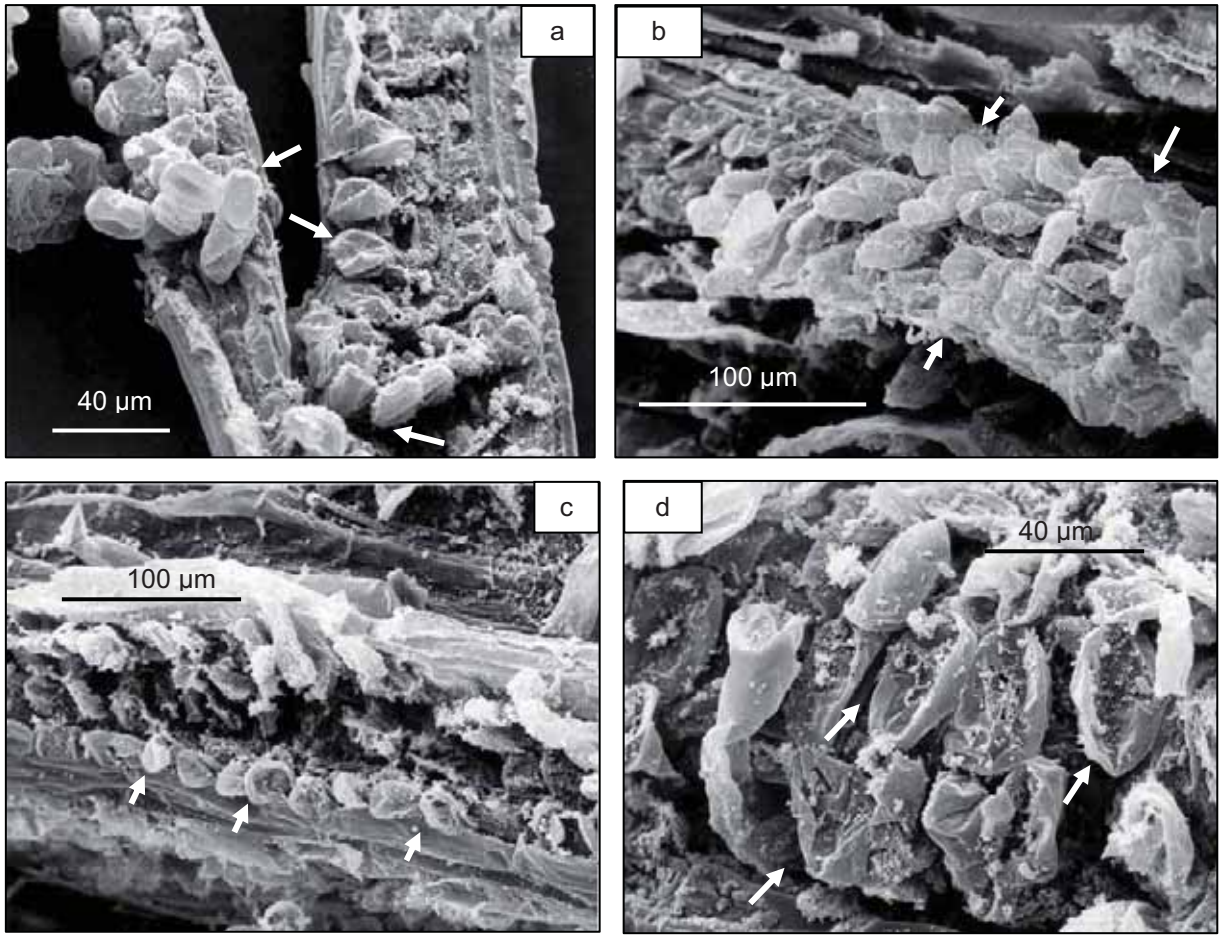

Figure 5. SEM micrographs showing rhizoids (arrows) of *Caecomyces* spp. on small macerated particles in ruminal digesta collected from a sheep fed forage.

Caecomyces spp. activity *in vivo* are likely to lead to increased intake and thus productivity.

This study also provides the first observations on structures moving from ruminal fungi to plant cell walls. The behaviour of the structures is consistent with their containing enzymes responsible for cell wall degradation so we conclude that they are multi-enzyme assemblages functionally equivalent to bacterial cellulosomes. Access to these should now allow their properties to be determined. This will provide new information for biotechnologies aimed at improving rumen function. We recognise that our evidence is indirect and experimental proof for the 'cellulosomes' is required. Unfortunately, further studies such as an immuno-cytochemical investigation using labelled monoclonal antibodies targeted at cellulosome components (cellulases, xylan-esterases, dockerins etc) were beyond the scope of our work.

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Effect of Ethyl Linolenate on Rumen Fermentation and Microbial Community in Sheep Fed Diets with Different Forage to Concentrate Ratios

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ABSTRACT

A two-way factorial arrangement was conducted to investigate the effect of ethyl linolenate (LNE) on rumen fermentation and microbial community in sheep fed diets with different forage to concentrate ratios (F:C). Four male Hu sheep were fistulated and each was paired with a non-fistulated animal, and then the four pairs of animals were fed a forage-based or a concentrate-based diet without or with LNE. Addition of LNE decreased methane (CH_4) emission by 17.3 and 33.8% in forage- and concentrate-based diets respectively, with a significant interaction between the diet and LNE ($P < 0.05$). Total volatile fatty acids were little affected by the diet, but decreased in the LNE-added group ($P < 0.05$). Addition of LNE decreased the molar proportions of acetate and butyrate, and increased the molar proportion of propionate in concentrate-based diet ($P < 0.05$), but this was not the case with the forage-based diet. Microbial protein mass was decreased significantly by inclusion of LNE ($P < 0.05$). Reducing the F:C ratio significantly decreased the population of fungi and *R. albus*, but had a minor effect on methanogen protozoa, *R. flavefaciens* and *F. succnogen*. Addition of LNE significantly decreased the population of methanogen and protozoa, but had a minor effect on fungi and *F. succnogen*. It is inferred that interactions of fat with the basal diet have to be taken into consideration when developing effective CH_4-abatement feeding strategies.

Key words: *ethyl linolenate, sheep, fistulated, rumen, volatile fatty acids, microbial protein, methane*

INTRODUCTION

Methane is produced as an unavoidable by-product of organic matter fermentation in the rumen and represents a two to 12% loss of gross energy intake (Johnson and Johnson, 1995). The concentration of CH_4 in the atmosphere has increased at a rate of 10 nL/L per year since the preindustrial revolution (Moss et al., 2000). Domesticated ruminants are estimated to produce about 80 Tg of CH_4 annually (1 Tg = 1 million metric tons), accounting for about 22% of CH_4 emissions from human-related activities (NRC, 2002). Therefore, reducing CH_4 emission from ruminants has implications not only for global environmental protection but also for efficient animal production.

Many potential strategies have been suggested to reduce CH_4 production (Moss et al., 2000). However, many of these options are at an early stage of development, or in the case of ionophores, are proscribed by European legislation. For increasing animal productivity and thereby reducing CH_4 production per unit of animal product, the main avenue available is the alteration of ruminal fermentation patterns through dietary manipulation, primarily the substitution of structural with non-structural carbohydrates and the dietary inclusion of fatty acids (Moss et al., 2000) which are normally added to increase energy density, enhance milk production, or modify the fatty acid composition of milk (Zheng et al., 2005; Sanz Sampelayo et al., 2007). In a previous study with different types and levels of octadecacarbon fatty acids, it was found that linolenic acid had the most efficient CH_4-suppressing effect *in vitro* (Zhang et al., 2008). However, there are few studies on dietary interactions with linolenic acid. In this trial, the effect of ethyl linolenate (LNE) on CH_4 emission and rumen fermentation was investigated in sheep given diets differing in the forage to concentrate ratio (F:C) using simple open-circuit respiratory chambers.

MATERIALS AND METHODS

Experimental Design, Animals and Feeds

The experimental design was a 4 × 4 Latin square with 2 × 2 factorial arrangement of four dietary treatments. Four male Hu sheep were fistulated and each was paired with a non-fistulated animal at the beginning of the experiment and the pairing of animals was maintained throughout the trial. Four pairs of sheep were fed a forage-based diet without (F; F:C = 70:30, dry matter [DM] basis) or with LNE (FL; F:C = 70:25, 5% LNE); a concentrate-based diet without (C; F:C = 30:70) or with LNE (CL; F:C = 25:70, five percent LNE), respectively. The LNE was purchased from Henan Linuo Biochem Co., Ltd., China and its α-linolenic acid content is above 70%. The LNE was poured onto the feed and mixed into the ration manually at the time of feeding. The feed amount of the experimental diets was adjusted to the live weight at the start of experiment and was kept constant afterwards. Each sheep was fed one kg of total feed (DM) including concentrate and alfalfa hay/d and were consumed without refusals. Diet ingredient and chemical composition are shown in

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Table 1. Ingredients and chemical composition of diets fed to sheep.

Diet[1]	Forage-based		Concentrate-based	
Item	F	FL	C	CL
Ingredient, g/kg				
Alfalfa hay	700	700	300	300
Ethyl linolenate	0	50	0	50
Corn	152	90	450	388
Soybean meal	78	90	100	112
Rapeseed cake	30	30	50	50
Wheat bran	26	26	86	86
Salt	4	4	4	4
Vitamin and mineral premix	10	10	10	10
Chemical composition, g/kg DM				
Crude protein	153.1	153.0	153.2	153.0
Neutral detergent fibre	394.4	390.2	253.9	249.8
Acid detergent fibre	22.8	22.3	45.9	45.4
Calcium	9.2	9.1	4.5	4.6
Phosphorus	3.2	3.2	2.3	2.2
DE, MJ/kg DM	93.3	102.0	118.0	126.7

[1]F — a forage-based diet without LNE; FL — a forage-based diet with 50 g/kg LNE; C — a concentrate-based diet without LNE; CL — a concentrate-based diet with 50 g/kg LNE.

Table 1. The diets were given in equal portions twice daily at 0800 h and 1600 h. During the whole experiment, the sheep had free access to fresh water.

Sampling Procedures and Measurements

Methane Measurement

Each period lasted for 25 d. During the first 22 d of each period, each pair of sheep was housed, untethered in individual pens. The pens were located in a sheltered, unheated barn. Before the morning feeding on d 23, the first two pairs of sheep were moved to one of chambers for measurements of CH_4. Because only two chambers were available, two pairs of animals were used at the same time. Within each chamber, the animals were untethered and had free access to fresh water. The first day within the chamber was considered an adjustment period, allowing the sheep to adapt before measurements were recorded for two consecutive 24-h d starting at 0800 h. After the morning measurements, the sheep were removed from the chambers and transported to their individual stalls. Then another two pairs of sheep were used for measurements. The gas sampling and monitoring techniques were as described previously (Yuan et al., 2007). Briefly, during the two consecutive d when the sheep were housed in chambers, air samples were taken hourly from each chamber with an airproof syringe, the volume of the air that flowed through the chamber was recorded, and CH_4 concentrations were analysed using a gas chromatograph (GC-2100, Shimadzu) equipped with a flame ionisation detector (FID) (Hu et al., 2005a).

Rumen Sampling

On the last d of each period, rumen fluid samples were taken from fistulated sheep before morning feeding using a vacuum bump. Immediately after collection, the samples were strained through four layers of compressed gauze and the pH was determined using a pH meter (Model PB-20, Sartorius). The fluid was sampled to determine ammonia-N, volatile fatty acids (VFA) and microbial protein (MCP) using the methods described by Hu (2005a). For determination of the relative quantity to total bacterial 16S rDNA of methanogens, protozoa, fungi, *R. flavefaciens R. albus* and *F. succinogenes*, six aliquots of one ml rumen fluid were sampled and stored immediately at -80 °C .

Total DNA Extraction and Real-time Quantitative PCR

Total DNA was extracted from rumen fluid by the bead-beating method as described by Zhang et al. (2008). The primers of total bacteria, methanogens, protozoa, fungi, *R. flavefaciens*, *R. albus* and *F. succinogenes* are as described by Denman and McSweeney (2006) and Denman et al. (2007). The species-specific real-time quantitative PCR was performed using the ABI 7500 real time PCR system (Applied Biosystems, USA) with fluorescence detection of SYBR green dye. Amplification conditions were as follows: one cycle at 95 °C for 10 s for initial denaturation, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Specificity of amplified products was confirmed by melting temperatures and dissociation curves after each amplification. Amplification efficiencies for each primer pairs were investigated by examining a dilution series of total rumen microbial DNA template on the same plate in triplicate.

Calculation and Statistical Analysis

Populations of rumen microbes were expressed as a proportion of total rumen bacterial 16S rDNA according to the equation: relative quantification of target = $2^{-(Ct\ target\ -\ Ct\ total\ bacteria)}$, where Ct represents the threshold cycle.

All data were analysed as a four×four Latin square using the mixed procedure of SAS (1999). The statistical model included

sheep as random effect, period, LNE addition, F:C, and LNE×F:C as fixed effects. Main effects (F:C and LNE addition) and interactions between F:C ratio and LNE addition were considered to be significant when $P \leq 0.05$.

RESULTS AND DISCUSSION

Effects on Methane Emission and Fermentation Characteristics

Addition of LNE decreased CH_4 emission by 17.3 and 33.8% in forage- and concentrate-based diets, respectively (**Table 2**). This reduction may be attributed to several interrelated factors. Total rumen hydrogen supply would be reduced primarily through a reduction in the total amount of ruminally fermented organic matter (Beauchemin and McGinn, 2006; Jordan et al., 2006), together with a shift in the ratio of the end products of fermentation from acetate towards propionate (Wettstein et al., 2000). Rumen hydrogen supply available for reducing CO_2 to CH_4 would be further reduced by the LNE serving as an alternative hydrogen sink through bio-hydrogenation, although the total amount of metabolic hydrogen used in the bio-hydrogenation of unsaturated fatty acids is small compared with that used for reducing CO_2 to CH_4 (Czerkawski, 1986). In addition, by reducing the rumen ciliate population, interspecies hydrogen transfer would be reduced and overall CH_4 production lowered (Finlay et al., 1994; Hu et al., 2005b).

Reducing the F:C ratio of diets led to a significant reduction in CH_4 emissions in the current study ($P < 0.05$, **Table 2**). Methanogenesis is an important terminal step in the anaerobic fermentation of organic matter within the rumen. Carbohydrates are the main energy source for the rumen microbes and the production of CH_4 is closely related to their fermentation. Compared with structural carbohydrates, the fermentation of non-structural carbohydrates (starch, sugars, etc.) results in less CH_4 per unit of substrate fermented (Hungate, 1966). Thus, increasing the dietary proportion of concentrate, i.e. the proportion of easily fermentable carbohydrates, appears to be an effective feeding strategy in decreasing rumen methanogenesis (Lovetta et al., 2003). A decline in rumen pH in the lower F:C diet (**Table 2**) might also contuibute to the reduced CH_4 production, since rumen methanogenesis was shown to be a pH-dependent process (Van Kessel and Russell, 1996).

Highly significant interactions in CH_4 emission existed between the basal diets and LNE addition, with greater responses for diets with a lower F:C ratio ($P < 0.05$). This may be attributed to the different amount and structure of the dietary particulate matter between the two F:C diets (Machmüller, 2006). Harfoot (1974) demonstrated that fatty acids may attach either to rumen microbes or to feed particles. In the present study, the decrease in CH_4 emissions by LNE in the forage-based diet was about half of the decrease achieved with the concentrate-based diet. Since the two basal diets had little effect on average rumen pH, it can be assumed that this was mainly a result of the different amount and structure of the dietary particulate matter. With the forage-based diet, probably more LNE was attached to the feed particles and less LNE to the methanogens compared to the concentrate-based diet.

Diet type had no effect on total VFAs, but LNE addition decreased total VFAs significantly ($P < 0.05$, **Table 2**). Molar proportions of acetate and butyrate decreased, while molar proportions of propionate increased significantly by addition of LNE in concentrate-rich diet ($P < 0.05$), but they were not affected in forage-rich diet ($P > 0.05$). Thus, the acetate-to-propionate ratio was significantly decreased in the concentrate-rich diet. The LNE treatment affected rumen fermentation patterns in a manner similar to that shown previously by McGinn (2004) in a study in beef cattle, with a lower VFA concentration and a smaller acetate:propionate ratio. Ammonia-N concentration and MCP were reduced significantly by addition of LNE ($P < 0.05$), but diet type had little effect on MCP yield (**Table 2**). These results are consistant with most previous observations (Hristov et al., 2004).

Effects on Rumen Microbes

The influence of LNE and diet on ruminal microbial population is shown in **Table 3**. Methanogen and protozoan populations were decreased significantly ($P < 0.05$) by addition of LNE, but not affected by the F:C ratio or their interaction ($P > 0.05$). This indicates that LNE reduces CH_4 emission mainly by reducing the quantities of methanogens and protozoa quantity; this is in line with previous observations *in vitro* (Zhang et al., 2008). The low methanogen population relative

Table 2. Methane emission and ruminal parameters for sheep fed diets containing a forage-based diet without (F) or with 50 g/kg LNE (FL), and a concentrate-based diet without (C) or with 50 g/kg LNE (CL).

Diet	Forage-based		Concentrate-based		SEM	*P*-Value		
Item	F	FL	C	CL		F:C	LNE	Int
Methane (L/kg DM intake)	28.9^{a}	23.9^{b}	26.6^{a}	17.6^{c}	0.8	<0.01	<0.01	0.02
Ruminal pH	7.14^{ab}	7.33^{a}	6.90^{b}	7.13^{ab}	0.09	0.02	0.03	0.84
VFA (mmol/L)	68.6^{a}	54.6^{ab}	62.2^{ab}	49.7^{b}	4.1	0.26	0.02	0.88
Molar proportions (%)								
Acetate	77.4^{a}	76.9^{a}	71.7^{b}	69.3^{c}	0.7	<0.01	0.14	0.30
Propionate	13.7^{b}	14.8 ^{b}	16.6^{b}	22.6^{a}	1.3	<0.01	0.03	0.11
Butyrate	9.0^{ab}	8.3^{b}	11.7^{a}	8.1^{b}	0.8	0.15	0.02	0.10
A:P	5.74^{a}	5.35^{ab}	4.38^{b}	3.16^{c}	0.30	<0.01	0.08	0.34
NH_3-N (mg/dL)	12.3^{c}	10.7^{d}	20.7^{a}	16.8^{b}	0.4	<0.01	<0.01	0.15
MCP (mg/mL)	1.95^{a}	1.55^{b}	1.95^{a}	1.69^{ab}	0.11	0.25	0.01	0.76

$^{a, b, c, d}$ Means within the same row sharing no common capital letters are different at $P < 0.05$.

LNE — ethyl linolenate; Int — interaction between F:C and LNE; VFA — volatile fatty acids; A:P — acetate-to-propionate ratio; MCP — microbial crude protein.

Table 3. Ruminal microbes for sheep fed diets containing a forage-based diet without (F) or with 50 g/kg LNE (FL), and a concentrate-based diet without (C) or with 50 g/kg LNE (CL).

Diet	Forage-based		Concentrate-based		SEM	*P*-Value		
Item	F	FL	C	CL		F:C	LNE[a]	Int[b]
Methanogen	1.70[a]	0.67[b]	1.39[a]	0.43[b]	0.13	0.29	<0.01	0.88
Protozoa	2.84[a]	0.61[b]	2.80[a]	0.43[b]	0.25	0.57	<0.01	0.54
Fungi ($\times10^{-4}$)	43.63[a]	15.28[b]	2.54[b]	0.89[b]	3.50	<0.01	0.07	0.10
R. flavefaciens ($\times10^{-4}$)	2.79[b]	14.97[a]	3.34[b]	10.38[ab]	1.37	0.58	0.02	0.48
R. albus ($\times10^{-2}$)	1.33[ab]	5.45[a]	0.13[b]	1.39[ab]	0.94	0.03	0.03	0.20
F. succnogen ($\times10^{-2}$)	7.224[a]	4.034[ab]	3.516[ab]	0.004[b]	1.200	0.19	0.25	0.95

[a, b] Means within the same row sharing no common capital letters are different at $P < 0.05$.

LNE — ethyl linolenate; Int — interaction between F:C and LNE.

to total bacterial 16S rDNA associated with adding LNE may also be due to reduced hydrogen availability in the rumen. Methanogens live by consuming hydrogen in the rumen and have to compete with propionate-producing microbes that also consume hydrogen to form propionate. An increase in the molar proportion of propionate with LNE addition (**Table 2**) led to lower availability of hydrogen for methanogens.

Reducing the F:C ratio decreased the population of fungi and *R. albus* significantly ($P < 0.05$), but had minor effects on *R. flavefaciens* and *F.succnogen* (**Table 3**). Addition of LNE significantly decreased fungi number ($P < 0.05$), but promoted *R. flavefaciens* and *R. albus* populations ($P < 0.05$), with little effect on *F.succinogenes* ($P < 0.05$). No significant interactions between the F:C ratio and LNE addition were observed on populations of all the microbes ($P < 0.05$). Anaerobic fungi in the rumen mainly display strong cellulase and xylanase activity, and some of their plant cell wall degrading activities are through the physical action of rhizoid development, resulting in disruption of feed structure (Akin et al., 1989). Thus, fungi populations decreased significantly because of the markedly decreased structural carbohydrates with reducing F:C ratios. The different responses of fibrolytic microbes to LNE may be attributed to competition between them. Competitive and cooperative interactions between cellulolytic microorganisms may affect the degradation of fibrous feed and hence the energy provided to animals. Different substrates may affect the competitive status. Odenyo et al. (1994) observed that *R. flavefaciens* FD-1 competed with *F. succinogenes* S85 when cellulose was used as the carbon source, while the relative proportions of the two bacteria were similar until the substrate was depleted in alkaline hydrogen peroxide-treated wheat straw culture. In the study by Chen et al. (2008), sodium hydroxide treated rice straw increased liquid-associated *R. flavefaciens* and *R. albus*, but decreased *F. succinogenes* markedly in a mixed culture.

CONCLUSIONS

The feeding of low F:C ratio diets to Hu sheep is an effective way to reduce CH_4 emissions. By adding LNE to the diet, further reductions in CH_4 emissions can be achieved. There was a significant interaction between the basal diet and LNE addition in CH_4 production, with greater responses for diets with a lower F:C ratio. Addition of LNE significantly decreased populations of methanogen, protozoa and *F. succinogenes*, but promoted populations of *R. flavefaciens* and *R. albus*. Diet type had a significant effect on fungal growth, with minor effects on other microbes. Competition may exist among different fibrolytic bateria, resulting in greater populations of *R. flavefaciens* and *R. albus* in the LNA added diet than in the control. Interactions of fat with the basal diet should be taken into consideration to develop effective CH_4-abating feeding strategies.

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Effects of Essential Oil from *Cordia verbenacea* D.C. on In Vitro Rumen Fermentation

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ABSTRACT

The objective of this experiment was to determine the effects of *Cordia verbenacea* D.C. essential oil (EO) on ruminal fermentation by using the *in vitro* gas production technique. Two substrates were independently assessed: i) Coastcross (*Cynodon* sp.) hay, and ii) 80:20 concentrate:forage diet. Treatments were defined as: Control i.e. without monensin or EO; MON i.e. monensin at 3 mM as a positive control; COR37.5 i.e. 37.5 mL of EO in 75 mL of buffered rumen fluid; and COR75 i.e. 75 mL of EO in 75 mL of buffered rumen fluid. Considering both substrates, MON reduced gas and methane (CH_4) production, increased propionate concentration, and decreased acetate:propionate ratio when compared with the Control. The most promising effect observed with EO inclusion was related to the inhibition of methanogenesis using hay as substrate. Methane produced per unit of $OM_{incubated}$ was reduced by 30% when COR75 was compared with Control. Although not statistically different, CH_4 production expressed as mL/g $OM_{degraded}$ showed an intermediary value for COR75 (32.9) compared with the Control (38.9) and MON (25.8). No effects were observed with EO inclusion when the high concentrate diet was used as substrate. In this condition, the doses tested seemed too low to manipulate rumen fermentation. The results indicate that the EO from *Cordia verbenacea* D.C. was able to modify *in vitro* ruminal fermentation using hay as substrate and that doses greater than 1 mL/mL of buffered rumen fluid may decrease CH_4 production as much as monensin.

Key words: *gas production, methane, plant secondary compounds, rumen manipulation.*

INTRODUCTION

Ionophoric antibiotics are the most common commercial feed additives used to manipulate rumen fermentation and enhance feed efficiency (Russell and Strobel, 1989). However, based on public health concerns the European Union banned the use of antibiotics as animal growth promoters in 2006 (European Commission, 2003). Apart from the debate derived from this decision (Russell and Houlihan, 2003), other countries, especially world beef exporters, probably will be pressed to follow this legislation in the near future.

In an attempt to reproduce the benefits of ionophores (e.g. monensin sodium), research has been exploiting the antimicrobial properties of plant secondary metabolites (PSMs; Calsamiglia et al., 2007). Plant secondary metabolites have some advantages over antibiotics, mainly because they are well accepted by consumers and generally considered safe for human consumption by regulatory agencies. Moreover, the appearance of PSM-resistant microorganisms is very unlikely because PSMs are a complex mixture of active components possessing a broad mode of action (Acamovic and Brooker, 2005). Conversely, these characteristics also reduce the selectivity against specific microbial populations, which impairs rumen manipulation (Calsamiglia et al., 2007).

Cordia verbenacea D.C. (Boraginaceae) is a Brazilian bush which has antimicrobial properties attributed to its essential oil (EO). A previous report using the plate diffusion method showed that 89% of the gram-positive bacteria tested were sensitive to this EO, whereas 80% of gram-negative bacteria were resistant (Carvalho Jr. et al., 2004). Thus, the hypothesis behind the research described below is that the EO from *C. verbenacea* would modify rumen fermentation and could mimic the positive effects observed for ionophores on ruminal fermentation. The objective of the experiment was to determine the effects of EO from *C. verbenacea* on ruminal fermentation by using the *in vitro* gas production technique. Two substrates were independently assessed: Coastcross (*Cynodon* sp.) hay and a 80:20 concentrate:forage diet. Monensin (MON) at 3 mM was included as a positive control.

MATERIALS AND METHODS

Experimental Design

The study was conducted from June to September 2008 at the Centre for Nuclear Energy in Agriculture, University of São Paulo, Piracicaba, SP, Brazil. It used the *in vitro* gas production technique and adapted to a semi automatic system using a pressure transducer and a data logger. A complete randomised block design was used with six replicates for gas production variables (mL/g $DM_{incubated}$ and mL/g $OM_{incubated}$) and three replicates for all other variables. Two conditions were independently assessed: i) Coastcross (*Cynodon* sp.) hay as substrate + an inoculum from sheep on pastures, and ii) an 80:20 concentrate:forage diet as substrate + an inoculum from sheep adapted to this diet. Two different inocula (blocks) were used as source of variation for each incubation condition. Treatments were defined as Control: without MON or EO; MON: MON at 3 mM

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as a positive control; COR37.5: 37.5 µL of EO in 75 mL of buffered rumen fluid; and COR75: 75 µL of EO in 75 mL of buffered rumen fluid.

Incubation Conditions

Serum glass flasks (total volume 160 mL; head space 85 mL) were filled with 500 mg of air-dried substrate (Coastcross hay or high concentrate diet), 50 mL of incubation medium (Theodorou's medium described in Preston, 1995), and 25 mL of rumen fluid. Flasks without substrate served as blanks to correct for gas release (n = 4), CH_4 production (n = 2), and residual dry matter (DM) and organic matter (OM) (n = 2) originating from the inoculum. Flasks filled with a standard Coastcross hay (n = 4) were also included to monitor incubation conditions. The pre-warmed flasks containing substrate were filled sequentially with incubation medium, MON solution or EO, and inoculum. Flasks were sealed immediately with 20 mm butyl septum stoppers (Bellco Glass Inc, Vineland, NJ, USA), swirled manually, and incubated in a forced air oven (Marconi MA35, Piracicaba, SP, Brazil) at 39 °C. Incubation time was 24 h for the hay and 16 h for the high concentrate diet (Makkar, 2004). Gas pressure was recorded at 4, 8, 12, and 16 or 24 h. For CH_4 analysis, 2.5 mL gas were sampled at each incubation time using a 5 mL syringe (Becton Dickson, Indústria Cirúrgica LTDA, Curitiba, PR, Brazil) and stored in a 10-mL vacuum tube. After each gas sampling, flasks were vented, swirled manually, and returned to the oven. Fermentation was terminated by placing the flasks in cold water (4 °C).

Substrates

The two substrates used were:

- Coastcross hay (89.2% dry mater [DM], 9.7% crude protein [CP], 1.3% ether extract [EE], 7.9% ash, 60.2% neutral detergent fibre [NDF], and 30.6% acid detergent fibre [ADF]);
- 80:20 concentrate:forage diet (20.0% Coastcross hay, 62.7% ground corn, 15.0% soybean meal, 1.0% limestone, and 1.3% mineral premix on DM basis; 91.5% DM, 15.7% CP, 3.3% EE, 4.3% ash, 20.3% NDF, and 8.8% ADF). The diet was formulated to meet the NRC (2007) recommendations for growing lambs by using the Small Ruminant Nutrition System v.1.8.0 (Cannas et al., 2004).

Both substrates were ground by using a Wiley mill (Marconi, Piracicaba, SP, Brazil) to pass a 1 mm screen. The DM was determined by oven drying at 105 °C for 24 h, and OM after ashing at 550 °C for 4 h (AOAC, 1990). Ether extract was also determined according to AOAC (1990). The CP (N × 6.25) was determined using a Leco FP528 (Leco Corporation, St. Joseph, MI, USA) combustion nitrogen analyser (AOAC, 1997). Concentrations of dietary NDF and ADF were ash-corrected and determined by the non-sequential method using beakers according to Van Soest et al. (1991) and Goering and Van Soest (1970), respectively. The NDF analysis was performed with the addition of heat stable α-amylase (Ankom Technology, Tecnoglobo Equipamentos, Curitiba, Brazil) and sodium sulfite.

Inocula Preparation

The inocula from sheep on pasture were obtained using three rumen cannulated Santa Inês wethers (50 kg BWt) kept on pastures of signalgrass (*Brachiaria decumbens*) and elephantgrass (*Pennisetum purpureum*) with free access to a mineral premix and fresh water. Each animal was supplemented with 150 g/d of ground corn, 65 g/d of soybean meal, and 4.5 g/d of molasses. The inocula from feedlot sheep were obtained from a further three rumen cannulated Santa Inês wethers (50 kg BWt) that were penned and individually fed 1.2 kg/d of the high concentrate diet described previously. Feed was provided twice daily in equal portions. Penned animals also had free access to a mineral premix and fresh water. Adaptation to the feeding conditions lasted at least 15 d. Ruminal liquid and solid fractions were collected independently before morning feeding and kept in pre-warmed thermos flasks under anaerobic conditions. Similar volumes (50:50 v/v) of both fractions were mixed in a blender for 10 s, squeezed with two layers of cheesecloth, and maintained in a water bath (39 °C) under CO_2 flushing until the moment of incubation.

Monensin Sodium Solution

A stock solution of MON (M5273 – Sigma Aldrich Inc.) was prepared by diluting 15.6 mg MON (MWt = 692.85) in 1 mL of pure ethanol and preserved at –10 °C until use. In each incubation flask (liquid volume 75 mL), 10 mL of the stock solution were added just before inoculation in order to achieve a final MON concentration of 3 mM (2.08 mg/L). According to Selje-Assman et al. (2008), 11.25 µL of ethanol in 75 mL of buffered rumen fluid had no measurable effects on fermentation. Ethanol was not included in the Control.

Laboratory Analyses and Calculations

Total gas production was estimated according to the equation:

$$V = 7.365 \times p \ (n = 500;\ r^2 = 0.99$$

data not published) where V is the gas volume (mL) and p is the measured pressure (psi).

After incubation, the pH of buffered rumen fluid was measured with a pH meter (Digimed DM21, São Paulo, SP, Brazil). The truly degraded DM (TDDM) was determined including in each flask 70 mL of neutral detergent solution (Van Soest et al., 1991) without α-amylase and incubating at 105 °C for 3 h. The residue was filtered in pre-weighed crucibles, washed with hot water and acetone, being oven dried at 105 °C for 16 h. The truly degraded OM (TDOM) was determined by ashing at 550 °C for 4 h. The TDDM and TDOM were corrected for the DM and OM residues of the blanks. The partitioning factor (PF), expressed as the ratio of TDOM (mg) to the volume of gas produced (mL), was used to estimate the efficiency of microbial production (Blümmel et al., 1997).

Apparent DM and OM degradabilities were determined according to Getachew et al. (2000). The content of each flask was transferred into a pre-weighed (60 °C for 24 h) centrifuge bottle (Du Pont Company, Wilmington, DE, USA) and centrifuged (Sorvall Superspeed RC2-B, Newton, CT, USA) at 23 000 g for 15 min. The supernatant was stored at –18 °C until analysed for short-chain fatty acids (SCFA) and ammonia. The incubation flask was washed twice with 30 mL saline solution (0.9% NaCl), transferring the washing solution to the respective centrifuge bottle. Centrifugation was repeated and the bottle containing the pellet was frozen at –18 °C and lyophilised (Labconco ThermoSavant Modulyo D–115, Holbrook, NY, USA). Apparent DM and OM degradabilities were determined by difference considering the weight of the centrifuge bottle containing the pellet minus the weight of the empty bottle and correcting for the pellet weight of the blanks.

The SCFA were determined by gas-liquid chromatography (GC HP 5890 Series II/ integrator HP 3396 Series II/automatic injector HP 6890 Series, Agilent Technologies, Palo Alto, CA, USA) according to Palmquist and Conrad (1971). The internal standard was 2-metylbutyric acid. Each tube contained 100 mL of internal standard, 800 mL of sample, and 200 µL of formic acid. A mixture of SCFA with known concentrations was used as external standard for the integra-

tor calibration. The ammonia concentration was determined using a microKjeldahl steam distillator according to Preston (1995). A 1 mL supernatant aliquot was distilled with 10-mL 5% sodium tetraborate solution and the ammonia released collected in 30-mL 20% boric acid solution and immediately titrated using 0.01 N sulphuric acid.

Methane concentration was determined by gas chromatography injecting 1 ml of gas in a Shimadzu 2014 GC (Shimadzu, Tokyo, Japan) equipped with a Shincarbon ST 100/120 micro packed column (1/16" OD, 1.0 mm ID, 1 m length; Ref. No. 19809, Restek, Bellefonte, PA, USA). Temperatures of column, injector, and flame ionisation detector were 60 °C, 200 °C, and 240 °C, respectively. Helium at 10 mL/min was used as the carrier gas. In order to calculate the CH_4 concentration, a standard curve (0, 3, 6, 9, and 15% of CH_4) was prepared with pure CH_4 (White Martins PRAXAIR Gases Industriais Inc., Osasco, SP, Brazil; 99.5% purity). Methane production (mL) was calculated by multiplying the total gas volume plus 85 mL (headspace) by the CH_4 concentration and correcting for the CH_4 produced by the blanks (Longo et al., 2006).

Plant Description and Essential Oil Characterisation

C. verbenacea (leaves and stems) was cultivated at the Chemical, Biological and Agricultural Research Center, State University of Campinas, Campinas, SP, Brazil. The EO was obtained by hydrodistillation in boiling water for 4 h by using a Clevenger device and a condenser column. The EO separation was performed by density difference using a separation funnel and filtrating with anhydrous sodium sulphate to remove residual water. The identification of the compounds present in the EO was done by GC-MS (GC HP 6890; mass selective detector HP 5975; automatic injector HP 7673, Agilent Technologies, Palo Alto, CA, USA) using a HP–5 fused silica capillary column (30 m × 0.25 mm × 0.25 µm; stationary phase 5% methyl silicone). Helium was used as the carrier gas (1.0 mL/min flow rate). The mass spectrum was acquired by electron impact ionisation (scan mode) at an electron energy of 70 eV. Samples (1 µL) were injected in the split mode employing a split ratio of 1:40. The temperature column programme was 110 °C/2 min followed by heating to 300 °C (5 °C/min). Injector and detector temperature were 220 °C and 250 °C, respectively. The essential oil was diluted in ethyl acetate (15 mg/mL) before injection. The compounds were identified by comparing their mass spectra with the National Institute of Standards and Technology (NIST) system data bank with a minimum of 95% of concordance when compared with the literature (Adams, 2001). A standard solution of n-alkanes was co-injected with the sample in order to calculate the retention index and provide additional identification criteria.

Statistical Analysis

Data were analysed by the Proc MIXED (SAS Inst. Inc., Cary, NC) considering treatment as fixed effect and inoculum (n = 2) as random effect. The two substrates (hay and high concentrate diet) were analysed independently. Means were obtained by using the LSMEANS option and differences were declared significant by using the Tukey ANOVA test at $P < 0.05$.

RESULTS AND DISCUSSION

Essential Oil Composition

The major components of the EO from *C. verbenacea* were: 28.19% of *trans*-caryophyllene, 23.58% of alpha-pinene, 6.90% of aloaromadendrene, and 4.54% of alpha-humulene (**Table 1**). A similar composition of *C. verbenacea* EO has been previously published (Carvalho Jr. et al., 2004). Considering the major compounds in the *C. verbenacea* EO, alpha-pinene was the only one that had been previously tested on ruminal fermentation. According to Busquet et al. (2006), the addition of cade oil (*Juniperus oxycedrus*) containing 35% of alpha-pinene resulted in small effects on *in vitro* ruminal fermentation, arguing that the lack of effect was probably related to the low oxygenated hydrocarbons content in this compound.

Effect on Coastcross Hay Fermentation

The effects of MON and *C. verbenacea* EO on 24-h *in vitro* fermentation of Coastcross hay are shown in **Table 2**. Gas production, expressed as mL/g of DM or $OM_{incubated}$, was reduced ($P < 0.05$) by MON when compared with the Control, mainly because substrate degradation was depressed. The reduction in TDDM and TDOM ($P < 0.05$) recorded for MON is not considered a negative effect and represents a basic limitation of short-term *in vitro* experiments (Russell and Strobel, 1988). Monensin inhibits cellulolytic ruminococci and also a cellulolytic strain of *Butyrivibrio fibrisolvens,* but *Fibrobacter succinogenes*, another cellulolytic species, is able to grow in the presence of MON (Chen and Wolin, 1979). However, *F. succinogenes* has a long growth lag time and only under *in vivo* conditions is this species able to replace the sensitive species of cellulolytic bacteria (Russell and Strobel, 1988). The MON also reduced ($P < 0.05$) gas production expressed/unit of $DM_{degraded}$, which is consistent with the greater propionate concentration ($P < 0.05$) and PF value ($P < 0.05$) when compared with the Control. According to the stoichiometry of gas production, propionate formation is always associated with lower gas production (Cone, 1998; Makkar, 2004).

The COR37.5 showed no effect on gas production when expressed as mL/g DM or $OM_{incubated}$. However, TDDM and TDOM were affected negatively ($P < 0.05$) by COR37.5, showing that *C. verbenacea* EO inhibited the activity of rumen microorganisms. As a result, gas production expressed as mL/g DM or $OM_{degraded}$, was increased ($P < 0.05$) and PF value was reduced ($P < 0.05$) by COR37.5. These results indicate that the microbial production efficiency was reduced and less degraded matter was incorporated into microbial mass (Blümmel et al., 1997). The COR75 showed even more pronounced effects than COR37.5. Despite the reduction in degradability caused by both MON and EO, the most important effect was that MON increased ($P < 0.05$) whereas EO reduced ($P < 0.05$) the PF value when compared with the Control. This indicates that utilisation of EO from *C. verbenacea* may not benefit ruminant animals.

Methane production, expressed as mL/g DM or $OM_{incubated}$, showed a 48% reduction ($P < 0.05$) when MON was compared with the Control. Reductions of 48, 52, and 58% respectively were reported using hay as substrate when 2.5, 5.0, and 12.5 mg/L of MON where added *in vitro* (Russell and Strobel, 1988). This demonstrates that very high doses of MON do not promote further reductions in CH_4 production. The dose of EO used in COR37.5 was too low to produce any detectable effect on CH_4 production. However, CH_4 produced/unit DM or $OM_{incubated}$ was reduced ($P < 0.05$) by 30% when COR75 was compared with the Control. Although not statistically different, intermediary values of CH_4 production expressed as mL/g $OM_{degraded}$ were observed for COR75 (32.9) when compared with the Control (38.9) and MON (25.8). Thus, it is speculated that higher doses of EO from *C. verbenacea* may reduce CH_4 production as much as MON. To support this idea, a previous study showed that peppermint (*Mentha piperita*) oil progressively inhibited *in vitro* methanogenesis by 19.9, 46.0, and 75.6% at levels of 0.33, 1.0, and 2.0 µL/mL, respectively (Agarwal et al., 2009).

Compared with the Control, total SCFA and acetate concentrations were not affected by MON, COR37.5 or COR75. However, the lower total SCFA (69.47 mM vs. 73.81 mM) and acetate (50.96

Table 1. Major volatile compounds identified by GC-MS analysis of the *Cordia verbenacea* D.C. essential oil

RT (min)[1]	RI[2]	Compound[3]	Relative %[4]
4.95	927	Alpha-Tujene	1.41
5.18	936	Alpha-Pinene	23.58
5.47	948	Camphene	0.19
6.10	973	Sabinene	0.99
6.19	977	Beta-Pinene	1.07
6.54	992	Beta-Myrcene	0.52
7.72	1 029	Beta-Felandrene	1.20
7.79	1 031	1,8-Cineole	1.76
17.64	1 284	Bornile acetate	0.77
19.79	1 336	Delta-Elemene	1.55
20.50	1 353	n.i.	0.31
21.34	1 373	Alpha-Copaene	0.80
21.95	1 388	Beta-Cubebene	0.46
22.04	1 390	Beta-Elemene	1.32
22.70	1 407	Alpha-Cedrene	2.23
23.18	1 418	*trans*-Caryophyllene	28.19
23.64	1 430	Beta-Gurjunene	0.75
23.81	1 434	Alpha-*trans*-Bergamotene	0.37
24.13	1 442	Beta-(Z)-Farnesene	0.42
24.51	1 452	Alpha-Humulene	4.54
24.69	1 456	Beta-(E)-Farnesene	2.55
24.82	1 459	Aloaromadendrene	6.90
25.59	1 478	Germacrene D	2.07
25.76	1 483	n.i.	1.11
26.21	1 494	Bicyclogermacrene	2.71
26.54	1 502	Alpha-(Z)-Bisabolene	1.66
26.75	1 508	Beta-Bisabolene	3.02
27.19	1 519	n.i.	0.64
27.29	1 522	Delta-Cadinene	2.03
27.62	1 530	Gamma-(E)-Bisabolene	1.08
29.48	1 579	Caryophyllene oxide	0.93
32.77	1 668	n.i.	1.42
33.06	1 676	n.i.	1.47

[1] — Retention time; [2] — Retention index; [3] n.i. — non-identified; [4] = percentage relative to the total area integrated in the chromatogram.

mM vs. 54.29 mM) concentrations recorded for MON compared with the Control is related to the reduction on apparent DM degradability. A decrease in acetate concentration was expected because gram-positive bacteria, which mainly produce acetate, are sensitive to MON (Russell and Houlihan, 2003). Propionate concentration was increased ($P < 0.05$) by MON even with hay as substrate. In contrast, this same variable was reduced ($P < 0.05$) by COR37.5 and COR75 when compared with the Control. The inhibition of methanogenesis observed for MON is always coupled with an increase in propionate and a decrease in acetate concentrations (Russell and Strobel, 1989), but this was not recorded when *C. verbenacea* EO was used. It might therefore be speculated that this EO may affect methanogens directly since the mode of action of MON is indirect, with CH_4 being reduced by inhibiting hydrogen and formate-producing bacteria (Russell and Strobel, 1989). A previous *in vitro* trial showed that peppermint oil inhibited methanogenic microorganisms directly, and CH_4 production was reduced even with a decrease in propionate concentration and an increase in the acetate:propionate ratio (Agarwal et al., 2009).

The acetate:propionate ratio was decreased ($P < 0.05$) by MON mainly due to the reduction recorded in acetate concentration. In contrast, the ratio was not affected by COR37.5 or COR75. All other SCFAs were reduced ($P < 0.05$) by MON compared with the Control, with the exception of valerate. Butyrate is generally reduced by MON because this ionophore inhibits the major butyrate producer, the gram-positive bacteria *Butyrivibrio fibrisolvens* (Russell and Strobel, 1989). The reduced concentrations of iso-acids are indicative of lower deamination, since iso-acids are derived from catabolism of branched-chain amino acids (Mackie and White, 1990). In the case of

Table 2. Effect of monensin (3 µM) and *Cordia verbenacea* D.C. essential oil (37.5 or 75 µL in 75 mL of buffered rumen fluid) on 24-h *in vitro* fermentation of Coastcross hay.

Item[1]	Treatments[2]				SEM[3]
	Control	MON	COR37.5	COR75	
Gas					
mL/g $DM_{incubated}$	117.3^{a}	78.4^{c}	119.4^{a}	106.0^{b}	1.9
mL/g $OM_{incubated}$	125.6^{a}	83.9^{c}	127.9^{a}	113.5^{b}	2.0
mL/g $DM_{degraded}$	228.9^{b}	206.0^{c}	252.7^{a}	255.7^{a}	5.5
mL/g $OM_{degraded}$	252.7^{b}	230.7^{b}	278.5^{a}	284.9^{a}	5.9
TDDM (%)	49.58^{a}	39.21^{c}	45.85^{b}	41.91^{c}	0.79
TDOM (%)	48.08^{a}	37.51^{c}	44.54^{b}	40.26^{c}	0.71
ApDDM (%)	33.67^{a}	28.30^{c}	31.74^{ab}	29.48^{bc}	1.11
Partitioning factor	3.97^{b}	4.42^{a}	3.60^{bc}	3.57^{c}	0.10
Methane					
mL/g $DM_{incubated}$	17.5^{a}	9.0^{c}	15.9^{a}	12.2^{b}	0.7
mL/g $OM_{incubated}$	18.7^{a}	9.7^{c}	17.0^{a}	13.1^{b}	0.7
mL/g $DM_{degraded}$	35.2^{a}	23.0^{b}	34.7^{a}	29.5^{ab}	1.8
mL/g $OM_{degraded}$	38.9^{a}	25.8^{b}	38.2^{a}	32.9^{ab}	2.0
SCFA, mM					
Total	73.81^{ab}	69.47^{b}	74.42^{a}	73.32^{ab}	1.13
Acetate	54.29	50.96	55.13	54.36	1.11
Propionate	9.88^{b}	10.16^{a}	9.57^{c}	9.27^{d}	0.05
Isobutyrate	0.55^{a}	0.43^{b}	0.57^{a}	0.56^{a}	< 0.01
Butyrate	7.39^{a}	6.38^{b}	7.44^{a}	7.31^{a}	0.06
Isovalerate	1.11^{b}	0.98^{c}	1.19^{a}	1.19^{a}	0.01
Valerate	0.60	0.57	0.53	0.63	0.05
Acetate:propionate	5.50^{a}	5.02^{b}	5.76^{a}	5.86^{a}	0.12
NH_3, mg/100 mL	26.49^{b}	27.63^{ab}	30.46^{a}	27.91^{ab}	0.89
pH at 24 h	6.70^{c}	6.76^{ab}	6.73^{b}	6.77^{a}	< 0.01

[1] TDDM = truly degraded dry matter; TDOM = truly degraded organic matter; ApDDM = apparently degraded dry matter; Partitioning factor = mg $OM_{degraded}$/mL $gas_{produced}$; SCFA = short-chain fatty acids.

[2] Means followed by distinct letters within row differ by Tukey test ($P < 0.05$).

[3] SEM = standard error of the mean.

COR37.5 and COR75, isobutyrate, butyrate, isovalerate, and valerate concentrations did not differ from the Control.

Despite the reduced iso-acids concentrations, ammonia concentration was not reduced by MON as expected. *In vitro* studies have shown that MON decreases deamination (Russell and Strobel, 1989) and also inhibits a group called hyper-ammonia-producing bacteria which have a high specific activity for ammonia production (Russell et al., 1988). The lack of effect on ammonia concentration may be explained by the limited microbial growth and excessive microbial lyses which result in the high ammonia concentrations commonly observed for *in vitro* conditions (Cone, 1998). When the EO was used, ammonia concentration was not affected by COR75 but was increased ($P < 0.05$) by COR37.5. There is no clear explanation for this finding.

The pH value after 24-h incubation was increased ($P < 0.05$) by MON and EO inclusion. Higher pH values have been observed with MON addition and are generally attributed to the inhibition of lactate-producing bacteria (e.g. *Streptococcus bovis*; Russell and Strobel, 1989). Unfortunately, lactate concentration was not measured. The lower pH observed for the Control is also consistent with the greater TDOM verified for this treatment compared with the other treatments. However, the effect on pH must be carefully interpreted, since *in vitro* pH is controlled by buffering agents.

Effect on Fermentation of Concentrate Diet

The effects of MON and *C. verbenacea* EO on 16-h *in vitro* fermentation of the 80:20 concentrate:forage diet are shown in **Table 3**. In general, the doses of EO tested seemed to be too low to produce any effect on fermentation of the high concentrate diet. The interaction of EO and type of substrate was not considered statistically, but some differences occurred on the EO effect using hay or high concentrate diet, especially on CH_4 production. Interestingly, a previous study did not detect significant interaction between a commercial blend of EO and the type of diet (high concentrate or high forage) on DM degradation, SCFA profiles, and N metabolism in continuous culture fermentation (Castillejos et al., 2005).

Table 3. Effect of monensin (3 µM) and *Cordia verbenacea* D.C. essential oil (37.5 or 75 µL in 75 mL of buffered rumen fluid) on 16-h *in vitro* fermentation of an 80:20 concentrate:forage diet.

Item[1]	Treatments[2]				SEM[3]
	Control	MON	COR37.5	COR75	
Gas					
mL/g $DM_{incubated}$	213.1^{a}	199.8^{b}	212.5^{a}	206.5^{ab}	3.0
mL/g $OM_{incubated}$	223.0^{a}	209.0^{b}	222.4^{a}	216.0^{ab}	3.2
mL/g $DM_{degraded}$	278.5^{ab}	268.4^{b}	288.3^{a}	284.4^{ab}	4.6
mL/g $OM_{degraded}$	290.5^{ab}	279.3^{b}	300.0^{a}	298.4^{a}	4.6
TDDM (%)	76.67^{a}	73.68^{ab}	73.28^{ab}	71.03^{b}	0.99
TDOM (%)	76.89^{a}	74.08^{ab}	73.68^{ab}	70.83^{b}	0.98
ApDDM (%)	44.74^{c}	56.03^{ab}	48.52^{bc}	59.64^{a}	2.15
Partitioning factor	3.48^{ab}	3.59^{a}	3.35^{b}	3.36^{b}	0.05
Methane					
mL/g $DM_{incubated}$	31.1^{a}	22.8^{b}	30.3^{a}	31.9^{a}	1.1
mL/g $OM_{incubated}$	32.5^{a}	23.9^{b}	31.7^{a}	33.4^{a}	1.2
mL/g $DM_{degraded}$	40.6^{a}	31.1^{b}	41.4^{a}	45.0^{a}	1.8
mL/g $OM_{degraded}$	42.3^{a}	32.4^{b}	43.1^{a}	47.2^{a}	1.9
SCFA, mM					
Total	90.43^{ab}	91.22^{a}	83.83^{b}	93.32^{a}	1.63
Acetate	56.78^{a}	55.78^{ab}	50.33^{b}	58.26^{a}	1.43
Propionate	17.08^{b}	21.05^{a}	16.55^{b}	17.36^{b}	0.34
Isobutyrate	1.27^{a}	1.11^{b}	1.28^{a}	1.33^{a}	0.02
Butyrate	11.17^{b}	9.52^{c}	11.54^{ab}	12.04^{a}	0.16
Isovalerate	3.08^{ab}	2.76^{b}	3.10^{ab}	3.23^{a}	0.09
Valerate	1.06^{ab}	0.98^{b}	1.03^{ab}	1.10^{a}	0.02
Acetate:propionate	3.34^{a}	2.66^{b}	3.06^{a}	3.37^{a}	0.09
NH_3, mg/100 mL	45.19	54.26	53.52	52.77	2.64
pH at 16 h	6.55^{a}	6.53^{b}	6.57^{a}	6.56^{a}	< 0.01

[1] TDDM — truly degraded dry matter; TDOM — truly degraded organic matter; ApDDM — apparently degraded dry matter; Partitioning factor — mg $OM_{degraded}$/mL $gas_{produced}$; SCFA — short-chain fatty acids.

[2] Means followed by distinct letters within row differ by Tukey test ($P < 0.05$).

[3] SEM — standard error of the mean.

As verified for hay, gas production expressed per unit of DM or $OM_{incubated}$ was reduced ($P < 0.05$) by MON. However, this effect occurred to a lesser extent than verified for hay, which is explained by the similar values of TDDM and TDOM between Control and MON. Although not statistically different, the lower values of gas production expressed as mL/g DM or $OM_{degraded}$ are consistent with the greater propionate concentration recorded for MON compared with the Control. The inclusion of *C. verbenacea* EO at the two doses tested did not affect gas production variables.

The TDDM and TDOM were not affected by MON when compared with the Control, a result consistent with the observation that ionophores do not decrease starch digestion. The COR37.5 also did not affect TDDM and TDOM, but a reduction ($P < 0.05$) was observed for COR75 compared with the Control. It is important to highlight that the overestimation of TDDM and TDOM (i.e. starch solubilisation) by the utilisation of neutral detergent solution seemed to be negligible. Using the same conditions of this experiment, a previous trial determined that the undegraded residue after 16-h incubation contained only 5.96% starch (data not published). Unfortunately, the amount of starch solubilised by the addition of neutral detergent solution was not determined; nevertheless it is possible to assume that any interference on degradability estimation was very low, because calculated PF values of all treatments were between the theoretical PF range of 2.74 to 4.65 (Blümmel et al., 1997). Compared with the Control, PF values were not affected by MON or EO.

Methane production, expressed as mL/g $DM_{incubated}$ and mL/g $DM_{degraded}$, was reduced ($P < 0.05$) by 27% and 23% respectively when MON was compared with the Control. Using a similar MON concentration and corn meal as the substrate, a previous study reported a 32% decrease in CH_4 production with MON addition (Russell and Strobel, 1988). In contrast to the data obtained for hay, CH_4 production (all variables) was not affected by inclusion of *C. verbenacea* EO when the high concentrate diet was used as substrate. While it is known that some EO effects on rumen fermentation are pH-dependent (Cardozo et al., 2005), this idea is not plausible under the present experimental conditions because the *in vitro* pH was buffer-controlled.

Total SCFA and acetate concentrations did not differ between Control and MON. However, MON resulted in greater ($P < 0.05$) propionate concentrations, leading to lower ($P < 0.05$) acetate:propionate ratios when compared with the Control. The gram-negative bacteria in the rumen, which mainly produce propionate and succinate, are MON-resistant (Russell and Houlihan, 2003), which explains the increase in propionate concentration. It is important to note that the acetate:propionate ratio decreased in the hay fermentation because acetate concentration was reduced, whereas this ratio decreased in the high concentrate diet fermentation due to the greater concentration of propionate. As verified for hay, MON reduced ($P < 0.05$) the concentrations of butyrate and iso-acids, without affecting valerate. Once again, ammonia concentrations did not differ between MON and the Control, and no effect was recorded on SCFA variables when COR37.5 or COR75 were compared with the Control. The only exception was for butyrate, which had a greater concentration for COR75 than the Control. Surprisingly, COR37.5 resulted in lower total SCFA and acetate concentrations than COR75. As verified for MON, ammonia concentration was not affected by COR37.5 or COR75. This result indicates that EO inclusion was not effective in reducing deamination, although some effect may occur in the processes of proteolysis or peptidolysis. Moreover, a period of adaptation to the rumen environment is generally necessary to observe PSM effects on N metabolism (Calsamiglia et al., 2007), and such effects would not be possible to evaluate under short-term *in vitro* incubations.

CONCLUSIONS

The EO from *Cordia verbenacea* D.C. was able to modify *in vitro* ruminal fermentation. The most promising effect was related to the inhibition of methanogenesis using hay as substrate. This experiment indicates that doses greater than 1 µL/mL of buffered rumen fluid may decrease CH_4 production as much as MON. However, the negative effect on the reduction of microbial production efficiency should be carefully considered. No positive effects were observed with the inclusion *of C. verbenacea* EO when the 80:20 concentrate:forage diet was used as substrate. However, taking into account the results obtained for hay, doses greater than 1 µL/mL of buffered rumen fluid should also be evaluated under the high concentrate diet condition.

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Reduction in Methane Emissions from Ruminants by Plant Secondary Metabolites: Effects of Polyphenols and Saponins

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ABSTRACT

The effects of plant secondary metabolites (PSM), specifically polyphenols (tannins) and saponins on rumen fermentation and methanogenesis were investigated using the Hohenheim gas method. We evaluated the effects of: (1) polyphenol-containing plants, (2) simple phenols in the form of phenolic acids, (3) purified tannins, (4) saponin-containing plants, and (5) isolated saponin-rich fractions on rumen methanogenesis. Statistically significant negative relationships between total phenols, total tannins or tannin activity and methane (CH_4) production were observed, whereas no correlation existed between condensed tannins and CH_4 production. Cinnamic, caffeic, p-coumaric and ferulic acids decreased CH_4 production significantly when added at 5 mM. Addition of purified chestnut and sumach tannins (hydrolysable tannins) at 1 mg/mL to the *in vitro* rumen fermentation system containing hay:concentrate (70:30) decreased CH_4 production ($P < 0.05$), by 6.5 and 7.2% respectively. However, addition of mimosa and quebracho tannins (condensed tannins) at this concentration did not decrease CH_4 production. For studying the effects of saponins, leaves of *Sesbania, Knautia* and seeds of Fenugreek, and their saponin-rich fractions were evaluated. Addition of Fenugreek and *Sesbania* plant materials to hay or the hay-concentrate mixture increased partitioning factor (PF, expressed as mg truly degraded substrate/mL gas produced; a measure of efficiency of microbial protein synthesis) and decreased CH_4 production per unit substrate degraded. These plant materials and their saponin-rich fractions did not reduce CH_4 production in absolute amounts despite decreases in protozoal numbers by 40–50%. The saponins altered the microbial community towards proliferation of fibre-degrading bacteria and inhibition of fungal population. The results with saponin-containing plant materials and their isolated fractions indicated a weak association between anti-protozoal activity of saponins and methanogenesis. Nevertheless, the saponin-containing plants possess potential to partition higher proportions of the substrate to microbial mass production

Key words: *methane, polyphenols, saponins, microbial ecology, rumen fermentation.*

INTRODUCTION

The emission of greenhouse gases such as carbon dioxide and CH_4 is considered to be one of the most important global environmental issues (IPCC, 2001). Animals, particularly ruminants, produce CH_4 from anaerobic fermentation in their gastro-intestinal tracts as a pathway for the disposal of metabolic hydrogen produced during microbial metabolism. Ruminant livestock are responsible for about 15–20% of the total anthropogenic emission of CH_4 (Moss et al., 2000). The CH_4 produced from enteric fermentation of ruminants is not only related to environmental problems, but is also associated with energy losses and, hence reductions in their retention and use of energy. Typically 6–8%, but up to 12%, of the gross energy (GE) in feed is converted to CH_4 during microbial digestion in the rumen (Johnson and Johnson, 1995). Therefore, decreasing CH_4 production from ruminants is desirable for reducing greenhouse gas emissions and increasing utilisation of the digested energy. Plant secondary metabolites (PSM) have been suggested as effective alternatives to antibiotics to suppress rumen methanogenesis through their antimicrobial activity (Makkar et al., 2007; Jayanegara et al., 2009). Plant secondary metabolites constitute the group of chemicals present in plants that are not involved in the primary biochemical processes of plant growth and reproduction. The potential of these compounds and specifically of polyphenols (tannins) and saponins to reduce enteric CH_4 production has been recognised and extensive screening of a large range of plants and their secondary compounds is underway in several laboratories. The antimicrobial action and effects on rumen fermentation of these compounds depend on their nature, activity and concentration. We present here work conducted in our laboratory on the potential of various polyphenols (tannins), saponin-rich plants and isolated saponin-rich fractions to reduce CH_4 emission from ruminants.

RESULTS AND DISCUSSION

Polyphenols

The evaluation of polyphenols was conducted using the *in vitro* Hohenheim gas production method (Menke and Steingass, 1988) as modified by Makkar et al. (1995). We examined a number of polyphenol-containing plants, non-tannin simple phenolics, and purified tannins.

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Table 1. Correlation coefficients between tannin assays and *in vitro* rumen methane production (n = 17).

Assays[b]	Methane (ml/100 mL)	Decrease in CH_4 (%)	Increase in CH_4[a] (%)
TP	–0.59*	0.57*	0.78***
TT	–0.60*	0.54*	0.62**
CT	–0.07[ns]	0.09[ns]	0.24[ns]
Tannin bioassay	–0.75***	0.79***	0.92***

TP = total phenols; TT = total tannins; CT = condensed tannins.
[a] Methane increase on polyethylene glycol (MW 6 000) addition; [b] for assay protocols see Makkar (2003a).
[ns] not significant; * P < 0.05; ** P < 0.01; *** P < 0.001.

Table 2. Effect of addition of simple phenols on gas production, methane production and organic matter digestibility.

Treatment	Gas (mL)	CH_4 (mL/100 mL)	Decrease in CH_4 (%)	OMD (%)	CH_4/OMD (mL/100 mg)	Decrease in CH_4/OMD (%)
Control	76.2[c]	15.9[cd]	0.0[b]	76.1	5.05[bc]	0.0[ab]
Benzoic						
2 mM	77.0[c]	16.0[cd]	–0.6[ab]	75.2	5.19[c]	–2.8[a]
5 mM	74.8[bc]	16.0[cd]	–0.3[ab]	75.2	5.03[abc]	0.4[ab]
Cinnamic						
2 mM	75.3[bc]	15.5[abc]	2.6[bcd]	75.7	4.88[abc]	3.2[abcd]
5 mM	74.5[bc]	15.4[abc]	3.4[cde]	75.5	4.79[abc]	5.0[abcd]
Phenylacetic						
2 mM	73.3[abc]	15.9[bcd]	0.2[abc]	73.7	5.00[abc]	0.9[abc]
5 mM	74.3[bc]	16.4[d]	–3.1[a]	75.1	5.13[c]	–1.7[ab]
Caffeic						
2 mM	73.3[abc]	15.6[abc]	2.0[bcd]	73.2	4.94[abc]	2.1[abcd]
5 mM	71.0[ab]	14.9[a]	6.3[e]	73.4	4.57[a]	9.4[d]
p-Coumaric						
2 mM	72.5[abc]	15.5[abc]	2.4[bcd]	71.8	4.96[abc]	1.6[abcd]
5 mM	68.5[a]	15.1[a]	5.1[de]	71.0	4.61[ab]	8.5[cd]
Ferulic						
2 mM	72.5[abc]	15.9[bcd]	0.4[bc]	75.2	4.84[abc]	4.0[abcd]
5 mM	70.8[ab]	15.2[ab]	4.7[de]	71.4	4.77[abc]	5.5[bcd]
SEM	0.49	0.08	0.51	0.43	0.039	0.75

OMD = organic matter digestibility.
Values in the same column with different superscripts are different at P < 0.05.

Polyphenol-containing Plants

Using 17 polyphenol-containing plants (**Table 1**), statistically significant negative relationships between total phenols (TP), total tannins (TT) or tannin activity and CH_4 production existed, whereas the relationship between condensed tannins (CT) and CH_4 production was not significant. The highest correlation was found between tannin activity determined by the tannin bioassay and CH_4 decrease.

Since the correlations between TP and decrease in CH_4 or increase in CH_4 on addition of polyethylene glycol (a tannin-inactivating agent) were higher than those for TT, it seems that non-tannin phenols contribute to reducing CH_4 production. It would be interesting to obtain direct evidence by isolating non-tannin phenols and incubating them in the *in vitro* gas method. These results, if confirmed, could have wide application since non-tannin phenols are not likely to decrease the utilisation of proteins and other nutrients, but could also have beneficial effects (antioxidant, anticarcinogenic) associated with phenolic compounds (Makkar, 2003b; Makkar et al., 2007).

Although it was evident from these results that tannin-containing plants are able to reduce ruminal CH_4 emission, some reports suggest that tannins have no significant effect on rumen CH_4 production. For example, Oliveira et al. (2007) reported that there was no effect of tannin levels on CH_4 emission from diets containing sorghum silages. Beauchemin et al. (2007) also reported that feeding a diet containing an extract of quebracho tannins at a level up to 20 g/kg dry matter did not reduce enteric CH_4 emissions from growing cattle, although the protein-binding effect of the quebracho tannin extract was evident. The different results obtained using different tannins could be

attributed to their nature, structure or activity and to the concentrations at which they were used.

Non-tannin Phenolics

The above study indicated that non-tannin phenols play a role in CH_4 reduction. In the next study we evaluated six simple phenols (benzoic, cinnamic, phenylacetic, caffeic, p-coumaric and ferulic acids), as representatives of non-tannin phenols. All of these simple phenols were added at two different concentrations, i.e. 2 and 5 mM. The results are presented in **Table 2**.

In general, the addition of simple phenols decreased gas production although most of them were not significantly different and the effects were higher at higher concentrations. None of the simple phenols was effective in decreasing CH_4 production at the lower concentration (2 mM). Cinnamic, caffeic, p-coumaric and ferulic acids decreased CH4 production significantly ($P < 0.05$) when added at 5 mM. Caffeic acid at 5 mM was the most effective of the simple phenols tested, decreasing CH_4 by 6.3% compared with the control. The magnitude was higher (9.4% compared with the control) when expressed as decrease of CH_4 per unit organic matter digested. After caffeic acid, the order of simple phenols to decrease CH_4 was: p-coumaric > ferulic > cinnamic. Phenolic acid containing tri-hydroxy group (caffeic acid) had a higher CH_4 inhibitory effect than those containing di-hydroxy groups (p-coumaric acid and ferulic acid). The phenolics containing a single hydroxy group (benzoic, phenylacetic and cinnamic acids) had the least effect. These results suggest that phenolics with higher numbers of hydroxyl groups are expected to elicit higher CH_4 inhibitory effects.

The effect of phenolic acids on methanogenesis could be expected since they affect the activities of rumen microbes. The decrease in ruminal CH_4 production could be linked to their role in inhibiting fibre degradation and in decreasing protozoa to a certain extent. Inhibition of fibre degradation will shift short chain fatty acid (SCFA) composition away from acetate and hence less production of hydrogen and less CH_4 formation. On the other hand, the anti-protozoal effect of phenolic acids would decrease CH_4 production since a portion of methanogens is attached to protozoa (Vogels et al., 1980). These protozoa-associated methanogens have been reported to contribute up to 37% of total rumen CH_4 emissions (Klieve and Hegarty, 1999). Therefore reduced protozoal counts in the rumen are associated with the reductions in CH_4 production, however, this is not always the case since a weak association between protozoal numbers and methanogenesis was observed with saponin-containing plants (discussed in later sections).

Phenolic acids are common constituents of forages fed to ruminants, where they occur most commonly as hydroxycinnamic acids ester-linked to polysaccharide. Ferulic and p-coumaric acids, the major phenolic acids found in this form, may represent up to 2.5% by weight of the cell walls of temperate grasses (Hartley and Jones, 1977). In the present study, phenolic acid concentrations of 2 and 5 mM were equal to 1.9–3.1 and 4.8–7.7% of the substrate dry matter incubated, respectively, depending on the structure and molecular weight of each phenolic acid. The lower concentration used was therefore in a reasonable range, while the higher concentration might also be in a reasonable range for the tropical forages, which generally contain higher concentrations of lignified tissues and secondary metabolites than temperate forages. In the *in vivo* situation, rumen microbes might encounter such a high concentration of phenolic acids provided that all phenolic acids are released from plant tissues, which is normally not the case. However, the microbes attached to the plant tissues are likely to encounter higher concentrations of phenolic acids in their microenvironment.

Purified Tannins

Some studies have reported that feeding tannin-containing forages to ruminants reduces CH_4 emissions (e.g. Puchala et al., 2005). However, in most of those studies, the reduction in CH_4 was confounded by changes in forage composition and quality. Lower fibre diets are associated with lower CH_4 emissions. Other nutrients such as lipid (oil) affect CH_4 production. Similarly, higher digestible feed is known to produce less CH_4 per unit feed intake. Negative effects on ruminal fibre digestion, which may relate to decreased number of cellulolytic bacteria, formation of tannin-cellulose complexes that are resistant to enzymatic digestion, and/or impaired substrate adhesion by fibrolytic microbes, would reduce hydrogen availability to lessen methanogenesis (Carulla et al., 2005). Thus, there is considerable uncertainty about the effectiveness of tannin-containing forages to reduce enteric CH_4 emissions from cattle. In the present study, therefore, other confounding components were omitted by using the purified tannins and, hence, specific effects of tannins were obtained. Different levels of purified tannins from chestnut, mimosa, quebracho and sumach (0.5, 0.75 and 1.0 mg/mL) were evaluated for their potential to reduce rumen CH_4 production. Chestnut and sumach tannins represented the hydrolysable tannins, whereas mimosa and quebracho tannins represented the condensed tannins.

The addition of purified chestnut and sumach tannins at 1 mg/ml to a hay:concentrate (70:30) diet significantly decreased ($P < 0.05$) CH_4 production by 6.5 and 7.2% respectively. Lower concentrations (0.5 and 0.75 mg/mL) did not significantly decrease CH_4 production. The addition of mimosa and quebracho tannins (condensed tannins) did not significantly decrease CH_4 production, even at the highest concentration. For all tannins, increases in concentration led to increases in CH_4 reduction (**Figure 1**). The condensed tannins decreased gas production and organic matter digestibility (OMD) more than the hydrolysable tannins. The results suggested that the hydrolysable tannins are more effective in decreasing CH_4 emissions than the condensed tannins, while at the same time the hydrolysable tannins did not significantly decrease OMD. The condensed tannins appear to decrease CH_4 more through reduced fibre digestion (indirect effect), while hydrolysable tannins act more through inhibition of the growth and/or activity of methanogens and/or hydrogen producing microbes (direct effect).

The tannin concentrations of 0.5, 0.75 and 1.0 mg/mL were equal to 4.0, 5.9 and 7.9% of the substrate dry matter, respectively. Animals are likely to encounter such concentrations, especially in tropical regions, where they are exposed to high amount of tannins during the dry season. During the dry season, animals depend largely on fodder tree leaves and browses, and the tannin content in these feed resources is generally high (5–15%). However, it may be noted that the effects on the extent of CH4 reduction of tannins in the soluble form as in this study and in *in vivo* situations where tannins are a part of the feed might be different. Nevertheless, the *in vitro* studies give insight into the mechanism of action of various tannins, their comparative effects and possible *in vivo* effects.

Although it was evident from our research that polyphenols reduce rumen CH_4 production significantly, it should be noted that we used *in vitro* experiments to measure the effects. Flachowsky and Lebzien (2009) noted that it is extremely difficult to extrapolate from *in vitro* measurements to *in vivo* situations in ruminants, or to field conditions. This is because the relationship between CH_4 produced *in vivo* and *in vitro* is very poor ($r^2 = 0.264$). However, the *in vitro* studies should be considered as a starting point for screening of potential CH_4 inhibitors and should be combined with *in vivo* experiments. Therefore, Flachowsky and Lebzien (2009) proposed a three-step programme to assess the CH_4-reduction potential of feed additives or feeding measurements. This includes *in vitro* screening of substances,

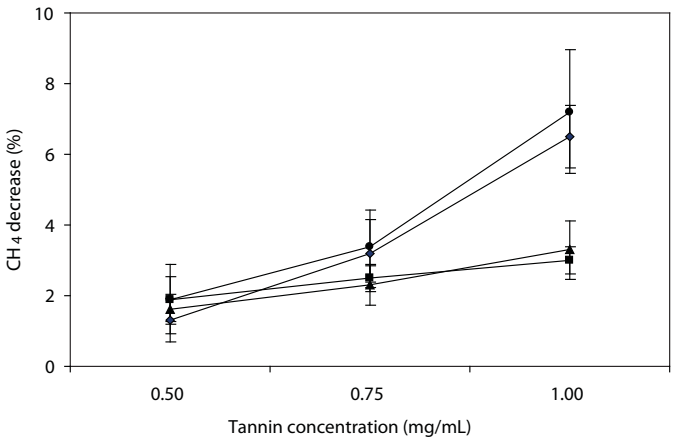

Figure 1. Effect of purified tannins from chestnut (-♦-), mimosa (-■-), quebracho (-▲-) and sumach (-●-) on CH_4 decrease when added to hay:concentrate diet (70:30 w/w) at concentrations of 0.5, 0.75 and 1.0 mg/mL

short-term *in vivo* experiments in target animals and finally *in vivo* long-term recording of CH_4 production together with other animal performance parameters. Following this programme will substantially increase the relevance of such studies to the industry and potential users. Also, short term *in vivo* studies could also be replaced by continuous fermentation studies as conducted by Goel et al. (2009). This would save time and resources.

Saponins

Saponins are natural detergents, chemically defined as high molecular weight glycosides in which sugars are linked to a triterpene or steroidal aglycone moiety. These compounds possess membranolytic properties, resulting in cell death by forming complexes with sterols on protozoal cell membranes (Cheeke, 1999). They modify ruminal fermentation by suppressing rumen protozoa and selectively inhibiting some bacteria. The symbiosis of protozoa with methanogenic bacteria in the rumen is well established and selective suppression of protozoa has been suggested to be a promising approach to reduce the CH_4 production (Dohme et al., 1999). Therefore, the plants rich in saponins have potential for enhancing flow of microbial protein from the rumen, increasing the efficiency of feed utilisation, and decreasing methanogenesis. We studied the effects of saponin-containing plants and their saponin-rich fractions for their anti-protozoal and CH_4 inhibition activities using the Hohenheim Gas Test (HGT). The first study was designed to observe the effect of saponin-rich plant materials on partitioning of nutrients from roughage- and concentrate-based feeds to CH_4, followed by another similar study with their isolated saponin-rich fractions. Fermentation parameters and microbial community structure were also investigated.

Saponin-containing Plants

The incubation of saponin-containing plant materials: leaves from *Sesbania* (*Sesbania sesban*) or seeds of Fenugreek (*Trigonella foenum-graecum* L.) as a sole substrate resulted in higher responses towards increasing the partitioning factors, PF (increasing efficiency of microbial mass synthesis) and increasing the reductions in CH_4 production (**Table 3a**). The plant materials when added to hay- or concentrate-based diets, did not produce substantial reductions in CH_4 production (**Table 3b**). The higher PF and CH_4 reductions were observed when the saponin-containing plant materials were added to concentrate-based diets. The crude plant extracts (in water and methanol/water (0.95:0.05, v/v) of the test plants had negligible effects on CH_4 production (data not shown). All different incubation materials: the sole plant material, plant material supplemented with hay- or concentrate-based diets or the plant extracts resulted in reductions in protozoal populations. However, these reductions did

Table 3. Effect of different plant materials on methane production.

a) Incubation with sole plant materials as substrate (380 mg/40 mL incubation fluid).

	Rumen liquor from roughage fed animal				Rumen liquor from concentrate-hay fed animal				
Substrate	PF	MR (%)	MR_{TD} (%)	Protozoa (% reduction)	Substrate	PF	MR (%)	MR_{TD} (%)	Protozoa (% reduction)
Hay	3.11a				Conc	3.16a			
Fenugreek	3.35a	-2.2	6.7a	68.1	Fenugreek	4.57b	20.5 b	29.1 a	73.2 b
Sesbania	4.63b	-3.4	30.4b	61.1	*Sesbania*	3.52a	5.4a	37.8 b	66.0 a
SEM	0.051	1.22	0.37	1.26	SEM	0.14	1.93	0.11	2.12

b) Supplementation of hay or concentrate (380 mg each) with the supplement (66 mg)

	Rumen liquor from roughage fed animal				Rumen liquor from concentrate - hay fed animal				
Substrate	PF	MR (%)	MR_{TD} (%)	Protozoa (% reduction)	Substrate	PF	MR (%)	MR_{TD} (%)	Protozoa (% reduction)
Hay	3.11^{a}				Conc	3.16^{a}			
Hay+F	3.28^{a}	0.56	5.18^{a}	49.5	Conc+F	3.33^{ab}	4.86^{b}	9.74^{a}	56.0
Hay+S	3.45^{a}	1.13	11.4^{b}	47.8	Conc+S	3.52^{b}	1.62^{a}	11.9^{ab}	50.7
SEM	0.096	0.124	2.028	0.98	SEM	0.132	0.221	1.948	1.03

PF = partition factor (mg of substrate truly degraded/mL gas); MR = methane reduction on volume basis; MR_{TD} = methane reduction on the basis of substrate truly degraded; F = Fenugreek seeds and S = *Sesbania* leaves.

Values in the same column with different superscripts are different at $P < 0.05$.

not accompany the decreases in CH_4 production in the incubations using rumen liquor from hay-fed animals; on the other hand, small reductions in CH_4 were produced in incubations with rumen liquor from concentrate-fed animals ($P < 0.05$). This observation indicates a weak association between the two parameters. The results from this study imply that the supplementations tested did not adversely affect the degradability of the basal feeds, hay or concentrate-hay mixture. Nevertheless, these plant materials when used as supplements, especially to the concentrate-based diet, have the potential to partition higher proportions of the substrate to microbial mass production and to elicit some CH_4 reduction per unit of substrate degraded. A weak association between protozoal number and methanogenesis was evident in this study and this association seemed to be diet dependent.

Saponin-rich fractions

Based on the results from saponin-containing plants, a further study was conducted with saponin-rich fractions prepared from the test plant materials: leaves of *Sesbania* (*Sesbania sesban*), *Knautia* (*Knautia arvensis*) and seeds of Fenugreek (*Trigonella foenum-graecum* L.). These fractions were evaluated for their effects on partitioning of nutrients from the concentrate-based diets to CH_4, SCFA and protozoa number. Additionally, changes in ammonia nitrogen (an important parameter in determining the N flow during substrate degradation which is used for microbial biomass production and absorption through the rumen cell wall), ammonia uptake and microbial community structure were also studied using real-time PCR assay (Denman and McSweeney, 2006). The lower concentration of saponin-rich fractions used in this study corresponded to the quantity of raw material of the test supplements which showed promising response in the former study, except for *Knautia* leaves, which were not evaluated in the earlier study. Saponins have been reported to alter rumen fermentation by affecting the digestibility (either increase or no effect) and increasing microbial protein synthesis (Makkar et al., 1998). In the present study the fractions did not affect digestibility, and a trend towards slightly higher gas production was observed, which might be due to the saponin-mediated increase in fiber degrading bacteria (discussed below). Increase in the PF was not observed on supplementation of any of the saponin-rich fractions (**Table 4**), while increased PF values were observed on supplementation of the plant materials from which these saponins were isolated. Different responses have been observed for different saponins, which could be attributed to the different nature and/or concentration of saponins. Therefore, caution should be exercised in generalising the effects of saponins.

The maximum CH_4 reduction was observed for higher concentrations of saponin-rich fractions of *Sesbania* (6.1%) and *Knautia* (6.4%). Saponin-rich fractions were not as effective as the original plant material which when used as equivalent to the lower concentrations of saponin-rich fractions from Fenugreek seeds and *Sesbania* leaves decreased the protozoal count by nearly 50%, while this inhibition was 39% and 36% only by the corresponding saponin-

Table 4. Effect of saponin-rich fractions of test plants on methane production and protozoal numbers.

Substrate*	Partition Factor	MR%	MR_{TD} (%)	Protozoa** ($\times 10^4$ mL/1)
S	3.25			19.54
S+F 5.62	3.12	1.82	–1.59	16.60 (15)
S+F 11.54	3.07	1.97	–4.47	11.93 (39)
S+Se 10.9	3.14	4.69	1.54	16.80 (14)
S+Se 21.8	3.08	6.14	1.71	12.41 (36)
S+K 3.88	3.16	5.50	3.23	16.83 (14)
S+K 7.76	3.16	6.43	3.94	14.66 (25)
SEM	0.122	1.821	1.112	1.224

*S = hay:concentrate (1:1), saponin-rich fractions (in mg) from: F = fenugreek; Se = *Sesbania*; K = *Knautia*. **Values in parentheses are the percentage reduction in protozoal number.

M — methane; MR — methane reduction on volume basis; TD — truly degraded substrate; MR_{TD} — methane reduction on truly degraded substrate basis.

Table 5. Effects of saponin-rich fractions on SCFA and ammonia uptake.

Substrate*	Total SCFA (µmol m/L)	C2:C3	Branched SCFA (µmol m/L)	NH_3–Nitrogen (mg m/L)	NH3-uptake (mg NH_3 m/L gas)
S	871.6	3.63	15.61	0.37	0.0747
S+F 5.62	1014.0	3.76	17.79	0.37	ND
S+F 11.54	837.2	3.39	17.12	0.35	0.0934
S+Se 10.9	849.1	3.76	12.12	0.36	ND
S+Se 21.8	911.9	3.56	14.30	0.36	0.0618
S+K 3.88	866.3	3.60	13.76	0.37	ND
S+K 7.76	1035.2	3.77	11.82	0.37	ND
SEM	10.11	0.045	1.234	0.056	

* — as in Table 1; SCFA C2:C3 — acetate:propionate; ** — iso-butyrate + iso-valerate; ND — not determined.

rich fractions, as observed in the present study. This reduction in the activity of saponin-rich fractions could be due to non-extraction of all the saponins or to a change of saponin activity during their extraction.

No difference was observed in the SCFA production amongst control and saponin-containing incubations (**Table 5**). A slight decrease in ammonia concentration was observed only with Fenugreek (11.54 mg/ 40 mL) and *Sesbania* (10.9 and 21.8 mg/40 mL buffer) saponin-rich fractions. Wide variations of the effects of saponins on ammonia concentration have been reported in the literature. In a review by Wina et al. (2005), 14 reports indicate no effect of saponins on ammonia while another 17 studies state a negative correlation between saponin and ammonia production. The slight decrease in ammonia concentration might be due to high anti-protozoal activities of Fenugreek and *Sesbania* saponins at their higher concentrations. The lower number of protozoa results in lesser bacterial lysis and therefore lower release of breakdown products of protein. The rate of NH_3-N uptake (an index of the efficiency of microbial protein synthesis) was calculated as the slope of a linear regression between the amount of NH_3-N (in mg) and net gas (mL) (Getachew et al., 1998). The higher slope values on supplementation of saponin-rich fraction from Fenugreek (**Table 5**) suggest increased ammonia uptake by rumen microbes which means that the nitrogen from feed is converted into microbial protein to a greater extent in the presence of these saponins. But this increase in microbial protein was not reflected in the PF values, probably due to the measurement of PF at an inappropriate time (24 h in this study) and erosion of PF differences by microbial lysis (Blümmel et al., 2003).

Saponin-rich fractions changed the microbial population as estimated by the comparative delta Ct method (Denman and McSweeney, 2006). *Sesbania* saponins decreased methanogen population by 78%. Reduced rumen fungal populations (20–60%) and increases in *Fibrobacter succinogenes* (21–45%) and *Ruminococcus flavefaciens* (23–40%) were observed (**Figure 2**). In absolute terms, increases were observed in total bacterial population as indicated by decreased threshold cycle (Ct) values on supplementation of saponin-rich fractions. This effect was expected due to decrease in protozoal numbers since there is no predation of bacteria by protozoa (Matheiu et al., 1996). The increased populations of *F. scuccinogenes* can be attributed to their resistance to saponins as observed by Wang et al. (2000), suggesting that this species has the ability to deglycosylate and therefore inactivate saponins. Vinogradov et al. (2001) also

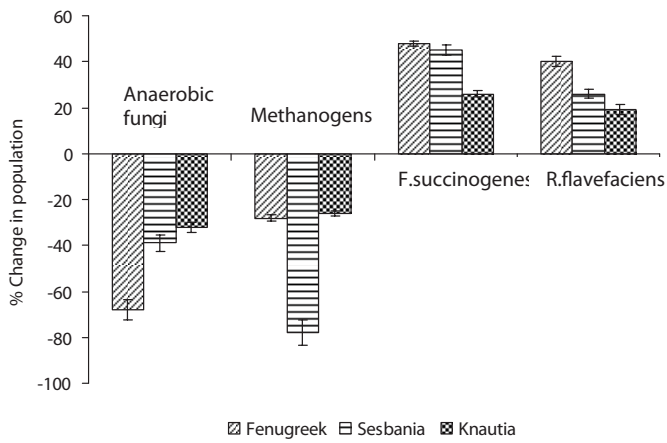

Figure 2. Effect of saponin-rich fractions on percent changes in microbial population.

reported that the presence of 2-aminoethylphosphoric acid and glycolipid in the membrane enhances the membrane stability of *F. succinogenes*. Saponin-rich fractions were inhibitory to ruminal fungi as well. The inhibition of protozoal population also resulted in inhibition of methanogens though the effect was more pronounced for saponin-rich fraction of *Sesbania*. The Fenugreek fraction being more inhibitory to protozoa did not result in higher suppression of methanogens, which again reconfirms the weak association between the protozoal population and methanogen numbers.

Results for the effects of saponin-rich fractions on methanogens and CH4 levels were unexpected. These fractions decreased protozoa numbers and methanogen populations but did not decrease CH_4 production. The association between methanogens and protozoa is not obligatory and there is considerable evidence that different groups of methanogens are not equally associated with ciliate protozoa. A weak relationship between methanogenesis and the methanogen population expressed as a proportion of total anaerobes was observed by Nollett et al. (1998) under both *in vitro* and *in vivo* conditions. In our study, the lack of inhibition of CH_4 production with decreases in methanogens could have been caused by (i) a slow rate of association between protozoa and methanogens due to higher generation time of protozoa as compared with methanogens, (ii) an increased metabolism of methanogenic microbes independent of species remained after addition of saponins, and/or (iii) by changes in composition of the methanogenic community (Machmüller et al., 2003) and their increased efficiency of CH_4 production. The two major groups of methanogens in rumen: methanobacteriaceae (99.1% of total archea associated with protozoa) and free living methanobacteriales (0.05%) differ in their physiological characteristics. Therefore, based on the present results, it is suggested that on inhibition of protozoa, the species belonging to methanobacteriaceae declined with an increase in the number of free-living methanobacteriales. The reduced rate of association of protozoa and methanogens could result in higher interspecies hydrogen transfer between increased population of both hydrogen producing bacteria (*R. flavefaciens* and *F. succinogenes*) and free-living methanobacteriales indicating no effect on CH_4 production.

CONCLUSIONS

Polyphenol or tannin containing plants decreased CH_4 production and, therefore, could be strategically used in diets for decreasing CH_4 emissions from ruminants. Amongst the tannin assays, tannin bioassay (a reflection of tannin activity) was the best predictor of the CH_4 reduction potential of a plant. Total phenols and total tannins were also good predictors of the CH_4 reduction potential. Methane decrease by addition of phenolic acids was relatively small (up to 6.3%), and the effect of phenolic acids on CH_4 reduction depended on the source and concentration applied. The order of simple phenols to decrease CH_4 was: caffeic acid > p-coumaric > ferulic > cinnamic. Hydrolysable tannins had greater ability to decrease CH_4 production and CH_4 production per unit organic matter digested than condensed tannins. The condensed tannins appear to decrease CH_4 more through a reduction in fibre digestion (indirect effect), while hydrolysable tannins appear to act more through inhibition of the growth and/or activity of methanogens and/or hydrogen producing microbes (direct effect).

The saponin-containing plants did not produce substantial reductions in CH_4 production but showed the potential to partition higher proportions of the substrate to production of microbial mass. The saponins tested possessed anti-protozoal activity but did not result in CH_4 inhibition suggesting that the uni-directional relationship between protozoal numbers and methanogenesis, as affected by

saponins, is not obligatory. However, the inhibition of methanogen population led to increases in fibre-degrading bacterial groups.

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Evaluation of *Leucaena leucocephala* and *Ziziphus mauritiana* as Sources of Tannins and their Interference with Nitrogen Utilisation in Goats

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ABSTRACT

Studies were undertaken to identify the roles of tannins in *Leucaena leucocephala* and *Ziziphus mauritiana* on ruminal degradability of sesame meal crude protein (CP) using nylon bags and on nutrient digestibility of nitrogen utilisation in goats. Four diets were used in an *in situ* study using a fistulated bull: S (sesame meal), S+L_1 (sesame meal and *Leucaena leucocephala* [25% of the diet]), S+L_2 (sesame meal and *Leucaena leucocephala* [50% of the diet]) and S+Z (sesame meal and *Ziziphus mauritiana* [50% of the diet]). Crude protein disappearance of S+L_1, S+L_2 and S+Z were significantly lower ($P < 0.001$) than that of S, indicating that supplementation with *Leucaena leucocephala* and *Ziziphus mauritiana* lowered degradation of sesame meal protein, thereby saving more protein. A digestion trial was carried out using four indigenous male goats allocated randomly to four dietary treatments using a 4 × 4 Latin Square design: RS (chopped rice straw and sesame meal), RSL_1 (chopped rice straw, sesame meal and *Leucaena leucocephala* at 25% of ration), RSL_2 (chopped rice straw, sesame meal and *Leucaena leucocephala* at 50% of ration) and RSZ (chopped rice straw, sesame meal and *Ziziphus mauritiana* at 50% of ration). Dry matter (DM) and organic matter (OM) intakes of all diets were relatively similar, but CP intakes of RSL_2, RSL_1 and RS were significantly higher ($P < 0.01$) than that of RSZ. Digestibilities of DM, OM, CP, neutral detergent fibre (NDF) and acid detergent fibre (ADF) for RSZ were significantly lower ($P < 0.01$) than those of other treatments. The proportion of faecal nitrogen:total nitrogen intake (Nf/NI, percentages) for RSZ was significantly higher ($P < 0.01$) than that of other diets while the proportion of nitrogen retention:total nitrogen intake (Nr/NI, percentages) for RSL_1 tended to be higher compared with RS, RSL_2 and RSZ. Supplementing the ration with 25% *Leucaena leucocephala* tended to promote nitrogen retention. The results suggest that tannins in *Leucaena leucocephala* interfere with protein degradation in the rumen and improve nitrogen retention.

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INTRODUCTION

Rice straw is a major feed source for ruminants in many tropical countries including Myanmar (Clark, 1982), especially during dry seasons. Like other fibrous residues, it is a poor quality feed but its nutritional limitations may be overcome by supplementation with concentrates, urea or green forage (Preston and Leng, 1984). Supplementation of rice straw with concentrate improves its utilisation (Trung et al., 1988). Supplementation using by-products may increase intake and/or digestion and/or utilisation of the basal diet by improving the microbial activity required to optimise rumen digestion when ammonia nitrogen in the rumen is adequate (Tin Ngwe et al., 1993).

In Myanmar, sesame meal is one of the common feed supplements for draught cattle and cross-bred dairy cows fed rice straw. However, since sesame meal is highly degradable (88.7%) in the rumen (Sampath and Sivaraman, 1987) several processing treatments (heat, tannins, formaldehyde, etc.) have been used to increase the proportion of dietary protein which is not degraded in the rumen (Chalupa, 1975). While protection of highly degradable feed protein by heat and formaldehyde treatment have already been reported, little information is available about the effect of including the tannins in tree foliages on protein protection.

Conventionally, tree foliages have been fed together with agricultural by-products (mainly crop-residues containing low levels of nitrogen) to enhance rumen microbial fermentation and hence animal productivity (Ni Ni Maw et al., 2002). Tanniferous trees and shrubs are important in animal production because they can provide significant protein supplements (Makkar, 1999). Forages containing tannins include *Leucaena leucocephala, Ziziphus mauritiana, Albizia chinensis, Manihot esculenta,* and *Terminalia oblongata* (Kumar, 1992), and tree legume forages offer a cheap alternative to concentrate diets in ruminant nutrition (Abdulrazak and Ondiek, 1998).

Among tanniferous trees and shrubs, *Leucaena leucocephala* and *Ziziphus mauritiana* are common feedstuffs for goats in Myanmar. *Leucaena leucocephala* is now widespread through most tropical and sub-tropical regions of the world and provides an important source of feed for ruminant livestock (Norton et al., 1994). It is a high quality fodder tree (Liu and Wang, 1998) with a crude protein content of 28.8% in Myanmar, and therefore with considerable potential to replace commercial protein feed resources in rations or be used as a supplementary feed (Abdulrazak and Ondiek, 1998).

Tannins are generally regarded as inhibitory to the growth of microorganisms but the mechanisms involved are poorly understood. Petroleum ether, chloroform, methanol, etc., are used to extract tan-

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nins sequentially from plant materials (Smith et al., 2001). However, detannification methods are very expensive and are not applicable in developing countries including Myanmar. Since tannins are widely distributed in tropical feedstuffs, if tree forages containing tannins were used to reduce protein degradability, savings on costs of providing dietary protein for ruminant production systems in developing countries may be expected.

Against this background, an experiment was undertaken to investigate the effect of tannins in *Leucaena leucocephala* and *Ziziphus mauritiana* on protein degradation of sesame meal using the nylon bag method in a fistulated bull. Another study examined the effect of *Leucaena leucocephala* and *Ziziphus mauritiana* on nutrient digestibility and nitrogen utilisation in goats.

MATERIALS AND METHODS

Experiment 1

Animals, Feed and Experimental Design

A fistulated bull (270 kg body weight [BWt]) was used to investigate the effect of *Leucaena leucocephala* and *Ziziphus mauritiana*, as sources of tannin on protein degradation. Four diets were used:

- Sesame meal (S)
- Sesame meal + *Leucaena leucocephala* 25% of the diet (S+L_1)
- Sesame meal + *Leucaena leucocephala* 50% of the diet (S+L_2)
- Sesame meal + *Ziziphus mauritiana* 50% of the diet (S+Z)

Before the study commenced a maintenance ration containing rice straw (4.75 kg), rice bran (220 g) and sesame meal (440 g) was fed 14 d. The experimental period for each diet lasted for 2 d. The experimental period lasted for 8 d.

Measurements

Dry matter, OM and protein degradation of each diet was measured by the nylon bag method (Orskov and McDonald, 1979). The sesame meal, *Leucaena leucocephala* and *Ziziphus mauritiana* were ground to pass through a 2 mm sieve. (Before incubation, the bags were dried in a hot air oven at 100°C for 4 h to a constant weight). Bags (13.5 cm × 8.5 cm; pore size 50 µm) were used in this study, with eight incubation times for each diet, and three nylon bags being introduced into the rumen for each incubation time. Thus, twenty four bags were required to complete incubation of each diet. About 5 g of ground sample were weighed into the bag which was closed with a plastic tie and tied with plastic string. The bags were then suspended in the rumen by tying the string to a bamboo stick placed outside the cannula. Bags were incubated in the rumen for 0.5, 1, 3, 6, 12, 18, 24 or 48 h., withdrawn and washed immediately with cold water for about 1 h under running tap water while rubbing gently between the thumb and fingers until the water was clear. They were then dried under sunlight for 5 h., and finally to constant weight at 60°C for 48 h in a hot air oven. After spreading on a table and allowed to equilibrate with the room temperature for 48 h the bags were weighed. Suitable amounts of residues were used to analyse for DM constituents.

Chemical Analysis

Dried residues were analysed for DM and OM as described by AOAC (1970). Nitrogen was determined using the Kjeldahl method (Foss 2020 digestor and Foss 2100 Kjeltec distillation unit), and CP calculated as 6.25×N (AOAC, 1970). All chemical analyses were carried out at the laboratory of Department of Physiology and Biochemistry, University of Veterinary Science, Yezin.

Statistical Analysis

The experimental results were subjected to one-way analysis of variance using ANOVA.

Experiment 2

Experimental Animals, their Management and Experimental Design

Four indigenous male goats aged about 5–7 months and BWt ranging from 19–29 kg were used to evaluate the effect of four dietary treatments on nutrient digestibility and nitrogen utilisation. Before starting the experiment, the goats were dewormed with Ivomec. During the study period, the goats were housed in individual metabolic stalls made of wood and an iron sieve and subjected to similar management practices. The feeding was done once daily at 09:00 h and the animals were given free access to water.

The experimental period for each dietary regime lasted 17 d. The goats were adapted to the test diet for the first 14 d and on the last 3 d of the experimental period samples of faeces and urine were collected from each goat for determination of nitrogen retention. The goats were randomly allocated to four dietary treatments using 4×4 Latin Square Design (**Table 1**). The dietary treatments were as follows:

- Chopped rice straw and sesame meal (RS);
- Chopped rice straw, sesame meal and *Leucaena leucocephala* 25% of ration (RSL_1);
- Chopped rice straw, sesame meal and *Leucaena leucocephala* 50% of ration (RSL_2);
- Chopped rice straw, sesame meal and *Ziziphus mauritiana* 50% of ration (RSZ)

All dietary treatments were adjusted to be isonitrogenous at the feeding level by calculating the crude protein content of each feedstuff contained in the diet. Dietary treatments were adjusted weekly by the supplement to levels of CP not less than 18% of the total diet.

Leucaena leucocephala and *Ziziphus mauritiana* (leaves and petioles) were harvested from the mature parts of the plant, collected, air dried and stored. The rice straws were also sun dried and chopped. Each diet was fed to goats at the level of 3.5% of BWt (as-fed basis). All feedstuffs used in the experiment were maintained as much as possible at a uniform composition throughout the trial period.

Amounts of all feedstuffs fed and refused were recorded daily for calculating intake levels. At the beginning of each period, animals were weighed before the morning feeding.

Measurements

Digestion trials were carried out by the conventional collection method. During collection periods samples of rice straw offered and refused and of sesame meal, *Leucaena leucocephala* and *Ziziphus mauritiana* were collected daily for chemical analysis. Residues were removed, weighed and sampled before the morning feeding.

Faeces voided and urine excreted were recorded daily during collection periods. The faeces from each goat were also weighed, sampled and put into a plastic bottle. After three consecutive d, 15% of faecal samples were dried under sunlight until constant weight was obtained. Urine was also measured, 10% sampled and stored in a refrigerator before chemical analysis. Five mL of 15% sulphuric acid was added to 200 g faecal samples before drying under sunlight and also added to the urine bucket before collection as preservative.

Table 1. Chemical composition (%) of diets incubated in the rumen of a fistulated bull.

Description	S	S+L_1	S+L_2	S+Z
DM[1]	91.8	92.2	92.0	92.3
OM	86.8	87.4	89.2	90.1
CP	40.8	34.4	33.4	25.7

S = sesame meal; S+L_1 = sesame meal + *Leucaena leucocephala* at 25% of diet; S+L_2 = sesame meal + *Leucaena leucocephala* at 50% of diet; S+Z = sesame meal + *Ziziphus mauritiana* at 50% of diet; [1] All values except DM are on DM basis.

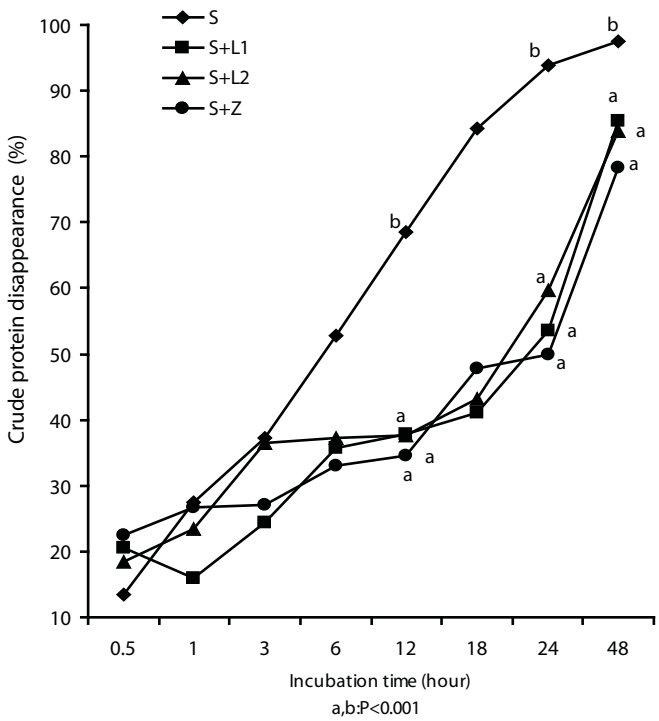

Figure 1. Disappearance of crude protein (%) of sesame meal supplemented with *Leucaena leucocephala* and *Ziziphus mauritiana* in the rumen of a fistulated bull

Chemical analysis

Ground samples of feed offered and of orts and faeces were analysed for dry matter (DM) and organic matter (OM) as described by AOAC (1970), and for neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL) and acid detergent insoluble nitrogen (ADIN) by the methods of Goering and Van Soest (1970). Faeces and urine were analysed for nitrogen using the Kjeldahl method (Foss 2020 digestor and Foss 2100 Kjeltec distillation unit) and crude protein (CP) was calculated as 6.25×N (AOAC, 1970). Estimates of tannin in *Leucaena leucocephala* and *Ziziphus mauritiana* were made by the sequential extraction of *Leucaena leucocephala* and *Ziziphus mauritiana* with acid detergent followed by neutral detergent.

Statistical analysis

Data were subjected to statistical analysis of ANOVA using Latin Square Design and the significance of differences between treatment means was compared by Duncan's Multiple Range Test (DMRT) (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Experiment 1

Chemical Composition and CP Disappearance

Table 1 and **Figure 1** show the chemical composition of the diets and disappearance of CP from them. Dry matter and OM levels were similar in all diets, but CP content was highest in sesame meal and lowest where sesame meal was supplemented with *Ziziphus mauritiana* at 50% of in the diet.

The CP disappearances of all diets were relatively similar at 3 h incubation, but CP disappearances of S+L_1, S+L_2 and S+Z were lower than that of S at all times thereafter, significantly so ($P < 0.001$) after 12 h, 24 h and 48 h of incubation. This would indicate that supplementation with *Leucaena leucocephala* and *Ziziphus mauritiana* lowered the degradation of the sesame meal protein, and that this might be due to the presence of tannins in these foliages. This is consistent with the observation of Khin San Mu (2002) who reported that CP disappearance was reduced by incubating a concentrate with tamarind seed husk as a source of tannins to growing female calves. Also, Sampath and Sivaraman (1987) showed that the disappearances of DM and CP were reduced by incubating heat-treated sesame meal in the rumen, and Chalupa (1975) reported that several processing treatments (heat, tannin, formaldehyde, etc.) increased the proportion of dietary protein which is not degraded in the rumen.

Degradation Constants

The degradation constants of DM, OM and CP for S, S+L_1, S+L_2 and S+ Z are given in **Table 2**. Degradation rates (c) of DM, OM and CP for all diets were relatively similar but the values of b and a+b for S were found to be higher than those of S+L_1, S+L_2 and S+Z. The higher degradation constants of S would also indicate that tannins in these tree foliages protect the protein from degradation. The lower CP disappearance and degradation constants of the treatments that included *Leucaena leucocephala* and *Ziziphus mauritiana* suggest again that tannins contained in these tree foliages interfered with protein degradation in the rumen of the fistulated bull.

Experiment 2

Chemical Composition of Feedstuffs

The chemical composition of the feedstuffs are presented in **Table 3** (all values except DM expressed on DM basis). The CP content of *Leucaena leucocephala* used in this experiment was 28.8% which was higher than reported earlier (Smith, 1992; Wheeler et al., 1994; Ni Ni Maw et al., 2002; Lwin Naing Oo, 2002; Met Aung, 2002; Moe Moe Khaing, 2003), but similar to values reported by others (Jones, 1979; Abdulrazak and Ondiek, 1998; Aregheore and Yahaya, 2001).

The CP content of *Ziziphus mauritiana* was 13.9% which was similar to the value of 14% reported by Nath et al. (1969) and lower than the 18.39% recorded by Ni Ni Maw et al. (2002). The NDF and ADF values of *Ziziphus mauritiana* used were 35.8% and 25%

Table 2. Degradation constants of diets in the rumen of a fistulated bull.

DM [1)]	Description			
	S	S+L_1	S+L_2	S+Z
a, %	5.0	16.0	12.0	2.0
b, %	88.0	54.0	60.0	56.0
c, h^{-1}	0.085	0.084	0.085	0.090
a+b, %	93.0	70.0	72.0	58.0
OM [1)]				
a, %	7.0	10.0	9.0	1.0
b, %	87.0	58.0	61.0	57.0
c, h^{-1}	0.088	0.088	0.090	0.088
a+b, %	94.0	68.0	70.0	58.0
CP [1)]				
a, %	5.0	1.0	9.0	10.0
b, %	93.0	63.0	58.0	51.0
c, h^{-1}	0.091	0.087	0.088	0.087
a+b, %	98.0	64.0	67.0	61.0

a — rapidly degradable fraction; b — slowly degradable fraction; a+b — potentially degradable fraction; c — rate of degradation; 1 as described in Table 1.

Exponential equation: $P=A+B\ (1-e^{-kdt})$.

Table 3. Chemical composition (%) of feedstuffs.

Description	Rice straw	Sesame meal	Leucaena leucocephala	Ziziphus mauritiana
DM	87.7	86.0	89.3	90.6
OM	81.4	84.8	91.0	92.2
CP	5.7	40.6	28.8	13.9
EE	1.7	10.5	8.2	4.2
NDF	68.3	17.4	22.7	35.8
ADF	41.2	9.6	13.7	25.0
ADL	—	—	3.7	8.3
Tannin	—	—	2.0	4.8
Silica	—	—	0.3	0.5

EE — ether extract; ADL— acid detergent lignin.

respectively, higher than the 30.0% and 19.78% reported by Ni Ni Maw et al. (2002).

The difference of 2.0% between ADF and NDF in the sequential analysis of *Leucaena leucocephala* for tannins was in agreement with 1.4 - 7.9% reported by Wheeler et al. (1994), while the difference of 4.8% between ADF and NDF in the sequential analysis of *Ziziphus mauritiana* for tannins agreed closely with the 5.3% reported by Bhatia et al. (1987).

The differences in chemical composition between *Leucaena leucocephala* and *Ziziphus mauritiana* used in this experiment and from other observations likely reflected differences between parts of the plants used, their maturity, soil, weather and environmental characteristics.

Digestibility coefficients

DM digestibility of RSL_2 was significantly higher ($P < 0.01$) than that of RS but CP digestibility was significantly lower ($P < 0.01$). This might be due to the greater amount of *Leucaena leucocephala* in the ration which resulted in an increased amount of the tannin. The OM digestibilities of RSL_1 and RSL_2 were not significantly different from that of RS (**Table 6**).

Although all dietary treatments were adjusted to be isonitrogenous at the feeding level, a significant decrease was observed in CP intake of the RSZ diet (**Table 5**). This was due to underestimation of the CP content of *Leucaena leucocephala* and overestimation of CP content of *Ziziphus mauritiana*. Therefore, it could be assumed that all nutrient digestibilities were significantly ($P < 0.01$) reduced (**Table 6**) due to decreased CP intake in RSZ diet (**Table 5**). It is generally agreed that intake and digestion by ruminants is limited when the roughage contains less than 7% CP (Doyle, 1987). However, the CP content of RSZ constituted 15.7% of the diet, well above the CP content that may have limited nutrient digestibility. Therefore, the reduced nutrient digestibility might be due to ADIN content in *Ziziphus mauritiana* (**Table 4**).

Table 4. Content of acid detergent insoluble nitrogen in *Leucaena leucocephala* and *Ziziphus mauritiana*.

Description	Leucaena leucocephala	Ziziphus mauritiana
Total N %	4.6	2.2
ADIN %	1.7	1.5
ADIN/total N, %	37.0	68.0

N: = nitrogen; ADIN: = acid detergent insoluble nitrogen.

Table 5. Nutrient intakes (g/kg $^{0.75}$/d).

Description[1)]	RS	RSL_1	RSL_2	RSZ
DM I	59.3	55.5	63.4	63.2
OMI	49.2	47.2	55.1	55.4
CPI	12.5	13.0	15.2	10.2

[1] DMI — dry matter intake; OMI — organic matter intake; CPI — crude protein intake.

Table 6. Digestibility of nutrients (%).

Description	RS	RSL_1	RSL_2	RSZ	SEM
DM digestibility	59.0^{B}	61.7^{Aa}	62.0^{Aa}	55.8^{C}	0.591
OM digestibility	66.6^{Aa}	67.7^{Aa}	66.8^{Aa}	60.2^{B}	0.533
CP digestibility	82.5^{Aa}	80.5^{ABa}	75.5^{Bb}	57.9^{C}	1.390
NDF digestibility	59.8^{Aa}	55.3^{ABb}	52.1^{Bb}	42.3^{C}	1.107
ADF digestibility	56.8^{Aa}	47.9^{ABb}	41.9^{Bb}	26.1^{C}	2.479

Significant differences between means over the whole experiment are indicated by dissimilar superscripts: $^{A,B,C}P < 0.01$ and $^{a,b}P < 0.05$.

Table 7. Nitrogen utilisation by goats fed different diets.

Description	RS	RSL_1	RSL_2	RSZ	SEM
Total NI, g/d	20.1^{Aa}	20.5^{Aa}	24.5^{B}	16.6^{C}	0.649
Faecal N, g/d	3.5^{Bc}	4.0^{Bc}	5.9^{Ab}	6.9^{Aa}	0.231
Urinary N, g/d	11.8^{ABa}	11.0^{ABa}	13.4^{Aa}	6.5^{Bb}	1.331
N retention, g/d	4.8	5.5	5.1	3.2	—
Nf/NI, %	17.5^{Aa}	19.5^{ABa}	24.5^{Bb}	42.1^{C}	1.327
Nu/NI, %	59.6	54.5	55.0	40.3	—
Nr/NI, %	22.9	26.0	20.5	17.6	
Nf/DNI, %	21.3^{Aa}	24.2^{Aa}	32.7^{Aa}	73.6^{B}	3.622
Nu/DNI, %	72.0	68.0	73.0	70.3	—
Nr/DNI, %	28.0	32.0	27.0	29.7	—

NI: = nitrogen intake; Nf: = faecal nitrogen; Nu: = urinary nitrogen; Nr: = nitrogen retention; DNI: = digestible nitrogen intake.

Significant differences between treatment means over the whole experiment indicated by dissimilar superscript: $^{A,B,C}P < 0.01$; $^{a,b,c}P < 0.05$

The NDF and ADF digestibilities of RSL_2 and RSZ were significantly lower ($P < 0.01$) than that of RS. Likewise, NDF and ADF digestibilities for RSL_1 were significantly lower ($P < 0.05$) than for RS, which is in accord with the report of Reed et al. (1990) that tannins have a negative effect on fibre digestibility (**Table 6**).

Nitrogen utilisation

Total nitrogen intakes of RS, RSL_1, RSL_2 and RSZ were 20.1, 20.5, 24.5 and 16.6 g/d, respectively (**Table 7)**. Total nitrogen intake of RSL_2 was significantly higher ($P < 0.01$) than those of other diets while total nitrogen intake of RSZ was significantly lower ($P < 0.01$) than those of other diets.

The proportion of faecal nitrogen to total nitrogen intake (Nf/NI, %) of RSZ was significantly higher ($P < 0.01$) than RSL_2, RSL_1 and RS. This might be due to the high content of acid detergent insoluble nitrogen (ADIN) in *Ziziphus mauritiana* (68% of total nitrogen) compared with that of *Leucaena leucocephala* (37% of total nitrogen) (**Table 4**). This is in agreement with the report of Nakamura et al. (1994) who showed that ADIN and nitrogen digestibility were correlated ($r^2 = 0.66$) and that ADIN was completely indigestible leading to underestimation of nitrogen digestibility.

The proportions of urinary nitrogen to total nitrogen intake (Nu/NI, %) of RS, RSL_1, RSL_2 and RSZ were not significantly different ($P > 0.05$). The Nu/NI, % of RSZ (40.3%) was numerically lower than those of RSL_1, RSL_2 and RS (59.6, 54.5 and 55.0% respectively), which might be due to the high content of tannins in the RSZ diet. However, the proportion of nitrogen retention to nitrogen intake (Nr/NI, %) of RSZ tended to decrease compared with other treatments which might be explained by an inadequate ammonia nitrogen concentration in the rumen for nitrogen utilisation because of the higher content of ADIN in *Ziziphus mauritiana* (68% total nitro-

gen) (**Table 4**). This was confirmed by the higher faecal excretion of nitrogen in RSZ diet (**Table 7**).

The proportion of nitrogen retention to nitrogen intake (Nr/NI, %) of RSL_2 was numerically lower than that of RSL_1, although the amount of tannin included in the RSL_2 was doubkle that of RSL_1. This would indicate an excessive amount of *Leucaena leucocephala* in the ration of RSL_2.

Although NDF and ADF digestibilities of RSL_1 were significantly lower than for RS ($P < 0.05$), the proportion of nitrogen retained to total nitrogen intake (Nr/NI, %) with the RSL_1 diet tended to be higher compared with other diets. Moreover, OM digestibility (**Table 6**) and the nutritive value of RSL_1 diet were also higher (**Table 7**). This observation is in agreement Dutta et al. (1999) who reported that the intakes ($g/kgW^{0.75}$) of DCP, TDN and the nitrogen balances of goats were significantly higher ($P < 0.05$) when *Leucaena* was fed. Norton (1994) also reported that nitrogen balance was apparently improved in animals that are fed low levels of tannins, although digestibility of forage fibre may be lowered.

No significant difference was observed in Nu/DNI, % and Nr/DNI, % among treatments suggesting that post ruminal nitrogen metabolism in goats fed on all diets was relatively similar.

Bhatta et al. (2000) reported decreased nitrogen excretion in urine with subsequent increased nitrogen retention in crossbred dairy cows fed with tamarind (*Tamarindus indica*) seed husks. Similar findings have been reported by Karda et al. (1998) in sheep fed with *Leucaena leucocephala,* by Barry et al. (1986) in sheep fed with *Lotus* and Pritchard et al. (1992) in sheep fed with *Acacia*, by Tin Ngwe (2003) in sheep supplemented with lablab bean (*Dlichos lablab*) husk, and by Lwin Naing Oo (2002) in goats supplemented with *Leucaena leucocephala*. In all these cases, the higher nitrogen retention was attributed to the tannin content of these legumes causing a reduction in protein fermentation in the rumen and an improvement in the efficiency of nitrogen utilisation.

Although tannins are regarded as antinutritional factors, certain types of tannins at low concentration are known to alter rumen fermentation of carbohydrates and protein (Barry and Duncan, 1984) and microbial protein synthesis (Makkar et al. 1995) to the benefit of ruminants. *Leucaena leucocephala* fed at the level of 25% of the ration used in the present experiment may have played roles favourable for nitrogen utilisation, while the higher level of ADIN content in *Ziziphus mauritiana* might have been a drawback as a ruminant feed.

CONCLUSIONS

Compared with other diets, the proportion of nitrogen retained to total nitrogen intake tended to be higher when the diet was supplemented with 25% of *Leucaena leucocephala*. The level of 25% of *Leucaena leucocephala* in the ration was found to be a good supplement to rice straw in terms of promoting nitrogen utilisation without reducing DM and OM digestibilities and could therefore be used as a source of tannins for protecting protein provided as concentrate from degradation in the rumen.

Ziziphus mauritiana reduced both fibre and protein digestibility because of its higher ADIN content and might therefore not be so suitable as a feed for goats.

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Application of Near Infrared Spectroscopy to Improve Animal Production in Developing Countries

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ABSTRACT

Near infrared (NIR) spectroscopy is an analytical technique measuring light absorption in the 780–2 500 nm region which is closely related to important chemical bonds (OH, NH and CH). NIR can be used to measure many nutritionally important constituents of concentrate and forage feeds, and from NIR spectra of faeces (i.e. dung) many constituents of the diet of grazing livestock. NIR depends on the development, in representative sets of samples, of mathematical relationships (calibration equations) between spectra and the constituents or attributes measured by conventional chemistry, and then application of these calibrations to estimate the constituents in unknown samples. These NIR calibration equations tend to be specific to the circumstances of the data used for their development. Application of NIR technology to livestock nutrition allows rapid, routine and economical analysis of feedstuffs and ingredients for compounded diets or supplements, thus improving stockfeed manufacture. Also NIR analysis of faeces allows estimation of the diet selected by grazing livestock and in small holder farming systems; this is not possible with any other technology. However, NIR instrumentation requires substantial capital investment, and considerable technical skills are required to develop and maintain calibration equations. Application of NIR technology allows established knowledge of the science of animal nutrition to be readily and objectively applied to improve productivity and cost-effectiveness of livestock production systems. Widespread use of this technology in developing countries would greatly improve quality control in manufacturing livestock feeds and application of existing nutritional knowledge to increase productivity and cost-effectiveness of livestock production.

Key words: *animal nutrition, ruminant nutrition, grazing animals, stockfeed.*

INTRODUCTION

Near infrared (NIR) spectroscopy is an analytical technique involving the measurement of absorption of electromagnetic radiation in the NIR region (780–2500 nm) of the spectrum of light (Osborne et al., 1993; Williams and Norris, 2001; Roberts et al., 2004a). An overview of the development and variety of NIR technology, and application in a wide range of industries and situations has been given by McClure (2003). Because absorption of NIR radiation is responsive to some chemical bonds (predominantly OH, NH and CH) the technique can be used to analyse many organic constituents of plant and animal tissues. During the last two decades NIR spectroscopy has developed for widespread, routine use in the food and agricultural industries in most developed countries, particularly for attributes of grains, forages, dairy products, and many other foods. Such measurements are commonly used for quality control, automated process control and valuation along marketing chains (Roberts et al., 2004a). It is also used extensively in the pharmaceutical, petroleum, textiles and other manufacturing industries and medical diagnostics (Flinn, 2007). This widespread development and application of NIR spectroscopy (NIRS) has been associated with improved sensitivity, ruggedness and reduced costs of instrumentation, and the parallel development of desktop computing essential for the spectral data analysis. NIR is only one of a suite of spectroscopy technologies developed in recent decades which may provide rapid and economical measurement in a wide variety of applications, but NIR has been most widely developed in the agricultural and other land-use sciences.

In the context of animal nutrition in developed countries, NIR spectroscopy is widely used for rapid and economical measurement of feedstuff ingredients and of forages for both monogastric and ruminant animals. A wide range of nutritionally important constituents (e.g. proteins, fibres, starches and sugars) and related functional properties (e.g. digestibility and voluntary intake by the animal) of feedstuffs and forages can be measured from their absorption characteristics (Givens and Deaville, 1999; Roberts et al., 2004b). It has been described as 'the most practicable and exciting analytical technique to hit the agricultural and food industries since Johann Kjeldahl introduced the Kjeldahl test' (Williams and Norris, 2001), and 'in the context of livestock industries in developed countries NIRS has revolutionised the analysis and nutritional evaluation of animal feeds by providing a rapid means of examination' (Givens and Deaville, 1999).

NIR analysis depends on the development of mathematical relationships (calibration equations) between absorbances at various wavelengths in the NIR region and composition of reference samples determined by conventional procedures such as wet chemistry. The NIR absorbance spectra of unknown samples are then used with these calibration equations to estimate constituents and functional properties. NIR spectroscopy allows rapid and economical analysis with minimal sample preparation and without the generation of wastes. Although conventional laboratories are still needed to devel-

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op and adapt general calibrations for local conditions and maintain NIR calibration equations, the number of samples requiring conventional analytical procedures can be drastically reduced and there is often opportunity to centralise laboratories. Furthermore some nutritional attributes cannot be routinely measured by any other procedure. Some classes of modern NIR instrumentation are designed to be rugged, reliable and portable, allowing use of the technology away from central laboratories and in the field, and with minimal training and technical support. For example such technology has been applied in the grains and horticulture industries using portable or handheld NIR spectrometers to provide real-time field measurement of maturity and quality of the crop.

In addition to direct measurement of feedstuffs, NIR spectroscopy of faeces (i.e. the dung) of ruminants has been developed to estimate many nutritional attributes of forage and forage-concentrate diets (Coates, 2004; Landau et al., 2006; Dixon and Coates, 2009). Prediction of diet attributes from the spectra of faeces is possible because there is sufficient information about the diet in the NIR spectra of faeces, despite the changes in chemical composition which occur during digestion in the gastrointestinal tract. However, because some constituents of the diet (e.g. the readily digested constituents) do not appear or appear in only low concentrations in faeces, the range of dietary attributes which can be measured from faecal spectra is more limited than the range which can be measured from the spectra of feeds. Nevertheless, faecal samples have the major advantages that they are usually easy to obtain and, because they represent the diet actually ingested, it is not necessary to obtain additional information on the proportions of the various dietary components which are actually selected and ingested by the animal.

Apart from the measurement of the nutritional attributes of feedstuffs and the diet, NIR spectroscopy also can be used to measure many aspects of animal physiology and health such as reproductive status, reproductive and stress hormones, parasite burden, mastitis and a variety of metabolites. It can also evaluate many quality attributes of animal products such as milk and meat (Shepherd and Walsh, 2007; Dixon and Coates, 2009). However, NIR spectroscopy is generally not suitable for analysis of minerals, and concentrations of organic constituents generally have to be substantial (e.g. > 1%) for this technology to be applied.

ANALYSIS OF GRAINS, CONCENTRATE FEEDSTUFFS AND COMPOUNDED FEEDS FROM NIR SPECTRA OF FEED

NIR has been developed to measure many constituents and functional properties of numerous species and cultivars of grains. There is a vast literature, both public and in-house to laboratories, reporting such developments (e.g. Williams and Norris, 2001; Roberts et al., 2004b). In addition, numerous studies have developed quantitative NIR analyses for other materials commonly used as ingredients for compounded feedstuffs (e.g. by-products of food processing, protein meals) for both monogastric and ruminant livestock. A number of studies (e.g. de Boever et al., 1995; Aufrere et al., 1996; Mentink et al., 2006; **Table 1**) have shown that NIR calibrations of sufficient reliability and accuracy for most animal nutrition purposes can be

Table 1. Example of the calibration equation errors associated with NIRS analysis of compounded feedstuffs. The feedstuff mixtures were based on commonly available concentrates such as cereal grains, legume grains, protein meals and by-products of food processing (n = 433). (Perez-Martin et al., 2004).

Constituent	Actual content (lab reference value)			Predicted content		
	Mean	Minimum	Maximum	R^2	SECV	RPD
Moisture (g/kg)	103	60	136	0.85	5.4	2.6
Crude protein (g/kg)	184	119	333	0.98	5.0	7.2
Fat (g/kg)	54	16	168	0.95	4.7	4.6
Crude fibre (g/kg)	72	17	253	0.98	4.6	8.1
Ash (g/kg)	85	39	172	0.90	6.1	3.1

SECV — standard error of cross validation; RPD — ratio of the standard deviation of the actual reference data to the SECV (Williams 2001).

Table 2. Example of the calibration equation errors associated with NIR analysis of forage. Samples were obtained over four years from a heterogeneous and botanically complex semi-natural grassland in a temperate environment. The spectra of samples were measured using a scanning monochromator and calibrations calculated following a second derivative transformation of the absorbance data (Garcia-Ciudad et al., 1993).

Constituent	Actual content (lab reference values) Calibration (n = 97)			Predicted content Validation (n = 140)		
	Mean	Minimum	Maximum	R^2	SEP	RPD
Crude protein (g/kg)	99	43	181	0.90	5.8	3.1
Neutral detergent fibre(g/kg)	509	358	755	0.86	2.4	2.6
Acid detergent fibre (g/kg)	334	250	415	0.76	14.2	2.2
Lignin (g/kg)	47	25	99	0.88	4.5	3.3
Cellulose (g/kg)	284	212	345	0.74	12.7	2.1

SEP — standard error of performance; RPD — ratio of the standard deviation of the actual reference data to the SEP (Williams, 2001).

developed for the major nutritionally important constituents of mixed compounded feeds. Although NIR is generally considered not to be suitable for minerals or constituents present at less than about 10 g/kg, in the study summarised in **Table 1** calibration equations were developed for ingredients such as dicalcium phosphate, sodium bicarbonate and organic acids used as preservatives. A further observation in this latter study and others was that NIR spectra can be used to correctly identify many of the ingredients used to prepare the mixed feed. For example, a prohibited ingredient such as meat and bone meal could be easily and unequivocally identified.

ANALYSIS OF FORAGES FROM NIR SPECTRA

Numerous studies have examined NIRS for measurement of the composition and functional aspects of forages, and this application of the technology has been comprehensively reviewed (e.g. Givens and Deaville, 1999; Roberts et al., 2004b; Andres et al., 2005). Accurate and reliable calibration equations have been developed to predict composition including protein and related N compounds, various carbohydrates, components of fibre (crude fibre, neutral detergent fibre, acid detergent fibre, lignin), lipids, the rate and extent of rumen and entire tract digestion of N and carbohydrate fractions, digestibility of organic matter and dry matter, and antinutritional factors such as tannins and alkaloids. An example is given in **Table 2**. Predictions of digestibility of organic matter or dry matter (DM) have usually been more accurate and reliable with NIRS than with conventional laboratory approaches based on *in vitro* digestibility or via correlations with forage components such as acid detergent fibre or lignin (**Table 3**; Givens et al., 1992; de Boever et al., 1996; Andres et al., 2005). A number of studies have shown that NIR can be used to estimate the major botanical and morphological (e.g. leaf-stem) components of mixed forage material, albeit often with 'lumping' of minority components or of similar species (e.g. grasses).

Studies have examined the development of NIR calibrations to measure constituents and attributes of fresh forages and silages of high moisture content to avoid difficulties of loss of some constituents during drying and to enhance rapid measurement and field analysis. NIR analysis of high moisture materials is generally more difficult, and associated with much larger error, than measurement of dried and ground forages. This is a general constraint in NIR spectroscopy since water has strong absorptive properties which often obscure the spectral characteristics associated with other constituents. Nevertheless, calibrations have been developed with comparable prediction error to analysis of dried samples (Givens and Deaville, 1999; Park et al., 1999; Cozzolino et al., 2006).

Voluntary intake of forage by ruminants is another functional attribute of forages which in many studies has been predicted more satisfactorily from the NIR spectra of the forage than from any of the chemical constituents examined (Lippke and Barton, 1988; Givens and Deaville, 1999; Deaville and Flinn, 2000). As these authors discuss, this may well be because NIR measures numerous aspects of the chemical properties of the forage rather than any single constituent or group of constituents. The standard error of performance (SEP) of calibrations for voluntary intake of forage derived from the NIR spectra of the forage have usually been in the range 5–10 g DM/kg $W^{0.75}$.d. SEP values have usually been higher for high moisture materials such as silages than for dried forages such as hays.

ANALYSIS OF ANIMAL DIETS FROM NIR SPECTRA OF THE FAECES

Since it is usually vastly easier to obtain representative samples of faeces than of the diet ingested by grazing herbivores, the NIR spectra of faeces has been examined *in lieu* of the spectra of the diet to predict dietary attributes of grazing animals. Faecal NIR analysis has generally used the instrumentation, sample processing and chemometrics established for forage analysis. The NIR spectra of faeces have been used in two fundamental ways to provide information about the animal and its diet.

In the first approach NIR has been used to measure the concentrations of various constituents of faeces, the advantages of NIR (relative to conventional chemical analysis) being associated with cost, convenience and timeliness, and reduced handling of unpleasant samples. The concentrations of faecal constituents have then been used directly to provide information such as high moisture or fat concentrations indicating digestive abnormality, or have been used with assumptions from other experimentation (e.g. that digestion of condensed tannins or alkanes or the digesta marker polyethylene glycol was negligible, or that there are relationships between the concentration of N in faeces and diet digestibility) to provide information about the diet. Unfortunately the relationships between faecal N concentration and diet constituents can vary widely with the pasture system and between years (Corbett, 1978; Wang et al., 2009). These relationships may be applicable in some specific circumstances, but

Table 3. An example of the errors associated with estimation of organic matter digestibility *in vivo* (g/kg) of silages using NIR, or by *in vitro* disappearance using rumen fluid, pepsin-cellulase, or disappearance *in sacco* from nylon bags suspended in the rumen, or relationships with acid detergent or lignin content of the forage. Relationships were developed with one population (n = 122) and tested for an external population (n = 48) (after Barber et al., 1990).

Method	Regression between observed & predicted values (n = 122)		Regression between observed & predicted values in an external population (n = 48)			
	R^2	RSD	R^2	SEP	Slope	Bias
NIR calibration	0.85	-[a]	0.76	26	0.93	-8
In vitro rumen fluid	0.74	0.32	0.64	36	0.89	-19
In vitro pepsin-cellulase	0.55	0.42	0.40	47	0.71	23
In sacco disappearance	0.68	0.36	-	-	-	-
Acid detergent fibre	0.34	0.51	0.14	53	0.48	12
Lignin	0.52	0.44	0.20	51	0.52	-6

a — no residual standard deviation (RSD) value given, but standard error of calibration = 0.25.

OMD — organic matter digestibility; SEP — standard error of performance.

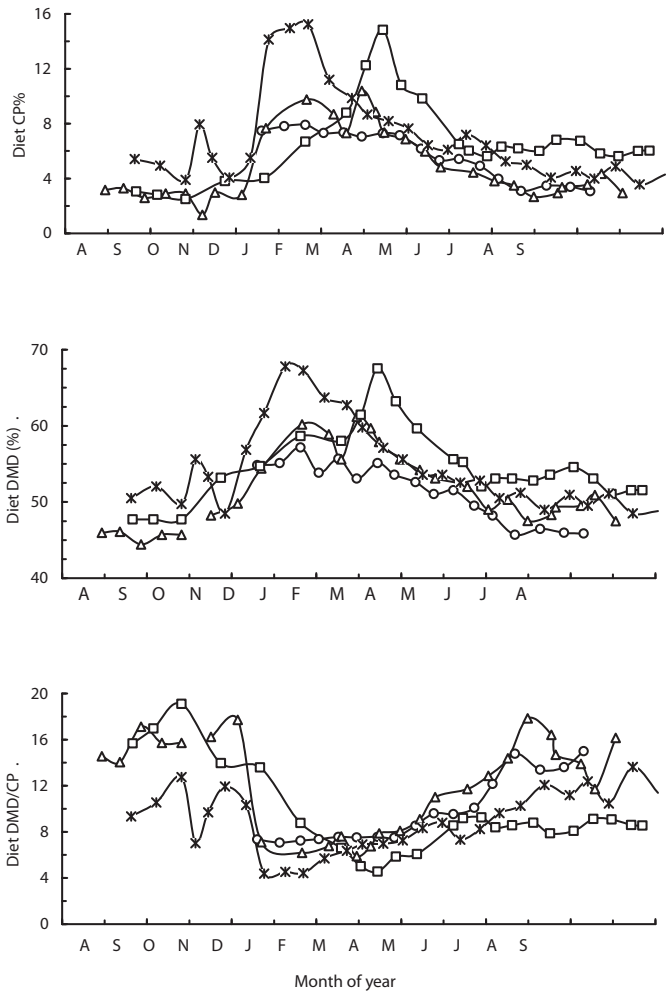

Figure 1. Dietary crude protein (CP; %) (A), dietary dry matter digestibility (DMD; %) (B), and the ratio of dietary DMD to CP (DMD/CP) in the diet (C) estimated from NIR spectra of faeces sampled at fortnightly intervals from four consecutive groups of *Bos indicus x Bos taurus* breeder cows grazing speargrass native pasture in a seasonally dry tropical environment at Millaroo, north Queensland, Australia during four annual cycles (2000-2003).
○ = Group 1; Δ = Group 2; □ = Group 3; x = Group 4 (after Dixon et al., 2007).

they are not generally useful. Other examples of the development of NIR to measure faecal constituents are the analysis of stress and reproductive hormones, of haemoglobin as a measure of some classes of parasite infection, and the ^{13}C:^{12}C ratio in faeces. This carbon isotope ratio is similar in faeces and in the diet. In tropical pastures the ratio in faeces allows estimation of the dietary proportions of grasses to non-grasses, the latter being comprised of dicotyledonous herbaceous plants and browses (Coates and Dixon, 2007 and 2008a). For a wide variety of tropical pastures the proportion of non-grass in the diet can be estimated from the NIR spectra of faeces with a standard error of performance of about five percentage units.

The second approach to application of NIR spectroscopy of faeces to predict diet has been to develop calibrations between the NIR spectra of faeces and the diet attributes of interest. In herbivores ingesting forage-based diets the NIR spectra of diet and faeces derived therefrom are similar, and sufficient spectral information is unchanged despite the processes of digestion to predict many dietary attributes (Brooks et al., 1984; Dixon and Coates, 2009). This is consistent with the observation that the faeces of herbivores consist principally of undigested plant material. Satisfactory calibrations can be developed to predict the crude protein and DM digestibility of forage and forage-concentrate diets with similar accuracy, reliability and limitations to NIR analysis of forages (Lyons and Stuth, 1992; Decruyenaere et al., 2009; Dixon and Coates, 2009). Limited studies indicate that it should be possible to develop general NIR calibrations for fibre fractions and condensed tannins in the diet. Prediction errors are likely to be greater for diet constituents of very high digestibility where there is little or negligible undigested residue in the faeces (e.g. soluble proteins or carbohydrates). Although most studies have been with cattle or goats, calibration equations have also been developed for non-ruminant herbivores (donkeys, kangaroos and ostriches) (Dixon and Coates, 2009). Also it is possible to predict the proportions of some plant species and groups of plant species (e.g. grasses), and the morphological components, at least in some circumstances.

Faecal NIR has been used to estimate the fluctuations in diet of grazing ruminants through annual cycles and also between years (**Figure 1**; Coates and Dixon, 2008b; Dixon, 2008). These data provide important information to enhance understanding of grazing livestock systems in the contexts of both the constraints of diet quality on the animal and the impact of the animal on the vegetation. For example, in the study shown in **Figure 1** animal responses to non-protein nitrogen supplementation would be expected when the ratio of DM digestibility to crude protein (DMD/CP) in the diet

Table 4. Examples of six studies with ruminants indicating the errors associated with calibration equations relating NIR spectra of faeces to the voluntary intake of diets of forage, or mixed forage and concentrates.

Animal species & class of diet	Units (VI/d)	n	Actual intake			Calibration		
			Mean	Min.	Max.	R^2	SECV	RPD
Cattle, pasture[A]	gDM/kgW	133	-	8	46	0.82	3.4	-
Cattle, forage [B]	gDM/$W^{0.75}$	139	101	58	157	0.98	6.8	4.6
Sheep, forage [C]	gOM/$W^{0.75}$	936	51	-	-	0.83	4.5	2.3
Cattle, forage [D]	gDM/kgW	472	16	4.2	28.6	0.85	1.9	2.4
Goats, mixed [E]	gDM	136	1031	552	1874	0.83	126	2.0
Goats, mixed [F]	gOM/$W^{0.75}$	305	28	-	-	0.90	5.4	1.9

References: [A]Coleman et al., 1995; [B]Decruyenaere et al., 2004; [C]Decruyenaere et al., 2009; [D]Coates, 2004; [E]Landau et al., 2004; [F]Landau et al., 2008.

VI = voluntary intake; DM = dry matter; OM = organic matter; W = animal live weight; SECV = standard error of cross validation; RPD = ratio of the standard deviation of the actual reference data to the SECV (Williams, 2001).

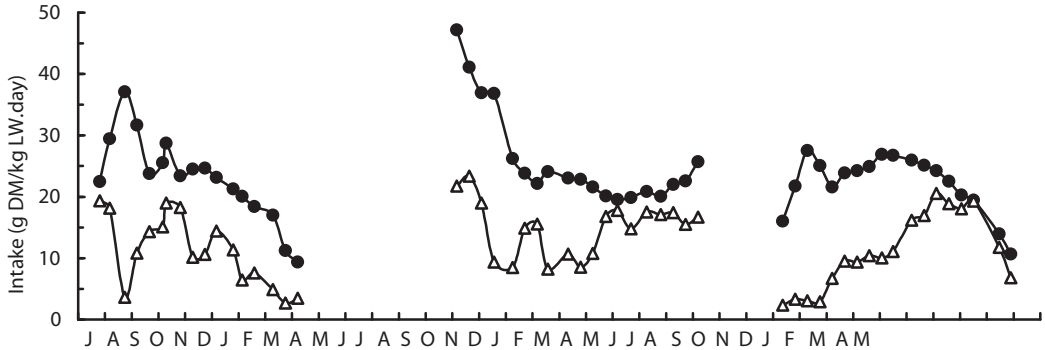

Figure 2. The estimated intakes of *Leucaena* dry matter (DM) (Δ) and total DM (□) (g/kg LW.day) in three consecutive groups of *Bos indicus x Bos taurus* steers grazing in three consecutive years a *Leucaena*-grass pasture located in a subtropical environment at Gayndah, SE Queensland, Australia (Dixon and Coates, 2008a). Total DM intake was calculated from the metabolisable energy intake estimated to be required for the measured live weight change and the DM digestibility of the diet, while the *Leucaena* intake was calculated from the total DM intake and the proportion of *Leucaena* in the diet. The DM digestibility and the proportion of *Leucaena* in the diet were estimated from NIR spectra of faeces. The difference between the intakes of total DM and *Leucaena* DM was grass DM.

was > 8 (Dixon and Coates 2005). Past understanding of the quality of the diet selected and such responses by grazing animals has usually depended on procedures which are difficult and costly to implement and which involve large errors. The application of faecal NIR estimates of diet quality to improve management (e.g. nutrition, supplementation, weaning) of grazing cattle in a seasonally dry tropical environment has been discussed and provides a practical tool for improved productivity and cost-effectiveness of production (Dixon and Coates, 2005; Dixon et al., 2007).

A major constraint in the development of NIR spectroscopy of faeces to estimate the diet is that the calibration equations require faecal samples to be paired to samples of the diet ingested so that laboratory analysis of the diet can be conducted. This usually requires animals to be hand-fed in pens for intervals of 1–2 weeks. Such experimental procedures are costly and labour-intensive, and many forages selected by the grazing herbivore cannot be fed in this manner (Coates and Dixon 2009).

Voluntary intake of DM and liveweight change of ruminants can be predicted satisfactorily from the NIR spectra of faeces under some, but certainly not all, circumstances (**Table 4**; Coleman et al., 1995; Dixon and Coates, 2009). It seems likely that the NIR spectra of faeces are estimating primarily the variability of the diet, and prediction of voluntary intake is primarily a consequence of the associations between voluntary intake and characteristics of the diet such as DM digestibility and physical resistance to fragmentation and breakdown. This hypothesis is supported by the observations of Decruyenaere et al. (2009) that the same regions of the NIR spectra were predominant in the calibration equations for both DM digestibility and voluntary DM intake. The prediction errors for voluntary DM intake of forages are generally comparable with, or smaller than, the errors associated with prediction of voluntary DM intake from the NIR spectra of forages, or from conventional laboratory analysis of forage such as *in vitro* digestibility, neutral detergent fibre or acid detergent fibre. Faecal NIR predictions of voluntary DM intake would be expected to be an estimate of the potential intake in the class of animals utilised in the calibration data sets, as limited by forage characteristics, rather than necessarily actual intake which will be influenced by numerous aspects of the physiological state of the animal and the availability of the forage.

Since principles of energy metabolism in animals determine that there is a broad relationship between metabolisable energy intake (approximated by DM intake multiplied by DM digestibility) and liveweight change of an animal, calibrations for animal live weight change derived from the NIR spectra of faeces are to some extent comparable with calibrations for voluntary DM intake and diet DM digestibility. Satisfactory calibrations have been developed for animal live weight change when data were restricted to a specific class of animal (young healthy growing tropically-adapted cattle), although the error was quite large (standard error of cross-validation; SECV = 0.16 kg/d) (Dixon and Coates 2009). However, because both voluntary DM intake and live weight change are influenced by many animal factors (e.g. maturity, lactation, compensatory growth, parasites and disease), thermal environment and forage availability, it will be difficult to develop calibrations for either voluntary DM intake or live weight change applicable to a wide range of animal and pasture circumstances. In this regard, although the Coates (2004) live weight change calibrations predicted satisfactorily where cattle grazed pastures comparable with those in the calibration data set (Dixon et al., 2007; Coates and Dixon, 2008b; Dixon, 2008), large errors sometimes occurred for cattle grazing different pasture systems which were not represented in the calibration data set (Dixon, 2008, Dixon and Coates, 2008a), or for animals in different physiological states such as lactation, maturity or compensatory growth (Dixon et al., 2007; Dixon and Coates, 2008b). Nevertheless, as has often been observed during development of NIR calibrations, inclusion of some data representing a new pasture system has often radically improved the calibrations (Dixon and Coates, 2008 a and b). In conclusion, NIR spectra of faeces cannot directly predict voluntary intake where intake is modified by animal or environmental factors or where intake is constrained by the availability of the diet.

Despite these difficulties and constraints, faecal NIR technology has been applied satisfactorily to enhance knowledge of the nutrient intake and production of grazing cattle. For example, Dixon and Coates (2008a) used the actual live weight gain of young steers grazing *Leucaena* (a palatable tropical shrub) - grass pasture to calculate the metabolisable energy intake of the cattle, and faecal NIR predictions of diet DM digestibility and the proportion of *Leucaena* in the diet to estimate intakes of *Leucaena* and grass DM through three growing seasons (**Figure 2**). Furthermore, there was reasonable

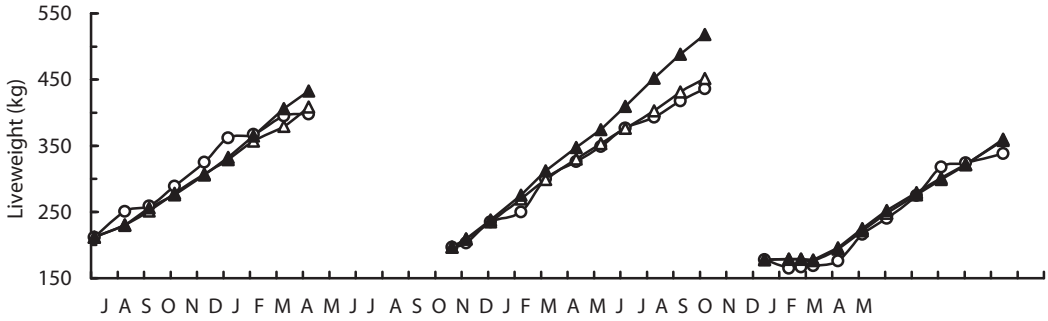

Figure 3. The actual measured live weight (○), and the cumulative live weight predicted from the NIR spectra of faeces calculated using two possible calibration equations (Δ, ▲) in three consecutive groups of *Bos indicus x Bos taurus* steers grazing a *Leucaena*-grass pasture located in a subtropical environment at Gayndah, SE Queensland, Australia (Dixon and Coates, 2008a).

agreement between the actual live weight gain of the cattle and the live weight gain predicted from faecal NIR calibrations provided that the calibrations were updated for the specific pasture system (**Figure 3**). There has also been reasonable agreement between observed and predicted live weight pathways of cattle grazing a number of other pastures in northern Australia (Coates and Dixon, 2008b; Dixon, 2008).

LIKELY ROLES OF NIR SPECTROSCOPY TO LIVESTOCK NUTRITION AND PRODUCTION SYSTEMS IN DEVELOPING COUNTRIES

In the stockfeed manufacturing industries, NIR spectroscopy can provide rapid and economical analysis of both concentrate and forage feedstuffs (including by-products) used as ingredients, and for quality control during manufacturing of products for both monogastric and ruminant animals. It thus allows quality control, and application of nutritional science in stockfeed manufacturing and thus animal production systems to an extent not previously practicable. In addition, NIR spectroscopy of faeces can provide routine estimation of the diet of ruminants in circumstances where this information is difficult or not possible to obtain using other technologies.

In developing countries NIR could be applied to understand the nutrition of grazing ruminants. It could also be readily applied to both ruminant and monogastric animals in small holder farming systems where diets will usually be derived from a diverse and changing array of local forages and feedstuffs, and where it is usually not possible to sample diet components adequately to estimate the diet consumed. Use of faecal NIR technology to better manage and improve livestock production systems in sub-Sahalan Africa and China have been demonstrated (Awuma, 2003).

In the context of developing countries NIR technology provides opportunities to:

- improve knowledge of the nutritional value, including the fluctuations through seasons and years, of regional and local resources used as livestock feedstuffs;
- apply the vast accumulated knowledge of livestock nutrition science to feeding livestock;
- use faecal analysis to simply and routinely monitor the diet and nutrition of livestock in local and regional production systems;
- improve nutritional management of livestock for improved productivity e.g. especially for milk production which is highly responsive to nutrition.

CONSTRAINTS TO IMPLEMENTATION IN DEVELOPING COUNTRIES

Numerous reviews such as those cited above have outlined and discussed the advantages of NIR technologies, and many of these advantages are as applicable to developing as to developed countries. In addition Shepherd and Walsh (2007) provide a comprehensive and thought-provoking overview and vision of the potential and possible role of such technologies for developing countries. Their focus, and many of the examples cited, relate to soil sciences and the African continent, but the vision is arguably equally applicable to other continents and other aspects of the agricultural and land-use sciences including livestock.

Major advantages of NIR technologies include:

- it provides rapid analysis, including with field-portable instruments in real-time;
- where appropriate calibrations equations are available the cost of analysis is much lower than that using conventional laboratory procedures;
- sample preparation is minimal (e.g. typically drying and grinding) or for some applications is not required (e.g. whole as-received grain or forage);
- no wastes are produced and no laboratory reagents are needed on a routine basis;
- routine analysis (e.g. as conducted in grain handling depots or on a factory floor in a country such as Australia) is possible by staff with minimal training or technical expertise (subject to type of instrument and application);
- some classes of modern instruments (e.g. diode array or [fourier transformed] FT-NIR) are rugged sealed units which should require little maintenance or repair. In some configurations such instruments are portable and suitable for field use. Data can usually be analysed by inbuilt electronics or down-loaded onto a laptop;
- a single NIR instrument and attached laptop can analyse a specific sample for a wide array of nutritional constituents or attributes thus reducing the need for a variety of laboratory instruments and lengthy procedures. Furthermore a single instrument (possible with several sampling attachments) can be used for a wide variety of feedstuffs and faecal samples, but also for a wide range of other agricultural materials and land-use related measurements (e.g. many foodstuffs, meat, milk, blood, soils).

The disadvantages of NIR technologies include:

- moderate to high capital cost of NIR instrumentation and software (e.g. US$15 000–150 000). This may be exacerbated by difficulties in obtaining technical support in developing countries;
- a high level of technical expertise, knowledge and skills are needed to develop or adapt existing calibration equations, for new and specific circumstances, and to trouble-shoot problems with development and maintenance of the instrument and data analysis. Considerable training and experience is usually required to become expert in chemometrics and the specialised software packages required;
- because calibration equations are usually quite specific for the product or material being measured they will usually need to be developed (or modified from elsewhere) for regional situations. This usually requires the analysis by both NIR and conventional chemistry of many hundreds of 'training' samples before analysis of unknown samples can commence. Although numerous laboratories and instrument companies have large data sets and calibration equations, these are usually regarded as intellectual property and often will not be made freely available;
- conventional laboratories still need to be maintained for high quality analysis of samples for development, and ongoing maintenance and refinement of calibration equations. Near infrared analysis can only be as accurate as the conventional laboratory analysis used to develop the calibration equations. Networks among laboratories are usually necessary to ensure quality control of conventional analysis and for conduct of ring tests. The advantage is that the number of analyses required in conventional laboratories should be greatly reduced;
- there will generally be a need for critical mass of scientific expertise across a range of disciplines to develop and maintain NIR spectroscopy groups.

An idealised design as a way forward to progress extensive development of infrared spectroscopy (both infrared and near infrared but where near infrared spectroscopy is likely to predominate) has been proposed by Shepherd and Walsh (2007). It involves developing regional centres of scientific and technical excellence to provide support for:

- high quality laboratory reference analysis;
- development of calibration databases and interpretation systems;
- upgrading of scientific and technical skills through training and education.

Key challenges for adoption of this design include:

- building human capacity in science and technology-based approaches, and developing understanding among both professional staff and clients in the industries and regions of interest on the role of these technologies. A particular need is to develop understanding and knowledge of spectroscopy among professionals as an essential basis for acceptance of the technology. Such knowledge is scarce in developed countries, far less in developing countries;
- development of rugged low cost infrared and near infrared spectroscopy instrumentation;
- development of decision support systems to interpret infrared spectroscopy data into management recommendations.

There are clear reasons that such centres of regional excellence in near infrared spectroscopy should not be limited to any one aspect of the technologies or to a single area or discipline of application. Many of the technical aspects of developing and improving instrumentation and use of data systems derived from this technology are likely to have much in common across disciplines and agricultural and land-use sciences.

CONCLUSIONS

NIR technology based on analysis of feedstuffs and faeces of livestock can rapidly and economically provide objective nutritional information on the diet of animals, their likely productivity, and of ingredients and processes for stockfeed manufacturing. It allows easy and comprehensive application of established nutritional science to the nutrition and management of livestock. This in turn improves the efficiency and productivity of livestock for food. Because a wide range of nutritional analyses can be conducted simultaneously with one instrument and a desktop computer, NIR spectroscopy can greatly reduce the capital investment, training and operational costs required for nutritional analysis and decision support. Nevertheless there are substantial obstacles to widespread implementation of NIR technologies in developing countries.

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Comparative Genomics for Prediction of the Relative Location of ESTs in the Goat Genome

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ABSTRACT

Worldwide the goat is an important agricultural species that is highly adaptable to many environmental conditions, and goat production is a rapidly growing industry within the USA. A better understanding of the goat genome could lead to new discoveries based on the genetic diversity and environmental adaptations important to ruminant health and production. An effort is underway to increase our understanding of the goat genome and develop a radiation hybrid (RH) map for stronger comparative genomic analyses. An embryo/uterine cDNA library was sequenced and about 12 800 expressed sequence tags (ESTs) added to the public database. In this study, comparative analyses among goat, sheep, and cattle maps were used to predict the location of the assembled EST contigs (n = 1 920) and singlets (n = 4 400) in the goat genome. Prediction of goat EST locations was determined through comparisons with the goat and sheep genetic maps using the bovine map as a backbone. Alignments of ESTs were predicted based on the relative location of mapped goat markers on the bovine sequence and refined by comparisons with the sheep maps. The predicted map attempts to localise the relative genomic position of the unique contigs and singlets developed from the available ESTs. Additionally, the degree of conservation among goat, cow, sheep, human, mouse, and rat genomes has been indicated and comparative maps generated. The predicted map will be a crucial resource for comparative genomic analyses and for the determination of EST and microsatellite markers during development of a goat RH map.

Key words: *goat genome, comparative genomics, radiation hybrid map, cDNA library, expressed sequence tags, markers.*

INTRODUCTION

Worldwide the goat is a primary source of milk, meat, and income to families and communities. The majority of the goats are found in developing countries, while in the USA the goat industry is relatively young and developing. Yet, the USA demand for goat products is greater than domestic production, which has led to an increase in the number of producers and animals over the last ten years. The majority of the world's goat producers are small, low-input farmers that need low-cost, effective mechanisms to address their individual disease and production needs. The discovery of the genes involved in phenotypic adaptations around the world can lead to the creation of inexpensive tests and when associated with local producer outreach programmes, can assist producers in the selection of animals that will be ideally suited to meet their environmental and production needs.

Comparative genomics is one of the more promising approaches for identifying the underlying causes for disease susceptibility and complex production traits. The worldwide phenotypic observations in the goat are an opportunity for scientists to utilise comparative genomics for identifying and understanding the underlying causes for a multitude of traits. However, the understanding of the goat at the genomic level is far behind other livestock species. A genetic map for the goat is available (Schibler et al., 1998), but it is somewhat dated and has a limited number of markers available for comparative mapping. With the current resources available, comparative analysis among the ruminant species is still challenging. However, development of a radiation hybrid (RH) map, which is available for sheep (Wu et al., 2007) and cattle (Womack et al., 1997), will allow for placement of a variety of marker types and comparisons with other species. The diversity of the goat populations and the development of a RH panel and map for the goat will allow scientists to use comparative analyses with the more developed bovine, sheep and human genomic resources to address the underlying genetic aspects of important traits. Additionally with the development of new technologies and the promise of reduced costs for genome sequencing looming in the future, the development of the RH map for the goat will provide a framework map which can be used to order the sequences.

The development of an RH map involves irradiating goat cells with 5 000 rad from a ^{60}Co source to randomly break the DNA. These irradiated cells are hybridised with hamster cells to form cells that contain both goat and hamster DNA segments. A panel of these hybrids is screened for the location of goat genomic markers. The relative frequency of markers in the panel can be used to determine their distance from each other and develop a RH map.

The development of the RH map for the goat will require the selection of markers throughout the genome to make the best use of the limited amount of DNA from the RH panel. The objective of this project was to develop a predicted gene map for the goat to assist in identifying genes and markers for developing a RH map.

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MATERIALS AND METHODS

Identification of EST Identities and Location

A total of 12 698 quality goat EST sequences were collected from the NCBI database. The sequences were previously clustered and assembled. From this process there were 1 921 contigs formed from 8 388 sequences, with 4 433 singles remaining (Sayre et al., 2006).

The 6 354 unique contigs and single sequences were subjected to a BLAST search of the NCBI RefSeq databases for sheep, cattle, human, mouse and rat. From the top hit for each EST sequence, the data collected was the percent of similarity of compared sequences, percent of the total EST sequence associated with the database sequence, the RefSeq accession number, and the description of the associated sequence from the database. The data were stored in Excel worksheets and Access databases.

The RefSeq accession data from the BLAST comparisons were used to withdraw information from the NCBI Gene database. The symbol, chromosomal location, and physical location data for each identified gene and species were extracted.

Development of the Predicted Goat Gene Map

To predict the location of the ESTs in the goat genome, a framework map based on the genetic maps was developed and followed with placement of the gene locations based on the cattle genomic map. The framework map was developed by localising the markers available on the goat genetic map with the sheep genetic and cattle genomic maps. The marker information was localised from the published genetic map for the goat (Schibler et al., 1998) and NCBI UniSTS for the physical location of the markers on the cattle genomic

Table 1. Total number of genes predicted and sequences conserved at 70% SI or greater in cattle, human, mouse and rat.

Chromosome	Genes	Cattle	Human	Mouse #[a] (%[b])	Rat	Overall
1	98	67 (68)	37 (38)	23 (23)	23 (23)	23 (23)
2	141	89 (63)	54 (38)	33 (23)	31 (22)	29 (21)
3	196	144 (73)	93 (47)	59 (30)	54 (28)	55 (28)
4	86	58 (67)	28 (33)	15 (17)	13 (15)	12 (14)
5	190	126 (66)	77 (41)	55 (29)	55 (29)	51 (27)
6	74	56 (76)	33 (45)	23 (31)	20 (27)	19 (26)
7	220	160 (73)	92 (42)	63 (29)	63 (29)	61 (28)
8	96	74 (77)	45 (47)	32 (33)	31 (32)	31 (32)
9	63	43 (68)	19 (30)	17 (27)	13 (21)	15 (24)
10	133	85 (64)	45 (34)	31 (23)	27 (20)	29 (22)
11	154	109 (71)	64 (42)	45 (29)	39 (25)	45 (29)
12	58	28 (48)	14 (24)	8 (14)	8 (14)	8 (14)
13	130	91 (70)	58 (45)	38 (29)	35 (27)	36 (28)
14	62	40 (65)	25 (40)	13 (21)	11 (18)	12 (19)
15	105	79 (75)	52 (50)	41 (39)	36 (34)	37 (35)
16	70	41 (59)	20 (29)	14 (20)	13 (19)	16 (23)
17	101	75 (74)	47 (47)	36 (36)	37 (37)	34 (34)
18	185	121 (65)	76 (41)	60 (32)	55 (30)	55 (30)
19	227	145 (64)	90 (40)	69 (30)	66 (29)	69 (30)
20	38	25 (66)	13 (34)	10 (26)	8 (21)	7 (18)
21	86	56 (65)	32 (37)	22 (26)	20 (23)	22 (26)
22	102	72 (71)	48 (47)	31 (30)	33 (32)	28 (27)
23	94	63 (67)	36 (38)	19 (20)	16 (17)	20 (21)
24	41	33 (80)	20 (49)	14 (34)	12 (29)	13 (32)
25	137	92 (67)	49 (36)	34 (25)	31 (23)	35 (26)
26	57	42 (74)	23 (40)	18 (32)	16 (28)	17 (30)
27	33	23 (70)	12 (36)	7 (21)	8 (24)	8 (24)
28	45	32 (71)	23 (51)	15 (33)	12 (27)	12 (27)
29	91	66 (73)	46 (51)	33 (36)	30 (33)	31 (34)
X	77	45 (58)	19 (25)	14 (18)	15 (19)	20 (26)
Overall	3 190	2 180 (73)	1 290 (43)	892 (30)	831 (28)	850 (28)

[a] Number of the total genes from each chromosome conserved within various species.

[b] The percent of gene conservation within various species ([# of genes conserved / total # genes on the chromosome] × 100).

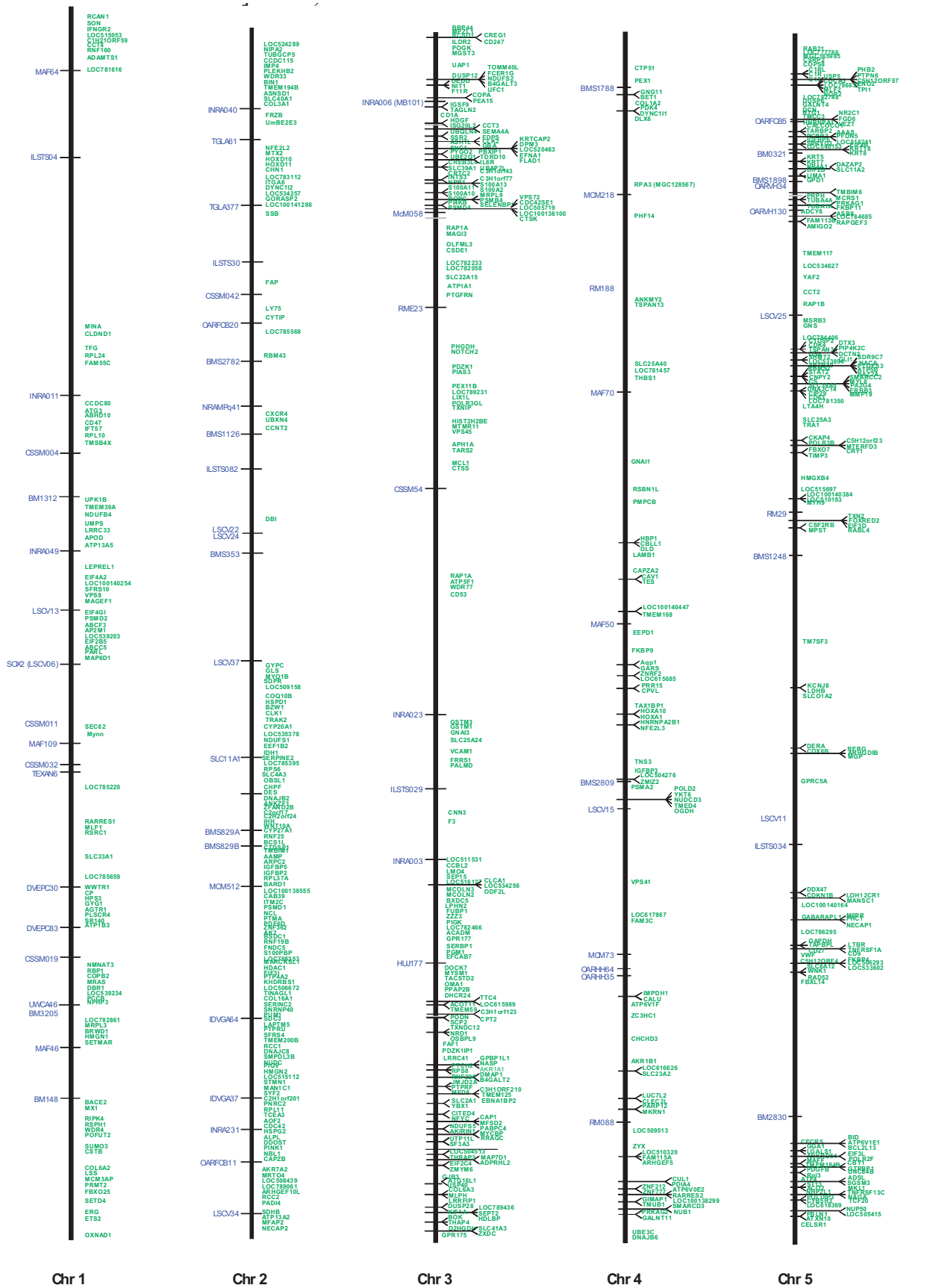

Figure 1. Predicted location of ESTs (green) on goat chromosomes 1–5 based on the location of known goat markers (blue).

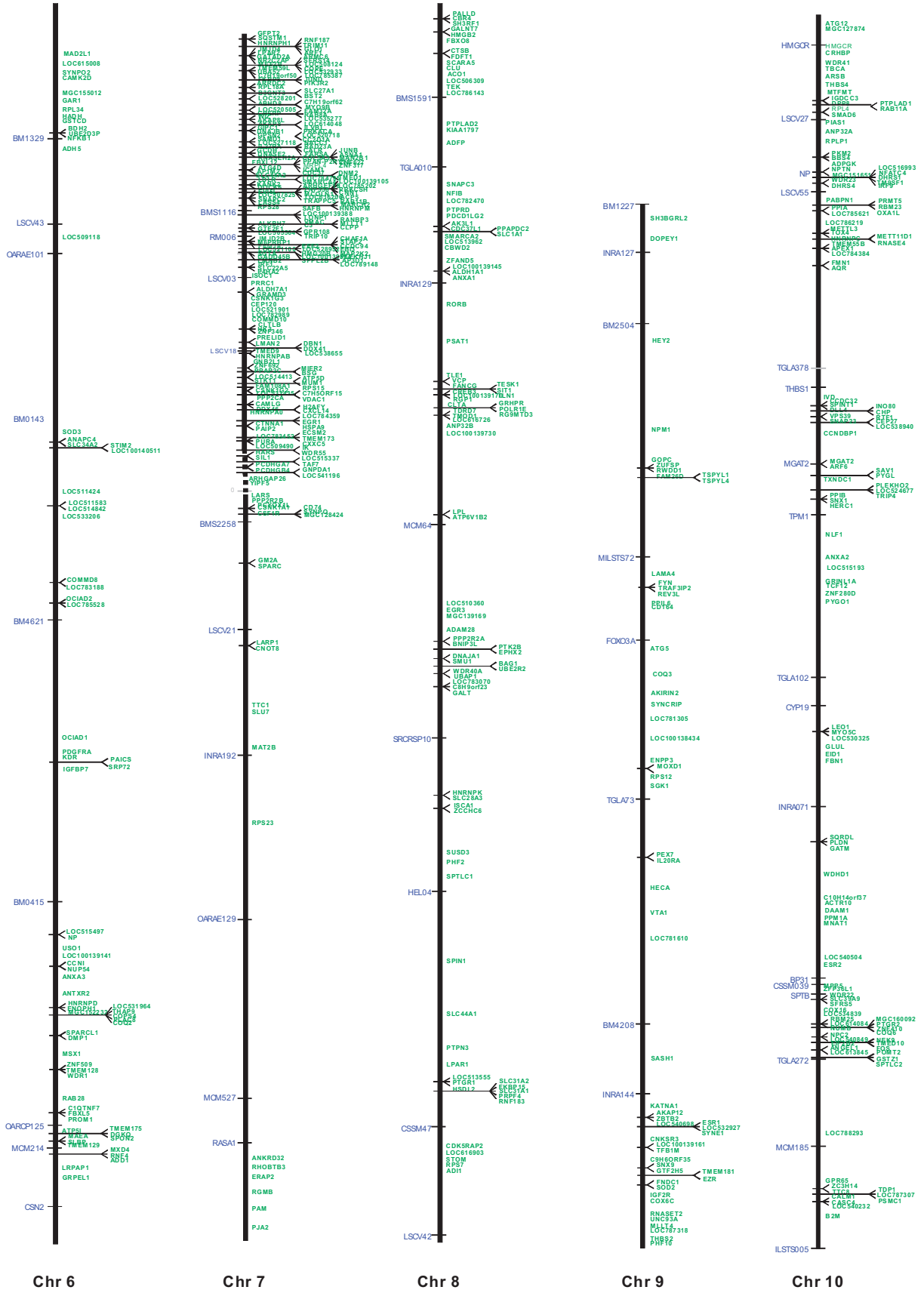

Figure 2. Predicted location of ESTs (green) on goat chromosomes 6–10 based on the location of known goat markers (blue).

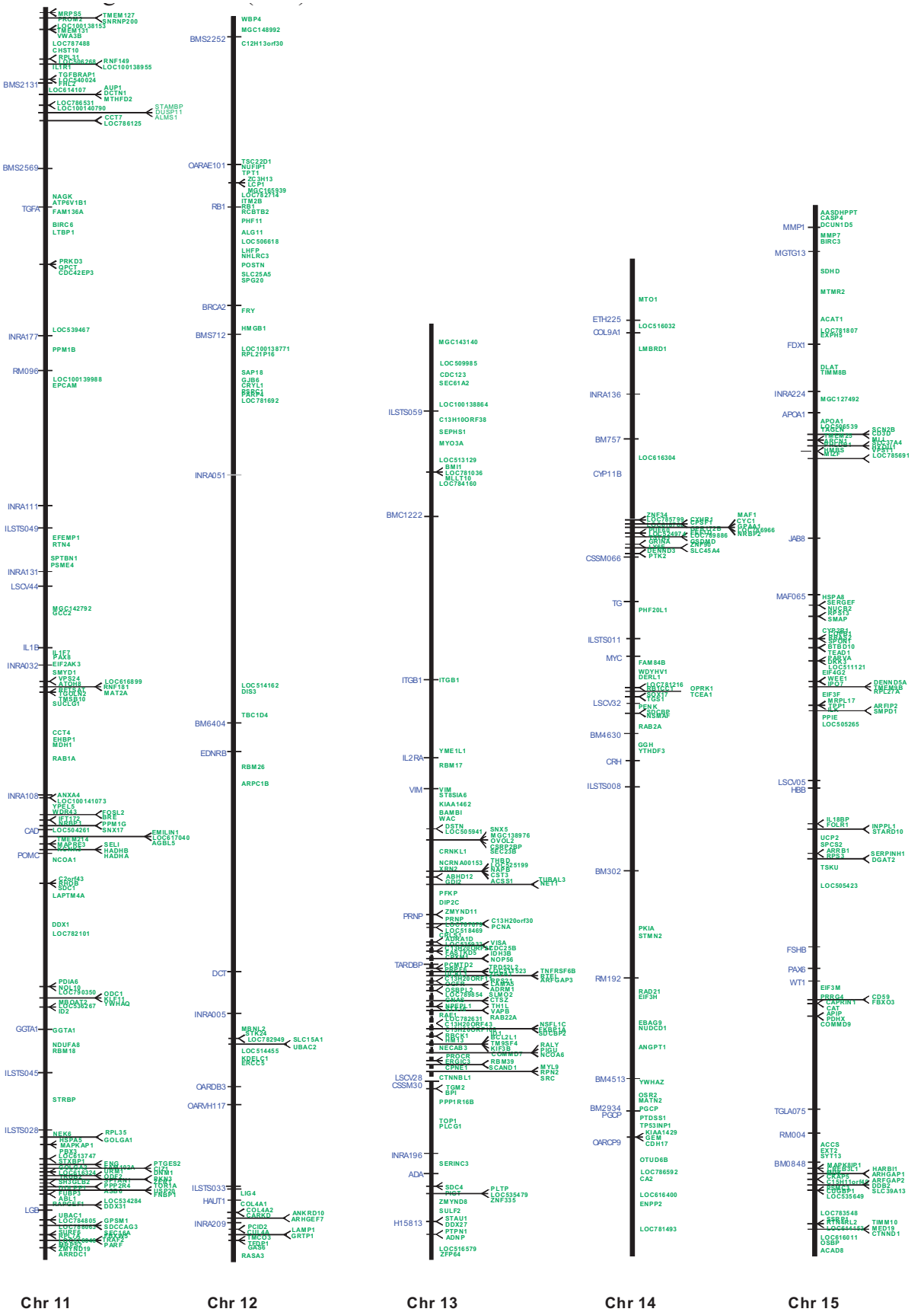

Figure 3. Predicted location of ESTs (green) on goat chromosomes 11–15 based on the location of known goat markers (blue).

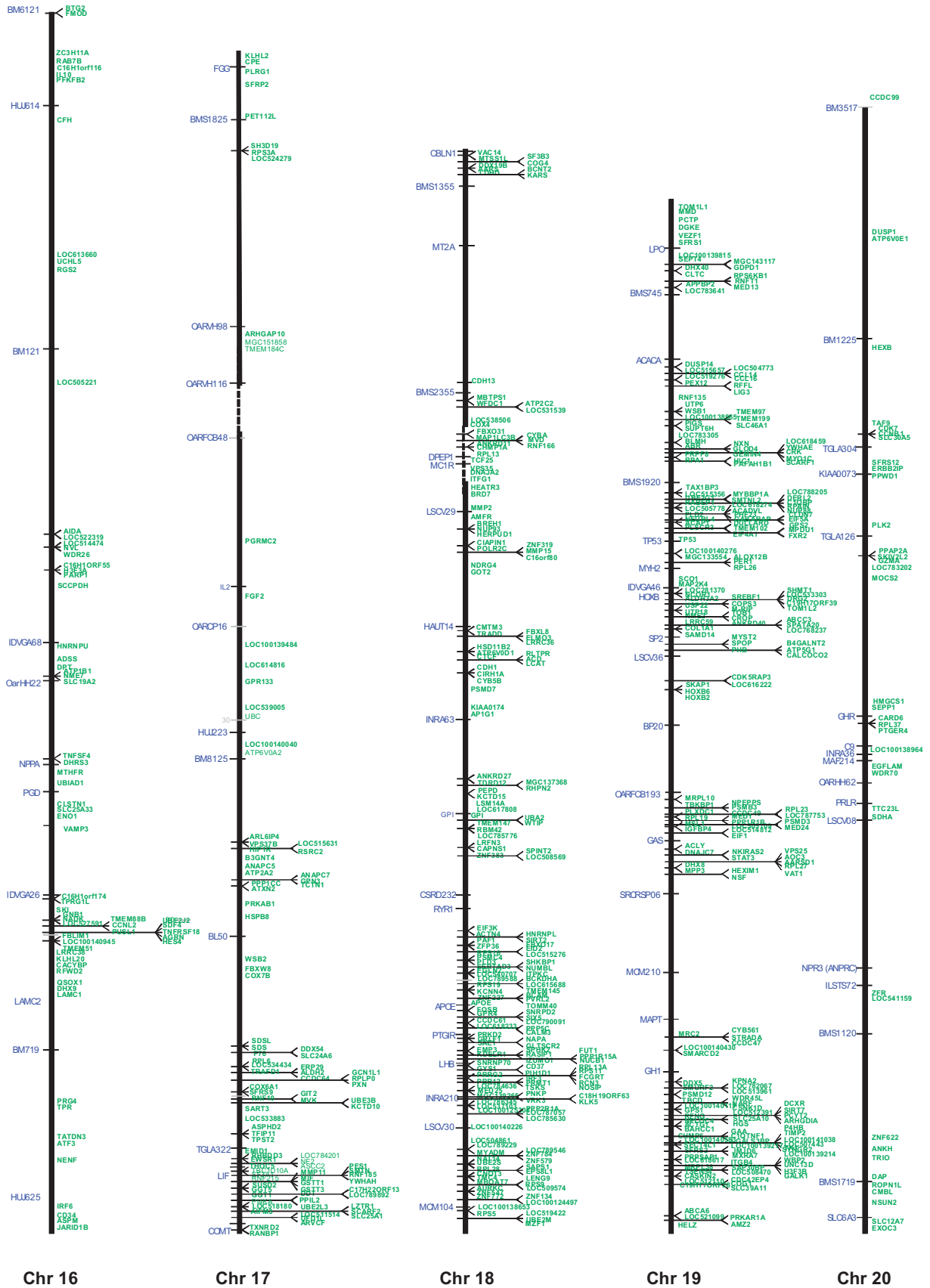

Figure 4. Predicted location of ESTs (green) on goat chromosomes 16–20 based on the location of known goat markers (blue).

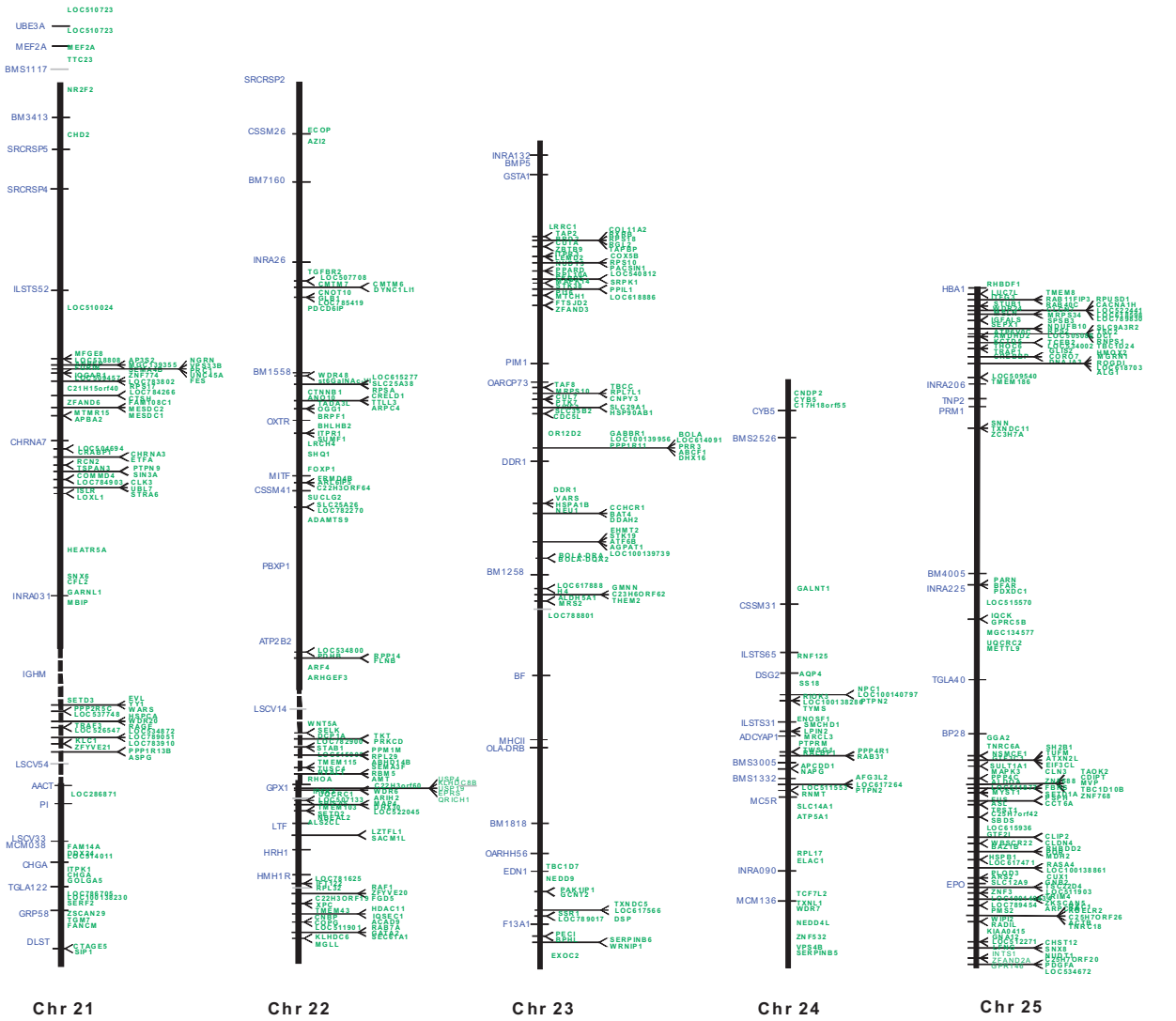

Figure 5. Predicted location of ESTs (green) on goat chromosomes 21–25 based on the location of known goat markers (blue).

map. The placement of the genes on this framework began with the localisation of the gene in the cattle genomic map and properly ordering the genes relative to the locations of the genetic markers. Then based on the framework map, the locations of genes were predicted on the goat genome map.

To make comparisons of the species based on conservation, a sequence index (SI) was created that took into account the percent sequence similarity relative to the percent association. The index was SI = association percent x (similarity percent/100) x 100. Based on the SI, the conservation of the sequences was compared among the species and individual chromosomes.

RESULTS

We predicted the possible identity and genomic location of 3 190 EST sequences. The number of predicted EST sequences and the number of those sequences with greater than 70% conservation to the cattle, sheep, human, mouse and rat sequences is displayed in **Table 1**. Conservation of gene sequences appeared to be evenly distributed across the chromosomes.

The predicted map includes 3 190 genes that have been distributed across the 29 autosomes and the X chromosomes in the goat (**Table 1**). The predicted locations can be visualised on the predicted map in **Figures 1–6**. The ESTs appear to be grouped to specific regions of the chromosomes while other regions have only few genes present. The gene symbols designating the EST identities can be found on the right side of the chromosome maps. The left side of the chromosome maps indicates the locations of the genetic and gene markers from the goat genetic map.

DISCUSSION

The map developed during this project will be used as a framework for the development of a RH map, which will be the first physical genomic map for the goat. The completion of the RH map will enable comparative analysis of marker association studies in the goat and the potential identification of genes related to particular phenotypes.

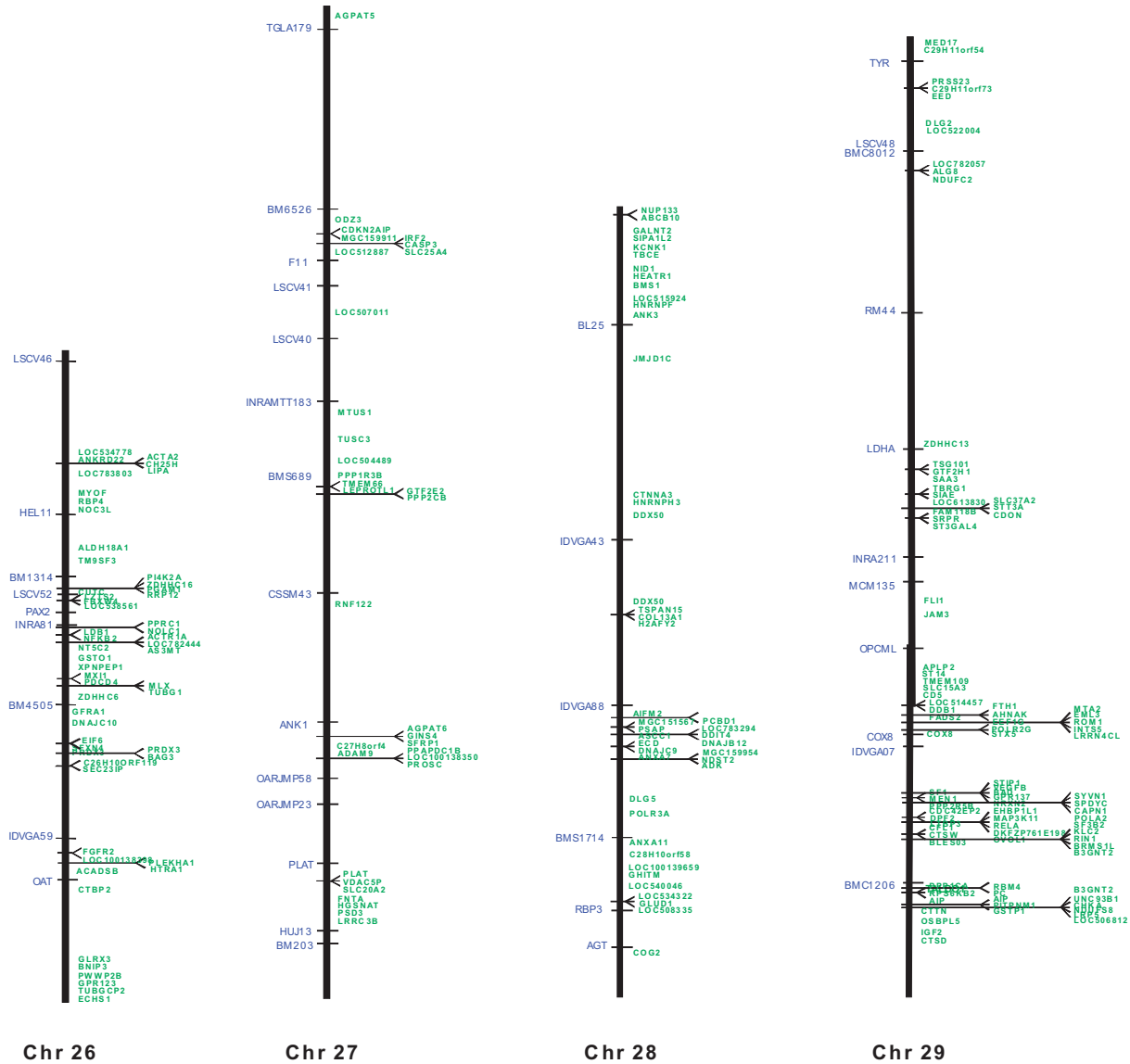

Figure 6. Predicted location of ESTs (green) on goat chromosomes 26–29 based on the location of known goat markers (blue).

Identification of the genes and markers can allow for local groups to assist goat producers in the selection of animals that will better survive and perform under their specific environmental conditions.

The predictive gene map was created to determine the potential location of the genes in the goat genome and relationships to the bovine sequence map. The sequencing of the goat genome would give researchers the best tools available for discovery of genes related to diseases and production issues. The development of this EST-based map will be useful in the assembly of the goat genome when sequencing commences. The EST-based map can provide the approximate locations of genes in the goat genome to assist in the ordering of sequence scaffolds during the genome assembly process. The production of a physical map for the goat would be the best comparative map. Until such time, comparative analyses among livestock species is one of the more promising approaches for identifying the underlying causes for disease susceptibility and production traits. The comparative analysis will be useful when identifying potential syntenic regions that may exist within quantitative trait loci (QTL) for similar traits.

CONCLUSIONS

This project identified the location of many of the goat ESTs and made comparisons with bovine, ovine, and human data. The development of this map is a valuable tool for development of physical and comparative genomic maps for the goat. Using comparative genomics, scientists can take advantage of the diversity of phenotypes found in the goat to address the underlying genetics of biomedical and production traits.

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Early Stirrings of Landscape Genomics: Awaiting Next-next Generation Sequencing Platforms before Take-off

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ABSTRACT

Landscape genomics is an emerging research field that bridges genetics and genomics to geo-environmental resources analysis. It aims to study genome/environment interaction to discover the genetic basis of adaptation by processing of many simultaneous DNA-environment association models, exploiting GIS (Geographical Information Systems) science and statistical methods. In this paper, we review the literature related to the recent history of this discipline, describing its application to livestock genetics, discussing its potential contribution to Farm Animal Genetic Resources (FAnGR) conservation and management, explaining its role in the analysis of the local adaptation of autochtonous breeds and showing how the upcoming next-next generation of DNA sequencing methods, in parallel with the availability of an increasing number of high quality environmental data sets, will allow a real take-off of this novel approach.

Key words: *landscape genomics, DNA sequencing, environment, geographical information systems, animal genetic resources.*

INTRODUCTION

Development of sustainable agriculture, including animal husbandry, based on adapted breeds is a priority for most countries in the world, and is of key importance to emerging countries in particular. The genetic basis and the level of adaptation of livestock breeds to their environment has to be investigated in order to reach a better understanding of the relationship between environment and adaptative fitness of livestock populations, in favour of production systems based on adapted local breeds. According to the Africa-based International Livestock Research Institute, landscape genomics seems to be a long term promising approach for understanding the genetic adaptation of livestock to the environment (ILRI, 2007).

LANDSCAPE GENOMICS

A wide definition considers this field as an "emerging area at the interface of natural resources management and the genome sciences" (Williams, 2004). Landscape genomics takes its roots in landscape genetics, a new approach described as the combination of landscape ecology and population genetics (Manel et al., 2003). Landscape genetics tries to facilitate our understanding of how geographical and environmental features structure genetic variation. Landscape genetics rapidly became a term used to describe all research about genetic data, exploiting their geographic dimension and spatial statistics (Storfer et al., 2007).

Implementation of the landscape genomics approach described by Luikart et al. (2003) was published by Joost et al. (2007) who described the detection of candidate loci for selection in insects (pine weevil) and in a livestock species (sheep). Population genomics, geo-environmental and statistical methods were combined to assess the level of association between specific genomic regions of living organisms and environmental factors. Association models (logistic regressions) between hundreds of environmental parameters and thousands of molecular markers from thousands of animals, were processed to identify genomic regions likely to be under natural selection.

Landscape genomics has also potential for supporting livestock conservation activities. The global purpose of this discipline is to uncover which genetic variations are likely to fit to environmental conditions, or to biogeographical regions worldwide. Indeed, autochthonous livestock are adapted to the landscape where they are bred. Since association models make it possible to go a step further to identify specific loci linked to environmental parameters compared with classical approaches (Joost et al., 2007), it is then possible to learn from the co-evolution of livestock and their production systems. In a subsequent step, acquired information can be used to better match different breeds with optimal production conditions (Long, 2008) and to produce, for example, maps of potential or optimal habitat (**Table 1**). Incidentally, the Host/Pathogen Interaction Programme funded by the Wellcome Trust and centred around bovine sleeping sickness in Africa initiated activities on landscape genomics with the goal of linking environmental factors, trypanosomiasis and cattle (http://www.genomics.liv.ac.uk/tryps/).

Landscape genomics is also of interest for fish biology (Nielsen and Hansen, 2008) and plant science (Holderegger et al., 2009), and the research strategy in the latter field uses of this promising approach. In a recent example from April 2009, the European Plant Science Organisation (EPSO) organised a workshop on landscape genomics (http://www.epsoweb.org/Catalog/epso_workshops/) to bring together ecologists, natural history collection experts and genomics specialists to discuss ideas and needs for future collaborative projects. This interdisciplinarity means that several research fields should contribute to future progress in landscape genomics.

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Table 1. Compulsory and optional components of livestock landscape genomics and expected outputs.

	Input	Output
Compulsory information to compute association models	Geo-environmental data (topographic and eco-climatic information, bio-physical information describing the production system e.g. type of pasture, soil, etc.) Geo-referenced genetic data (SNPs but also AFLPs, microsatellites, etc.)	Working hypotheses about the function of genome regions which are linked to the genetic markers Maps of potential habitat for breeds (Joost, 2008) Predictions about consequences of climate change (landscape change) Predictions about consequences of landscape change due to human activity (constructions)
Optional additional information on diseases and farming systems	Geo-referenced disease information Geo-referenced farming system data (socio-economic information on farms, management systems, goals, knowledge, resources, monitoring opportunities)	Working hypothesis on relationship among selected genomic regions, specific environmental configuration and disease occurrence

The first applications of landscape genomics were recently reported in wildlife (Joost et al., 2008), livestock (Joost et al., 2007; Pariset et al., 2009) and plants (Parisod and Joost, 2010). In livestock they are limited to studies carried out on sheep (Joost et al., 2007) and goat (Pariset et al., 2009), both being based on the use of SAM software (http://www.econogene.eu/software/sam/) as well as population genomics theoretical approaches for results validation (Foll and Gaggiotti, 2008). Far from fully exploiting the potential of landscape genomics, these reports are restricted to the simultaneous processing of approximately one hundred environmental parameters related to a small number of genetic markers (<1 000) produced from 2 000 animals at most. We will now discuss how these figures are expected to evolve over the short term, taking advantage of the availability of environmental data dealing with distinct characteristics, and considering the perspective offered by the next-next generation of molecular technologies.

ENVIRONMENTAL DATA

The environment in which livestock populations are reared directly affects animal health and production. Thus, geo-environmental data provide the framework for mapping and analysing disease occurrence, monitoring climate trends and characterising production environments in order to support evaluations and comparative analyses of livestock performance (FAO, 1998). Moreover, as mentioned above, this information is essential to understand the genetic basis of native livestock adaptation to their environments, and is therefore important for optimising the management of animal genetic resources (FAO, 2007).

Most of environmental global data sets are freely available from the Internet and can be used for a comparative description of production environments worldwide. The 'sustainable development' principle established during the United Nations Conference held in Rio de Janeiro (1992) promoted actions towards the collection of additional environmental data at different scales, and recommended that countries provide open access to the information for stakeholders and scientists involved in environmental decision-making processes (UN, 1992; Haklay, 2003). For instance, as a concrete consequence of this call, the Global Map project (http://www.globalmap.org/) proposes data sets comprising elevation, land cover, land use, and vegetation, as well as information on transportation, population and political boundaries. The project is supervised by the International Steering Committee for Global Mapping (Secretariat of ISCGM, 1998), with over 90 participating countries (Verdin and Jenson, 1996). Version 1.0 of the Global Map project consists of data contributed, updated and maintained by each country. The main international global environmental geodata sources are included into the Global Map project, and are available from the Secretariat for the ISCGM hosted by the Geographical Survey Institute of Japan.

In parallel to this action, several international or national agencies provided free access to geo-environmental data at different spatial resolutions and for different time periods. Among them, the most important are the European Environment Agency (http://www.eea.europa.eu/, EEA) and agencies in the USA like USGS or NASA, or LANDSAT satellite images (http://www.landsat.org) that offer global orthorectified Landsat data freely. Moreover, the Global Biodiversity Information Facility (GBIF, http://www.gbif.org/), is an international organisation working to make the world's biodiversity geodata accessible worldwide with the possibility to download livestock species-related data sets.

Finally, the United Nations Environment Programme (UNEP) documented the Global Environment Outlook (http://www.unep.org/geo) — a report presenting the challenges that countries face in safeguarding the environment and for moving towards a more sustainable future - and supplying a data compendium with a list of all key data providers who contributed to the elaboration of the action (http://geocompendium.grid.unep.ch/).

In summary, we do not expect a quantum leap in the short-term. The number of available environmental data bases (new satellites, new environmental monitoring capacities) will gradually increase, their quality will improve (better spatial resolution), and a growing number of geographic areas will be covered. However, most of these global data sets are already freely available and can be used for a comparable description of production environments worldwide.

MOLECULAR DATA

The 2.91 billion base pair (bp) consensus sequence of the euchromatic portion of the human genome was generated by an international consortium of researchers that exploited whole-genome shotgun methods and Sanger biochemistry in a 13-year and $3 billion project (Venter et al., 2001). The recent publication of the complete genome sequence, its annotation, and comparative analysis of the cattle genome by the The Bovine Genome Sequencing and Analysis Consortium (see Elsick et al., 2009) required a six-year effort and a significantly lower budget.

Over the past three years, various innovations of cyclic-array sequencing were introduced into commercial products (e.g. 454 Genome Sequencer –, Roche Applied Science; Solexa – Illumina; SOLiD – Applied Biosystems; Polonator - Dover/Harvard; HeliScope Single Molecule Sequencer - Helicos) and quickly replaced the first generation of Sanger sequencers for genome projects, reducing the cost of DNA sequencing by several orders of magnitude. This promoted the access to these biotechnologies to other groups of users than those restricted to major genome centers or large consortia (Shendure et al., 2004). These second generation sequencing methods operate in the same way: a set of oligonucleotide probes is used to capture the desired sequences from total genomic DNA. These sequences are then amplified in a single PCR reaction using common linkers or adaptors, originally attached either to the probes or to the genomic DNA as primers. Finally, PCR reaction 'enriched' for the desired target sequences is sequenced by synthesis.

In 2004, the US National Institutes of Health (NIH) launched a new challenge to the scientific community: sequence one human genome for US$1000 (Service, 2006). Although this objective has not yet been reached, the latest generation of sequencers (called 'third generation') will make it feasible. In particular, the theoretical potential of single-molecule/nanopore sequencing is undeniable (Tersoff, 2001; Branton et al., 2008). A nanopore-based device enables the detection of nucleobases by electrophoretically driving DNA or RNA molecules in solution through a nano-scale pore without PCR amplification or labeling, thereby providing a unique and inexpensive analytical capability. These 'third generation' instruments offer the prospect of sequencing a diploid mammalian genome for around US$1000 in 24 h (Blow, 2008).

Several alternative low cost sequencing technologies are also under way and even potentially more cost-effective; one example is the Pacific Biosciences technology that claims to sequence a complete diploid genome for less than US$100 (Levene et al., 2003; Eid et al., 2009). This means that in the immediate future, any research project in livestock genetics can take the opportunity of analysing the entire genome of an individual and generate Gigabases of DNA sequence data.

However, in this new era of rapidly evolving technologies and availability of exhaustive data sets, some near-term challenges should be considered to fully exploit the landscape genomics approach: the development of robust protocols for molecular data production, the availability of adequate computational platforms, the development of bioinformatic pipelines for data handling and analysis, and the reformulation of experimental design methods.

Regarding the last topic, large scale sampling of high perfomance standardised livestock breeds and native populations adapted to different endemisms, climates, and management systems should be designed to exploit geographic criteria. Enough biological material should be collected to meet the requirements of the new technologies and appropriate methodologies have to be elaborated or enhanced to carry out whole genome comparative analysis. The overcome of these current challenges will offer a priceless opportunity to detect the genomic regions and genes under adaptive selection or underpinning disease resistance.

COMPUTATIONAL ISSUES

A final and very important issue to enable landscape genomics 'take-off' is the design and validation of a formal methodology and related tools for studying genome-environment relationships. This should include robust sampling strategies across areas of traditional breeding, efficient computer infrastructure to handle whole genome data, as well as eco-climatic parameters, computing resources (computer grid facilities for instance) to provide enough processing power for a large number of users and easy-to-use software for the analysis and visualisation of the results.

The upcoming whole genome revolution shortened our time to pave the way. With regard to GIS, computational and statistical aspects, the importance must be emphasised of including the recording of geographic coordinates in any new project requiring animal sampling campaigns; it is also necessary to enhance methods for statistical analysis and develop adapted software solutions. Although specific methodological developments (capacity to process ordinal and nominal association models, or to develop spatial statistics for instance) and practical improvements (easy-to-use graphical interface, web-based platform) are still necessary, the calculation process for association models is rather straightforward. Thus, the challenge will mainly consist of improving the efficiency of algorithms, in making software applications usable by both supercomputers and computer grids, and in providing users with a centralised access to analytical tools, as well as to environmental data. These are prerequisites for fully exploiting the opportunity to analyse the complete genome of hundreds of thousands of animals worldwide, and to associate genetic variations with the hundreds of variables that are progressively constituting enhanced environmental data sets. In other words, we must be prepared to handle models potentially composed of millions of single nucleotide polymorphisms (SNPs) and hundreds of eco-climatic parameters corresponding to hundreds of thousands of individuals.

CONCLUSIONS

The landscape genomics approach is promising. Applied to livestock, it should integrate geographical distribution of breeds, their genetic diversity, as well as climatic, ecological, epidemiological and production system information related to the place where animals are reared. It can be used to understand the genetic basis of animal adaptation to the environment, as well as useful information towards optimised breed conservation and management strategies (Long, 2008). It is likely to favour a better management of farm animal genetic resources (FAnGR), as described in the First International Technical Conference on Animal Genetic Resources for Food and Agriculture in Interlaken, 2007 (ILRI, 2007).

Methods, tools and data are now available to detect the footprint of selection driven by environmental parameters. On the basis of existing data, and before the advent of the imminent 'paradigm change in genomics' (see Conference on Next Generation Sequencing: Challenges and Opportunities; http://ngs2009.uab.es/), it is already possible to characterise - to a certain extent - the landscapes to which livestock breeds are best adapted. Together with the integration of global warming models, it is also possible to forecast the consequences of climate changes on breed surviving ability, and to simulate different scenarios for predicting population demographic trajectories and adopting appropriate measures to reduce the risk of extinction. However, forthcoming whole genome data sets will really made us turn to a new dimension of analysis, and therefore lead to new ways to assess genetic diversity. From then on, all conditions will be achieved to enable the real landscape genomics take-off.

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Tandem Inhibin Gene Immunisation to Induce Sheep Twinning

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ABSTRACT

To study the effect of sheep twinning after tandem inhibin gene immunisation, the recombinant plasmid of tandem inhibin were constructed with inhibin α-subunit (1–32) of pig and complement 3d (C3d) of sheep by real-time polymerase chain reactions (RT-PCRs) and used to immunise sheep. The results showed that the recombinant plasmids, pcDNA-DPPISS-DINH and pcDNA-DPPISS-DINH-sC3d3 were constructed successfully. Expression products were secreted after BHK-21 cells were transfected with the recombinant plasmids. After immunisation with 0.4 mg DINH and 0.3 mg DINH-sC3d3, twinning rates were 12.5% and 25.0% respectively, which were significantly higher ($P < 0.05$) than the control group. However, there was no significant association between twinning rate and immunisation dosage with either antigen. On the basis of these preliminary studies, it is concluded that recombinant plasmids of tandem inhibin gene form a sound theoretical and technical basis for developing an inhibin-based-gene as a vaccine for increasing twinning and reproductive efficiency in sheep. However, further investigations involving more animals are required to determine the most effective dosage and timing of vaccination as well as choice of adjuvant for eliciting an optimal immune response for increasing twinning rates.

Key words: *inhibin, gene immunisation, twinning rate, sheep.*

INTRODUCTION

Inhibin is a type of glycoprotein hormone secreted by testicular sertoli cells and ovarian granulose cells. The protein is structurally a heterodimer composed of two sub-units α and β. Inhibin influences mammalian reproductive performance by regulating secretion of follicle stimulating hormone (FSH) (De Kretser et al., 2000; Medan et al., 2007; Padilla et al., 2007). The development of animal follicles and fertility could be improved by inhibin immunisation. Active immunisation against inhibin increased FSH secretion and ovulation rate in females (Anderson et al., 1998; Medan et al., 2003; Sasaki et al., 2006), and passive immunisation also increased FSH secretions in young adult male Shiba goats (Araki et al., 2000). While the use of active and passive immunisation against inhibin in animal production is restricted by difficulty in preparation and high cost, development of a gene vaccine offers potential to make it practical and effective to improve the reproductive performance of sheep by inhibin immunisation.

Immune technology involving inhibin gene is now becoming a hot research area for improving lambing rates as its efficiency, stability, ease of production and delivery offer potential for circumventing the deficiencies of traditional methods like genetic selection, embryo transfer, superovulation, hormonal induction etc. Different types of inhibin gene vaccines have been constructed and used to immunise mice (Jiang et al., 2002), rats (Mao et al., 2004), sheep (Zhang et al., 2004) and cattle (Cui et al., 2006), with the aim of improving follicular development, ovulation and the number offspring produced, but results to data have been somewhat disappointing. It is therefore necessary to explore novel methods of inhibin production and new strategies involving immunologically-based reproductive technology. To examine the feasibility of developing the inhibin gene as a vaccine for sheep, we constructed a recombinant plasmid of the tandem inhibin gene and investigated its effect on sheep twinning after immunisation.

MATERIALS AND METHODS

Experimental Animals

Sixty adult Gansu Alpin Merino (GAM) ewes were randomly selected from a sheep population of 758 individuals from the Huangcheng sheep breeding enterprise in Gansu Province. These ewes were healthy, being subjected to routine vaccinations and anthelmintic treatments while grazing on wild grassland. The reproduction and twinning rates of the animals averaged 68.3% and 1.3%, respectively.

Gene Sequences and Synthesis

The gene sequence ecoding the α-subunit (1–32) of inhibin in pig was synthesised by AugCT Biotechnology (China, Beijing) and used as the template in the PCR. For this, two pairs of primers containing endonuclease sites were designed according to the gene sequence. The forward inhibin (FINH) gene fragment was obtained from PCR by primers F1 (5'-**AG**GAATTCATGTCCACCGCC-3', containing a *EcoR* I site) and R1 (5'-**AC**TCTAGA TCTGTGGCAGT C-3', containing a *Xba* I site), while the reverse inhibin (RINH) gene fragment was obtained by primers F2 (5'-**TT**TCTAGATCCA CCGCCCCTCTG-3', containing a *Xba* I site) and R2 (5'-**CG**AAGCTTTTA TCTGTGGCAGTCGGC-3', containing a *Hind* III site). The amplicons of both genes were later cloned into the plasmid vector T-easy via restriction endonucleases and then transformed into host strain JM109. The positive recombinant bacteria were identified and the plasmids extracted. The tandem inhibin gene was obtained through relevant endonuclease digestion and the recombinant plasmid pcDNA-DPPISS-DINH constructed by

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N.E. Odongo, M. Garcia & G.J. Viljoen (eds), Sustainable Improvement of Animal Production and Health. Food and Agriculture Organization of the United Nations, Rome, 2010: **191–194**

incubating the tandem inhibin gene with pcDNA3.1 and dog preproinsulin signal sequence (DPPISS) in a solution of T4 DNA ligase.

The complement 3d (C3d) gene sequence of sheep was amplified by real-time PCR (RT-PCR) from the mRNA in liver tissue and the primers used were designed according to the sequence (GenBank: EF681138) retrieved from GenBank (Yue et al., 2008). The complement 3d gene fragment was inserted into pGEM-T Easy vector and the pGEM-sC3d obtained. The recombinant plasmid pcDNA-DPPISS-DINH-sC3d3 (pcDNA dog preproinsulin signal sequence double inhibin sheep C3d was constructed by linking pGEM-sC3d with pcDNA-DPPISS-DINH.

The recombinant plasmid pcDNA-DPPISS-DINH-sC3d3 was extracted and used to transfect BHK-21 cell line by liposome-mediated transfection. The expression level of fusion proteins DINH and DINH-sC3d3 were determined using Western blot.

Immunisation Schedules

The sixty adult ewes were randomly divided into five groups, each with 12 individuals. Animal treated with pcDNA-DPPISS-DINH were divided into two groups and immunised respectively with doses of 0.2 mg and 0.4 mg. Similarly, sheep transfected with pcDNA-DPPISS-DINH-sC3d3 (two groups) received either 0.3 mg or 0.6 mg. The control group was injected with 2 mL of saline water. Animals received three gene immunisations at 20-d intervals, the first immunisation being carried out 60 d before mating. Immunisation was by intramuscular injection and artificial insemination was conducted after oestrus.

The results obtained were subjected to SPSS11.5 analysis.

RESULTS

Tandem Inhibin Gene Cloning and Recombinant Plasmid Selection

The forward inhibin (FINH) and reverse inhibin (RINH) gene fragments amplified by PCR were both approximately 115bp (**Figure 1**). Meanwhile, the amplified tandem inhibin gene (DINH) varied between 200bp and 250bp (**Figure 2**), indicating that the two gene fragments amplified were linked together. Thereafter, the tandem inhibin gene (DINH) was subjected to digestion with *EcoR* I and *Hind* III. The two fragments obtained had similar lengths of those obtained by PCR amplification (**Figure 3**).

The recombinant plasmids pcDNA-DPPISS-DINH and pcDNA-DPPISS-DINH-sC3d3 were subjected to digestion with *EcoR* I and *Hind* III, respectively. As shown in **Figures 4** and **5**, the fragments

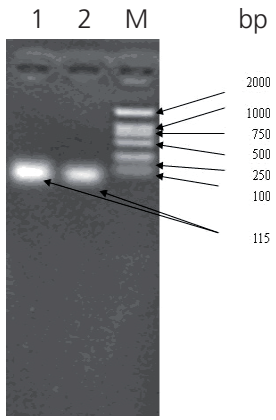

Figure 1. PCR amplification of FINH and RINH. 1 — FINH; 2 — RINH; M — Marker.

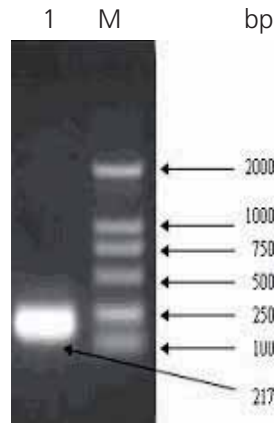

Figure 2. PCR amplification of DINH. 1 — DINH; M — Marker.

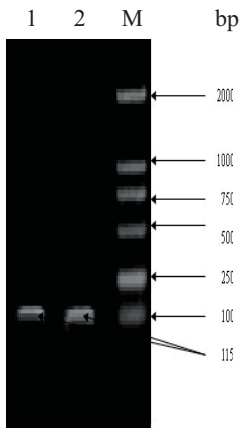

Figure 3. PCR amplification of FINH and RINH. 1 — FINH; 2 — RINH; M — Marker.

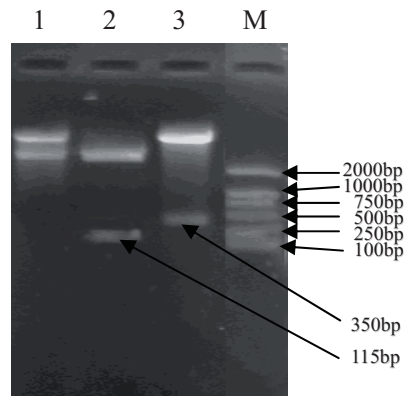

Figure 4. Identification of recombinant plasmid pcDNA–DPPISS–DINH. M — DNA marker, (2000bp); 1 — pcDNA–DPPISS–INH–C3d3, (*Bam*H I/*Xba* I); 2 — pMD19–INH, (*Bgl* II/*Xba* I); 3 — pcDNA–DPPISS–DINH, (*Hind* III/*BamH* I).

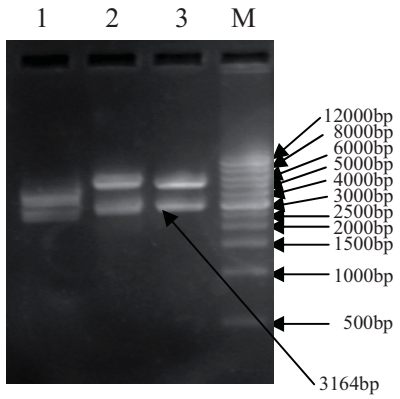

Figure 5. Identification of recombinant plasmid pcDNA–DPPISS–DINH–sC3d3. M — DNA marker, (500bp ~ 12000bp); 1 — pcDNA–DPPISS–DINH–sC3d3, (*EcoR* I/*Xba* I); 2 — pSG–sC3d3, (*Bgl* II/*Xba* I); 3 — pcDNA–DPPISS–DINH–sC3d3, (*BamH* I/*Xba* I).

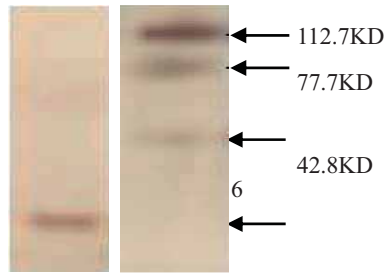

Figure. 6. Western blot analysis of expressed proteins of pcDNA–DPPISS–DINH and pcDNA–DPPISS–DINH–SC3d3 in BHK-21 cells.

obtained were conformed to the design requirements of recombinant plasmids.

Detection of Protein Expression by Recombinant Plasmids

Endonuclease digestion and DNA sequencing confirmed the correctness of recombinant plasmid construction. As indicated in **Figure 6**, the fusion protein DINH of DINH-sC3d3 was expressed in the cell line BHK-21 transfected with the recombinant plasmids pcDNA-DPPISS-DINH and pcDNA-DPPISS-DINH-sC3d3. The expressed proteins from the two different constructs showed differences between the two lanes.

Twinning Rate in Immunised Sheep

The results of sheep twinning after tandem inhibin gene immunisation are shown in **Table 1**. Chi-square test indicated that the twinning rates of sheep in the immunised groups were significantly higher ($P < 0.05$) than those in control group (animals which did not lamb excluded form analysis). Twinning rates were not different between sheep immunised with 0.2 mg and 0.4 mg, but rates in the groups immunised with 0.3 mg and 0.6 mg were significant in statistical difference ($P < 0.05$).

DISCUSSION

Anderson et al. (1998) immunised Merino ewes with different inhibin alpha subunit peptides conjugated to human serum albumin, and found that immunisation with synthetic inhibin peptides 10–26, 13–26, 7–13, 1–6 resulted in lower inhibin antibody titres and ovulation responses which were associated with increased FSH or ovulation rate, compared with that of longer peptides 1–32, 1–26, 7–16, 8–30. Immunisation with inhibin α-subunit (1–32) has been shown to be better than others (Mayo et al., 1986; Anderson et al., 1998), indicating that the inhibin α (1–32) fragment is an effective antigen when conjugated with a large protein (Mao et al., 2003). In our study, we compared the amino acid sequences of the α-subunit (1–32), mature peptide and precursor protein of inhibins from pigs, cattle, sheep and the mouse. These comparisons indicated higher homologous amino acid sequences in the α-subunit of porcine inhibin than in the α-subunit of bovine, sheep, mouse and rat inhibin; amino acid sequences in α-subunit also showed higher homology with other protein sequences in the pig. In this sense, with the complete gene sequence of the α-subunit of porcine inhibin being used for immunisation, immune-reactions should occur between the antibody produced and other types of protein in the body. However, the amino acid sequence of α-subunit (1–32) not only exhibited immunogenicity of inhibin, but also elicited no immune-reaction with other body proteins. Therefore, its nucleotide sequence is an ideal region for

Table 1. The reproduction rate of sheep in different groups after three immunisations.

Group	Twins	Singles	No lambing	Total	Number of lambs born	Twinning rate
0.2 mg DINH	1	8	3	9	10	11.1[b]
0.4 mg DINH	1	7	4	8	9	12.5[b]
0.3 mg DINH-sC3d3	2	6	4	8	10	25.0[c]
0.6 mg DINH-sC3d3	1	6	5	7	8	14.3[b]
Control	0	8	4	8	8	0[a]

Note: Value in the same column with different superscripts mean significant difference values ($P < 0.05$); same superscripts mean no statistical difference ($P > 0.05$).

gene cloning and recombinant plasmid construction. In this work, the recombinant plasmid constructed contained the nucleotide sequence of the N-terminal α-subunit (1–32) of inhibin. Furthermore, we improved the immune efficiency by using two tandem antigen determinants and we expected to improve the reproductive efficiency through gene immunisation by using recombinant plasmids.

As is well known, immune reactions can be promoted by injecting an adjuvant together with the antigen. C3d is a fragment produced by the pyrolysis of C3 during complement activation. As a molecular adjuvant, C3d could decrease the activation threshold of B cells and improve the processing and presentation of antigen. C3d could also promote antibody production and affinity. On the other hand, C3d could transform the immune response from one involving TH1 cytokines to a TH2-type cytokine response, thereby promoting humoral immunity. In this work, the nucleotide sequence of C3d was amplified by RT-PCR, and the primary and advanced structures of cloned gene sequences were predicted using specific computer programs (Yue et al., 2008). By immunising sheep with recombinant plasmids containing the molecular adjuvant C3d, the maximum twinning rate of sheep was as high as 25%, which provides a theoretical and technical basis for developing the inhibin gene as a vaccine for sheep.

Inhibin gene immunisation could counteract the levels of inhibin produced in the body, thereby increasing the level of FSH secreted and in turn promoting multiple ovulation and improving the reproductive performance of mammals (Medan et al., 2007). Currently, research on inhibin gene immunisation involves mainly single-copy gene immunisation (Zhang et al., 2004; Cui et al., 2006), with multiple-copy gene immunisation being rarely reported. However, Cao et al. (2008) constructed the recombinant plasmid of bi-copy inhibin gene (*pcISI*) and successfully used this to immunise rats. After immunisation, the twinning rates of sheep weres 12.5% and 25.0%, respectively, which was significantly higher ($P < 0.05$) than the control group. We therefore suggest that tandem inhibin gene immunisation can regulate the level of FSH secretion in sheep, promote follicular development and maturation, stimulate multiple ovulation and induce sheep twinning. However, the twinning rates recorded need further improvement, possibly by changing, the dosage of inhibin gene for immunisation and/or the selection of adjuvant.

CONCLUSIONS

In this experiment, the α-subunit (1 to 32) gene in tandem inhibin was cloned. Also, a new type of molecular adjuvant, C3d, was cloned from the liver tissue of sheep. Recombinant plasmids, pcDNA-DPPISS-DINH and pcDNA-DPPISS-DINH-sC3d3 were constructed and expressed successfully. After immunisation, the twinning rates of sheep varied between 12.5% and 25.0% which was significantly higher ($P < 0.05$) than the control group. The construction of recombinant plasmids of tandem inhibin gene provides the theoretical and technical basis for developing the inhibin gene as a vaccine for sheep.

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Factors Affecting Age of Puberty and the Response of Syrian Female Awassi Sheep to FGA and eCG Treatment

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ABSTRACT

Two experiments were conducted on Syrian female Awassi sheep to characterise certain parameters during various reproductive stages. In experiment 1, 18 ewe lambs were tested at 5 months of age to assess pubertal parameters and affecting factors. The overall average age at puberty was 18.0 months, occurring between May and August (during the normal breeding season). There were no significant differences in time to reach puberty between ewe lambs in terms of the month of birth, type of birth (single or twin) or weaning weight. The average live weight (LWt) and serum progesterone concentration of ewe lambs at puberty were 53.7 kg and 6.32 nmol/L, respectively. A positive and significant correlation ($r = 0.72$, $P < 0.001$) was found between progesterone concentration and LWt of lambs. In experiment 2, 16 nulliparous cyclic Awassi ewes, 21 months of age, were treated with intravaginal sponges containing 40 mg of flugestone acetate (FGA) for a period of 14 d during the breeding season. Eight animals (Group P) were then injected intramuscularly at sponge withdrawal with 500 IU of equine **c**horionic gonadotropin (eCG), the remainder (Group C) acting as controls. All females exhibited oestrus and were mated within 3 d of sponge withdrawal. Twinning rates were 37.5% and 12.5% respectively for the animals in Groups P and C ($P < 0.05$). It is concluded that it is possible to improve the twinning rate of nulliparous Syrian Awassi ewes in their first pregnancy using eCG with no adverse effects on either the ewes or the lambs born.

Key words: *Awassi sheep, puberty, progesterone, intravaginal sponges, equine chorionic gonadotrophin, twinning.*

INTRODUCTION

There is a threshold of LWt necessary for the attainment of puberty in the first breeding season, and when LWt was below that threshold, the first ovulation in Mouflon and Manchega ewe lambs did not occur until the beginning of the next breeding season, despite minimal further growth (Moreno et al., 2000). Galmessa et al. (2003) indicated that Horro ewe lambs tended to breed at similar LWt, but attained puberty at different ages. Nakada et al. (2002) suggested that the development of capacity to secrete LH in response to gonadotrophin-releasing hormone (Gn-RH) before puberty is one of the factors for deciding the time at puberty in heifers.

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However, recent research suggests a pivotal role for the hormone leptin (Pittroff, et al., 2008). Leptin has been reported to be required for the normal onset of puberty (Chehab et al., 1997), and to have direct effects through steriodogenesis on the ovary (Ryan et al., 2002). Yu et al. (1997) found that leptin not only stimulates luteinising hormone releasing hormone (LHRH) in the rat but also stimulates the release of luteinising hormone (LH) and follicle stimulating hormone (FSH) from anterior pituitary cells *in vitro*.

Differences have been reported regarding age at puberty and LWt of ewe lambs in different breeds (Parawan et al., 1987). Also, some researchers found relationships between the onset of puberty and the type of birth (Younis et al., 1978) or weaning weight (Mukasa-Mugerwa et al., 1991), and some have reported no effect of the lambing season on the age of puberty (Lopez-Sebastian et al., 1985), whereas such an effect was reported by others (Papachristoforou et al., 2000).

Synchronisation of oestrus has been recently and widely performed in small ruminants to improve reproductive efficiency and management (Al-Merestani et al., 1999). For this purpose, intravaginal sponges containing synthetic progestagens, namely MAP (medroxyprogesterone acetate) (Kausar et al., 2009) and FGA (flougestone acetate) (Letelier et al., 2009) are used. *Equine chorionic gonadotropin* has been used with the sponge treatment to improve fecundity (Lamrani et al., 2008), and Saloia ewes in Portugal (Silva et al., 2003) and Awassi ewes in Syria (Zarkawi and Soukouti, 2009) treated with eCG had a higher number of follicles over 5 mm in diameter in the ovaries than untreated animals.

The Awassi is a fat-tailed triple purpose and the most important sheep breed in Middle Eastern countries. Its desirable traits, such as the popularity of its meat and milk, high adaptability to different ecosystems, resistance to disease and tolerance to extreme temperature, and endurance of adverse management and feeding conditions (Sleiman and Abi Saab, 1995; Abi Saab and Sleiman, 1995; Salhab et al., 2003) have encouraged breeders in many countries to raise Awassi sheep.

Syrian Awassi sheep (about 23 million, AODA, 2009) are seasonal breeders, mate between June and September (Zarkawi, 1997), and normally lamb once annually. Moreover, they have a relatively poor reproductive performance and a low twinning rate (Thomson and Bahhady, 1988). However, there are no available data on age at puberty in Syrian Awassi ewe lambs using reproductive hormones, such as delineating the age and LWt at which the first elevation in progesterone concentration occurs, followed by a normal oestrous cycle. The availability of such data is essential for studying the reproductive physiology of this breed. Moreover, the effects of intravaginal

N.E. Odongo, M. Garcia & G.J. Viljoen (eds), Sustainable Improvement of Animal Production and Health. Food and Agriculture Organization of the United Nations, Rome, 2010: **195–200**

sponges on certain parameters of the young Syrian Awassi ewes in their first reproductive cycle have yet to be determined.

The main objectives of the current study were therefore to determine the LWt and age at puberty in ewe lambs as well as some affecting factors, and to assess the response of nulliparous ewes following insertion of intravaginal sponges and injection of eCG.

MATERIALS AND METHODS

Location and Experimental Animals

The two experiments were carried out at the Division of Animal Production, Der Al-Hajar area, 33 km south-east of Damascus (33°21'N, 36° 28' E; 617 m above sea level). This is a dry area with an annual rainfall of about 100 mm, and in many respects resembles the Syrian steppe region where the majority of sheep are raised.

Animal Housing and Feeding

Animals were kept indoors at night and outside for most of the day. Indoors, the animals were offered diets based on barley and wheat straw supplemented by vitamins (High Vet, Safco Vet Products, Damascus). Outdoors, they had free access to natural grazing. Water and mineral licks (Phosphadin, Al-Shark Vet Products, Damascus) were available *ad libitum*.

Experiment 1

Experimental Animals

Eighteen Syrian Awassi ewe lambs (9 singles and 9 twins), born between December and March were used for a period of 16 months, starting at an age of 5 months and an average LWt of 24.6 ± 4.6 kg. The average birth and weaning weight at 3 months of age of these ewe lambs were 4.7 ± 0.8 and 22.5 ± 5.5 kg., respectively.

Experiment 2

Experimental Animals and Hormonal Treatments

Sixteen cyclic Syrian Awassi ewe lambs (8 singles and 8 twins), aged 21 months and an average LWt of 55.5 ± 6.5 kg., were used for a period of 8 months. Females were randomly allocated in August (during the breeding season) into two equal groups, an experimental (P) and a control (C). Animals in both groups were treated with intravaginal sponges containing 40 mg of FGA (Chronogest®, Intervet International B.V., The Netherlands) for a period of 14 d. However, only females in the P group were injected intramuscularly at sponge withdrawal with 500 IU of eCG (Folligon, Intervet International B.V., The Netherlands).

Oestrus Detection and Mating

Three fertile Awassi rams were introduced daily (08.00 h–14.00 h) into all females in both groups 24 h after sponge withdrawal for oestrus detection and mating (all females were mated within 3 d). Rams were separated from the females until the following day. All females that were in oestrus and mated were recorded.

Blood Sampling and Progesterone Analysis

Blood samples (10 mL) were taken from the jugular vein of all animals twice weekly (at 10.00 h) starting at 5 months of age and continuing for a period of 16 months in the first experiment (n=18) and from the ages of 21 months until 29 months in the second experiment (n=16). Serum was prepared by centrifugation of blood at 3 000 rpm for 20 min., and stored at –20 °C until assayed using validated progesterone RIA kits (COAT-A-COUNT, DPC, USA). The intra-assay coefficient of variation was 7.2% and the inter-assay coefficient of variation was 7.4%. Progesterone levels equal to or exceeding 3.18 nmol/L were indicative of normal luteal function, while levels under 3.18 nmol/L were indicative of anoestrous, follicular, or the early luteal phases of the oestrous cycle (Zarkawi, 1997).

Statistical Analysis

Data were subjected to analysis of variance (ANOVA) using a Statview-IV programme (Abacus Concepts, Berkeley, CA, USA) on an IBM system. In addition, a correlation analysis was used to determine the relationship between blood serum progesterone concentrations and LWt of ewe lambs used in Experiment 1.

RESULTS

Experiment 1

The different parameters related to puberty of the ewe lambs are illustrated in **Table 1**. Based on the first elevation in serum blood progesterone to a concentration exceeding 3.18 nmol/L, as an indicator for active corpora lutea (Zarkawi, 1997), followed by the appearance of regular oestrous cycles as a criterion for the attainment of puberty, it was found that puberty was reached during the second breeding season after birth between May and August at the following rates: May (16.7%), June (27.8%), July (33.3%) and August (22.2%).

The data presented in **Table 2** also indicate that neither the type of birth (singles or twins), nor the month of birth (December, January-March) had a significant effect on the time to attain puberty. Likewise, a high weaning weight of ewe lambs had no significant effect on the age of puberty despite the significant difference ($P < 0.05$) between the two weights (26.3 kg and 17.8 kg respectively) at three months of age.

Relationship between Serum Concentration of Progesterone and Live Weight

Average serum progesterone concentration and LWt of the lambs during the period from 5 months of age until puberty are shown in **Figure 1**. A positive and significant correlation ($r = 0.72$, $P < 0.001$) was found between these parameters during the experimental period.

Experiment 2

Table 3 gives some reproductive parameters for the groups of nulliparous Awassi ewes (P and C), as affected by the eCG intramuscular

Table 1. Live weight, age, and blood serum progesterone concentration (mean ± SD) in 18 Syrian Awassi ewe lambs at 5 months of age and at puberty.

	At 5 months of age	At puberty
Body weight (kg)	24.6 ± 4.6	53.7 ± 7.2
Age at puberty (month)		18.0 ± 1.0
Progesterone concentration (nmol/L)	0.3 ± 0.3	6.3 ± 3.7

Table 2. Effects of litter size, month of birth and weaning weight on age at puberty in Syrian Awassi ewe lambs.

	At 5 months of age	At puberty
Litter size	Single Twin	18.1[a] 17.9[a]
Month of birth	Before 1st January After 1st January	18.4 [a] 17.6[a]
Weaning weight	High (Mean: 26.3 ± 2.9 kg) Low (Mean: 17.8 ± 3.8 kg)	18.3[a] 17.7[a]

Means within a parameter with different superscripts are significantly different ($P < 0.05$).

Table 3. The effect of intramuscular injection with eCG on some reproductive parameters of the nulliparous Syrian Awassi ewes employed in experiment 2.

Parameter	*Group P (n = 8)	**Group C (n = 8)
Mating weight (kg)	56.0 ± 6.1[a]	55.5 ± 8.0[a]
Mating rate (%)	100[a]	100[a]
Lambing rate (%)	100[a]	100[a]
Weight after lambing (kg)	61.5 ± 6.3[a]	60.8 ± 6.4[a]
Duration of pregnancy (d)	151.4 ± 1.8[a]	151.0 ± 1.5[a]
Twinning rate (%)	37.5[a]	12.5[b]

[a,b] Means, within a row, followed by different small letters are significantly different ($P < 0.05$).

* The experimental group receiving a muscular injection of 500 IU eCG at sponge withdrawal.

** The control group that was not injected with eCG after sponge withdrawal.

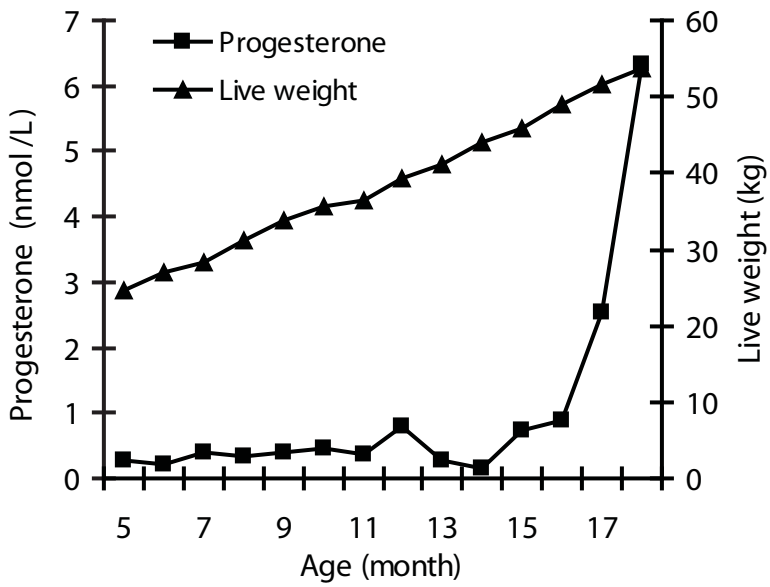

Figure 1. Average blood serum progesterone concentration and mean live weight of Syrian Awassi ewe lambs.

injection. All the females exhibited oestrus and were mated within 3 d after sponge withdrawal (87.5% within 2 d).

Serum progesterone concentration at sponge withdrawal was very low averaging 0.43 ± 0.19 nmol/L. However, this basal concentration increased within 5 d to levels exceeding 3.18 nmol/L, remained high throughout pregnancy to term and decreased sharply to concentrations below 2.0 nmol/L just after lambing.

Twinning rate was 37.5% in animals that received eCG (Group P) as compared with 12.5% for those in the control group C; the difference was significant ($P < 0.05$).

There were no problems during delivery and the lambs born together with their mothers were healthy; mortality from birth to weaning at 3 months of age was zero in both groups.

DISCUSSION

This study provides additional information on the age at puberty in local Awassi female sheep as well as other related information and adds to previous observations (Zarkawi et al., 1999; Zarkawi, 2000; Zarkawi, 2004).

Blood progesterone concentrations have been widely used by researchers in many countries as a valuable indicator to monitor the age at puberty in some animal species, such as Shiba goats in Japan (Sakurai et al., 2004), Braford and Brahman x Angus heifers in the USA (Cooke and Arthington, 2009), Murrah buffaloes in India (Haldar and Prakash, 2005), and in some sheep breeds such as Charollais x Awassi, Romanov x Awassi in Jordan (Kridli et al., 2006) and Karagouniko in Greece (Valas et al., 2006).

Like in many other small ruminants, to attain puberty in Awassi ewe lambs, targets in both age and LWt have to be achieved, since both are involved in activating the Gn-RH pulse generator in the brain and trigger puberty (Adam and Robinson, 1994). Ewe lambs in the current study did not reach puberty during the first breeding season (at 6 - 9 months of age). This can be explained by the animals' failure to reach a threshold of LWt and/or age necessary for the attainment of puberty in the first breeding season. The onset of puberty is associated with an increased frequency of luteinising hormone (LH) pulses, stimulating follicular development, a sustained increase in oestradiol secretion, a preovulatory LH surge, and ovulation (Foster et al., 1985). In ewe lambs below the LWt threshold, initiation of frequent LH pulse secretion is inhibited (Rhind, 1992).

Based on the first rise in serum blood progesterone concentration >3.18 nmol/L, followed by a regular oestrous cycle, the average LWt and age at puberty in the current study were 53.7 kg and 18.0 months, respectively. Using a similar criterion, Abella et al. (2005) reported that the age and body weight at puberty was similar in three genotypes of ewes (Fec^BFec^+, Fec^+Fec^+ Booroola x Merinos d'Arles and Merinos d'Arles) (332.5, 334.8, 330.8 d and 34.1, 34.1, 34.9 kg, respectively).

Breed-related differences in both the age and LWt of ewe lambs at puberty have been reported. Parawan et al. (1987) reported an average of 13.2 months and 19.5 kg at puberty in Philippine ewe lambs, whereas the corresponding figures for Iranian Mehraban ewe lambs were about 8 months and 44 kg (Bathaei and Leroy, 1997). Kridli et al. (2006) reported that crossing Awassi ewes with either Charollais or Romanov sires in Jordan improved the reproductive characteristics of the F_1 crossbred by advancing age at puberty.

The present data indicate that ewe lambs born between December and March reached puberty in the same breeding season indicating that there was no effect of lambing month on the attainment of puberty. Spanish Mouflon lambs born in March/April and that reached a minimum threshold body weight (23.8 kg) in their first breeding season reached puberty at 8 months of age whereas in those with slower growth rates, the prepubertal period was extended throughout the first breeding and non-breeding seasons, with puberty being reached during the breeding season of the following year at 19 months of age and 27 kg body weight (Santiago-Moreno et al., 2001).

In our study, all females treated with FGA plus eCG showed oestrus behaviour and were mated within 3 d after withdrawal of the sponges, became pregnant to term and lambed normally with no adverse effects on either themselves or their lambs. This indicates that nulliparous Syrian Awassi ewes could respond to the above treatments at an early age (21 months) with no effect on mating rate, duration of pregnancy, health of lambed ewes and lambs born or on the weight of the lambs. Similar results were reported by Zarkawi (2001) on adult Syrian Awassi ewes treated with MAP + eCG. Hamra et al. (1988) treated Iraqi Awassi ewe lambs aged 8–10 months with intravaginal sponges, and found that 77% of the treated lambs showed oestrus behaviour and were mated, but none of them became pregnant, indicating that they had either not attained the proper LWt and/or were not old enough; thus, the ovarian follicles had not reached the preovulatory stage.

Syrian Awassi ewe lambs responded in their first mating to 500 IU of eCG injection, twinning rates increasing from 12.5% in untreated ewe lambs to 37.5% in eCG-injected ones. Most probably this would have a positive impact on farmers' incomes. The fact that all ewe lambs started cycling after FGA + eCG treatment without problems is a further advantage of using such treatment. An increase in twinning rate from 20% in sponge-treated adult Syrian Awassi ewes without eCG intramuscular injection to 50% in eCG-injected ewes was reported by Zarkawi (2001) and using a similar procedure (sponges + eCG), lambing rates increased from 153% in untreated to 206% in treated Suffolk ewes (Tetuska et al., 1988) and from 100% to 134% in Karaman, Tuj and Turkish Awassi (Atsan et al., 2007).

In the current study, there was no significant difference in the duration of pregnancy between treated (151.4 d) and untreated (151.0 d) young ewes. A similar duration of pregnancy (152.0 d) was reported by Zarkawi (1997) in untreated adult Syrian Awassi ewes during the breeding season, confirming that the hormonal treatment had no effect on the duration of pregnancy.

CONCLUSIONS

Syrian Awassi ewe lambs attained puberty stage during the second breeding season after birth at an age of about 18 months and a LWt of around 54 kg. The month of lambing, birth weight, type of birth and weaning weight had no effect on the attainment of puberty.

Nulliparous Syrian Awassi ewes responded well to hormonal treatments (FGA + eCG) in terms of oestrus synchronisation and mating, with no effect on the duration of pregnancy, birth or weaning weight. Injection of eCG could be safely employed to improve twinning rate.

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Reproductive Performance following Artificial Insemination in Sanga and Crossbred (Friesian × Sanga) Cows in the Accra Plains of Ghana

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ABSTRACT

The performance records of Sanga and Friesian-Sanga crossbred cows kept at the artificial insemination (AI) Centre of the Amrahia dairy farm in the Accra plains of Ghana between the period January 1998 to December 2007 were evaluated. The intervals from calving to first AI, calving to conception, and calving interval were prolonged especially in the Sanga cows inseminated with Friesian semen, averaging 158.8±8.9, 177.5±9.5 and 517.9±13.5 d respectively. In Friesian-Sanga cows bred with Friesian semen these intervals were respectively 115.7±19.2, 138.7±16.3 and 510.3±41.0 d. These parameters were not affected (P > 0.05) by season of calving preceding AI and season of insemination. The conception rates at first AI service and for all inseminations were low in the Sanga (42.6% and 46.0% respectively) and in crossbred cows (53.5% and 53.4% respectively). They were not affected (P > 0.05) by the season of insemination. Improving the nutritional status of the cows through strategic supplementation coupled with effective heat detection techniques, appropriate timing of AI, as well as efficient methods of storage, transport and handling of semen should improve the reproductive performance of cows.

Key words: *reproductive performance, artificial insemination, Sanga, Friesian × Sanga, conception, calving intervals.*

INTRODUCTION

The Ministry of Food and Agriculture in Ghana began a five-year National Livestock Services Project in 1994 with the objective of increasing meat and milk production through breed improvement using AI to meet the protein needs of the population as well as reduce the country's increasing dependence on livestock and livestock products. The introduction of AI for breed improvement has, however, met some difficulties in Ghana. These include lack of appropriately designed breeding programmes and technical shortcomings including poor management practices, inadequate nutrition and occurrence of reproductive disorders.

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A major factor affecting the success of AI is the conception rate which in turn is influenced by several factors and their interactions including those related to the cow, management of animals, AI services, semen quality, bull fertility (Nordin et al., 2007) and high environmental temperatures or heat stress (Chebel et al., 2004). The main objective of this study was to evaluate the reproductive performance of Sanga and Friesian-Sanga (crossbred) cows kept at an AI Centre. This would enable the development of measures to improve the efficiency of the AI service provided to cattle farmers.

MATERIALS AND METHODS

Location of Experiment

The study was based on AI carried out between the period 1998–2007 on Sanga and Friesian × Sanga (crossbred) cows kept at the AI Center of the Animal Production Department's Amrahia dairy farm located at latitude 05° 46' N and longitude 00° 08'W in the Accra plains of Ghana. Total rainfall for the study period was 900.9 mm with an average daily temperature of 29°C. Rainfall was bimodal with peaks in June and October, April to July being the major rainy season, and September to November the minor rainy season. The driest months were January–March, August and December.

Management of Animals

The Sanga cows were grazed from 08.00 h–15.00 h on natural pastures comprising *Panicum maximum*, *Stylosanthes haemata, Sporobolus pyramidalis* and *Vertiveria fulvibarbis* which constitute the dominant grass species in the grazing area. They had access to water from a dam twice daily in addition to water provided in the animal house *ad lib.* The crossbreds were zero grazed. They were provided with *Panicum maximum,* sorghum and spent malt, in addition to a concentrate mixture based on maize, wheat bran, palm kernel cake with or without soyabean meal. Salt lick was always provided. The crossbreds had access to water in the animal house *ad lib.* Oestrus (heat) was observed for the two groups of cows twice daily at 06:00 h and 18:00 h. A cow standing to be mounted (standing heat) was used as the main criterion for the cow to be on heat and therefore ready for insemination. Cows observed to be on heat in the morning were inseminated in the evening of that day, while those which demonstrated signs of heat in the evening were inseminated the following morning. Friesian semen was used for insemination.

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The semen was imported from South Africa, and kept frozen under liquid nitrogen. Four sires were used in the AI programme.

Data Collection

Artificial insemination records from 126 Sanga and 35 Friesian × Sanga cows were used. These covered a 10-y period (January 1998 to December 2007). Parameters studied included interval from calving to first AI, interval from calving to conception, calving interval and conception rate. The effect of season of calving preceding AI and season of AI on calving to first AI, calving to conception, calving interval and conception rate were evaluated.

Conception rate at first service was estimated from the following equation:

$$\text{Conception rate at first service} = \frac{\text{No. of conceptions at first service}}{\text{Number of first services}} \times 100$$

$$\text{Conception rate} = \frac{\text{Number of conceptions}}{\text{Number of services}} \times 100$$

Statistical Analyses

The general linear models (GLM) procedure of the Statistical Analysis Systems Institute (SAS, 1999) was used in investigating d from calving to first AI, d from calving to conception, calving interval and the effect season of calving preceding AI and season of insemination on these parameters. The following model was applied:

$$Y_{ijk} = \mu + S_i + C_j + e_{ijk}$$

where Y_{ijk} = *d* from calving to first artificial insemination, *d* from calving to conception, calving interval.
μ — overall mean
S_i — effect of ^{i}th season of calving preceding AI
C_j — effect of the ^{j}th season of insemination
e_{ij} — a random error associated with each observation

Differences between means were tested by PDIFF/SAS.

The effect of season of insemination on conception rate was assessed using the Chi-square test.

RESULTS

Calving Intervals

The overall mean intervals from calving to first AI, calving to conception, and calving interval in the Sanga cows were 158.8±8.9, 177.5±9.5 and 517.9±13.8 d respectively. These parameters were not affected (P > 0.05) by season of calving preceding AI and season of insemination.

The overall mean interval from calving to first AI averaged 115.7±19.6 d in the Friesian × Sanga crossbred cows, while the mean interval from calving to conception was 138.7±16.3 d and the calving interval averaged 510.3±41.0 d. Neither season of calving preceding AI nor season of insemination influenced (P > 0.05) these variables.

Conception Rate (CR)

The CR at first service for the Sanga cows was 42.6%, and for all services it was 46.0%. The number of services per conception averaged 2.3. Season of insemination did not affect (P > 0.05) conception rate and number of services per conception.

The CR at first service for the crossbred cows was 54.5% and for all services it was 53.5%. The mean number of services per conception was 1.9. The season in which cows were inseminated did not affect (P > 0.05) conception rate and number of services per conception in crossbred cows.

DISCUSSION

The average calving to first service intervals of 158.8±8.9 d and 115.7±19.2 d obtained in this study for the Sanga and crossbreds respectively were long compared with periods considered to be economically desirable. This delay of first service after calving, particularly in the Sanga cows, may be due to prolonged postpartum anoestrus (interval from calving to the resumption of ovarian cyclicity), most likely a result of inadequate nutrition and suckling management (Jolly et al., 1995; Diskin et al., 2003).

The Sanga cows were grazed mainly on natural pastures and were not supplemented with either crop residues, agro-industrial by-products or energy or protein concentrates. During the dry season, the limited pasture available on the Accra plains is of poor quality; protein levels are low and the grasses become fibrous and highly lignified affecting their digestibility. In addition, there was lack of restriction on suckling by calves, cows being allowed to suckle their young until they were weaned naturally between six and nine months of age (Obese et al., 1999 and 2009). The low nutritional status of animals coupled with the prolonged suckling stimulus could delay normal resumption of ovarian cycles by interfering with the synthesis and secretion of hormones especially luteinising hormone and insulin-like growth factor-I which are important in ovarian follicular development and function in cattle (Williams et al., 1996; Diskin et al., 2003; Thatcher et al., 2006). Poor heat detection and silent heat could be additional factors accounting for the prolonged intervals from calving to first service in both the Sanga and crossbred cows.

The interval from calving to first AI was more prolonged in the Sanga cows and this may account for their extended calving to conception intervals (177.5±9.5 d) compared with the crossbred cows (138.7±16.3 d). Furthermore, the prolonged interval from calving to first AI obtained for the Sanga and crossbreds may have contributed to the extended calving to conception and calving intervals. Eduvie and Oyedipe (1991) reported that the main determinant of long calving intervals is a prolonged postpartum anoestrous interval.

The calving to conception and calving intervals obtained for Sanga cows inseminated with Friesian semen in this study were higher than the 155.2±4.5 d and 444.3±16.5 d respectively reported for the Sanga breed on smallholder peri-urban dairy farms on the Accra plains of Ghana (coastal savanna zone) (Obese et al., 1999). They were also higher than the values of 149.7±5.8 d and 43±6.7 d reported for the same breed on smallholder farms in the humid forest zone in Ghana (Osei et al., 1993).

The extended overall mean estimates for calving to first service, calving to conception and calving intervals obtained for the Sanga and crossbred animals studied here are unfavourable for profitable livestock production. Better management practices including improving the nutrition of cows by strategic feed supplementation especially during the dry season, as well as early weaning or restricted suckling of calves should shorten the postpartum anestrous period and subsequently reduce calving to conception and calving intervals in these herds. Treatments to synchronise oestrus can provoke an increase in plasma LH concentrations and hasten the onset of ovulation and ovarian cycles and thus improve the efficiency of AI programmes. Results from this study indicated that generally the reproductive performance of animals at the Amharia farm was lower than in cows owned by smallholder farmers in the Accra plains. This may be due to the fact that, whilst the private farmer seeks to maximise profit and therefore strive to provide adequate resources for farming, state-

owned farms tend to suffer from bottlenecks including the lack and timely release of funds and resources for farm operations. This tends to delay the implementation of the kinds of interventions suggested above for improved animal productivity.

The overall CR to first service was poor especially in the Sanga cows. The major reason for this low CR may be poor heat detection, inappropriate timing of AI, poor insemination technique or poor semen quality. The timing of insemination in relation to first detection of heat is critical for achieving high conception rates (Peters and Ball, 1995; Tjiptosumirat et al., 2007) as are factors relating to the transport, storage, handling and thawing of semen in the field (Peters and Ball, 1995). Putting in place very effective heat detection mechanisms could reduce undetected oestrus, while more appropriate timing of AI coupled with better transport, storage, handling and thawing of semen should improve the conception rate of cows.

CONCLUSIONS

The intervals from calving to first service, calving to conception and calving intervals were prolonged in Sanga and Friesian × Sanga crossbred cows kept on a government farm in Accra Plains of Ghana. Possible factors involved include prolonged postpartum anoestrus, a consequence of poor nutrition and suckling management especially in the Sanga cows. Conception rates were poor, probably due to one or a combination of management factors including poor heat detection, inappropriate timing of AI, poor insemination technique and low semen quality.

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Evaluation of Semen Quality of Three Boar Genetic Lines Reared in Intensive Units in Romania

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ABSTRACT

This paper reports the results of studies to establish the reproductive performance of breeder lines belonging to one of the most important providers of genetic material in Romania (the Pig Improvement Company, P.I.C.), within a top swine husbandry unit in Romania. Parameters measured in three PIC boars lines (PIC 1075, PIC 402, PIC 408) were sperm volume, spermatozoal concentration in sperm, total numbers of spermatozoa, number of doses produced etc. Ejaculate volumes ranged between 224 mL and 235 mL at between eight and 12 months of age, between 310 mL and 366 mL at 13–24 months old, between 330 mL and 348 mL between 25–36 months old and between 304 mL and 404 mL when 37–42 months of age. There were significant differences between both boar genetic lines and age periods. Comparing levels of spermatozoa in the semen of boar lines, PIC 1075 had an average of 372 × 10^6 spermatozoa/mL and PIC 402 had 311.5 × 10^6 spermatozoa/mL, a difference of 16%, or compared to PIC 408 (302.3 × 10^6 spermatozoa/mL) a difference of around 19%. The highest number of doses (21) was produced by the PIC 402 and PIC 1075 lines, but differences between groups were not significant. It is concluded that due to the high sperm concentration per ejaculate throughout the exploitation period, the use of the PIC boars studied could be improved by decreasing the interval between sampling.

Key words: *boar, genetic lines, sperm, ejaculate volume, motility.*

INTRODUCTION

Alignment with European standards requires pigs breeders in Romania to adopt new strategies for increasing the national swine herd and improving its genetic potential for increasing meat production. Especially important is putting in place systems to maximize exploitation of the genetic potential of hybrid stock in Romania.

The aim of genetic selection is to improve performance and ultimately profitability by incorporating the beneficial traits from a breed type while eliminating undesirable traits. Terminal sire lines have been shown to affect the reproductive traits of the sows with which they are mated. Young boars are used for reproductive activity from the age of 10–12 months until they are 36–42 months old. In some cases, however, they begin their reproductive activity at the age of 8–9 months (Bogdan et al., 1999; Păsărin, 1997; Stoica, 2003) with good reproductive performances.

The principal objective of this study was to examine the quality of semen produced by different boar types available in Romania at different ages.

MATERIALS AND METHODS

The study was carried on 15 boars, each line (PIC 1075, 402 and 408) being represented by five boars. The semen obtained from the 1075 boars is used to artificially inseminate PIC 1050 sows from the hybridisation farm of the unit, whereas semen from the boars of PIC 408 and PIC 402 lines, is used to artificially inseminate Camborough sows, with the resulting piglets earmarked exclusively for slaughter.

Accommodation and other conditions were similar for all boars and the age differences between them were minimal. Semen quality of the boars was assessed from the onset of reproductive activity until culling. The three first weekly series of ejaculations obtained when the animals were eight months old were not evaluated. Intensity of use was semen collection at 9 a.m followed by 5 d resting. A metallic dummy (1 m long and adjustable in height) was used for collecting semen using the gloved-hand technique.

After collection, each ejaculation was submitted to quantitative and qualitative evaluation in the company laboratory. Measurements included: ejaculate volume (ml), motility (percent), sperm concentration (× 10^6/mL), sperm/ejaculate (x 10^9), number of doses/ejaculate.

Semen concentration was determined using a Spermaque photo densitometer, motility was assessed by microscopy and based on the proportion of spermatozoa which moved straight forward. The semen extender used for preparing doses was the XCLL.

Age groups used for data analysis were between 8–12 months, 25–36 months and 37–42 months, when the boars were culled.

RESULTS AND DISCUSSION

Knowledge of the yield dynamics and quality of boar sperm allows specialists to optimise its use for maximising the production potential of the flock. The results obtained for the boar genetic lines investigated here are shown in **Table 1**.

Ejaculate volume of the three bloodlines of boars fell within the limits described by others (Feredean, 1974; Păsărin, 1997; Bogdan et al., 1999). It reached values that ranged between 224 mL and 235 mL when the animals were 8–12 months old, between 310 mL and 366 mL at 13–24 months, between 330 mL and 399 mL at 25–36

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Table 1. Semen characteristics of three PIC boar genetic lines reared in intensive units in Romania.

Variable[1]	Line[2]	Age (months)				Mean
		8–12	13–24	25–36	37–42	
Volume (mL)	1075	232 ± 8^{a}	310 ± 7 ^{b}	330 ± 8^{c}	304 ± 7^{b}	294
	408	224 ± 7^{a}	358 ± 9^{b}	383 ± 7^{c}	404 ± 12^{d}	342
	402	235 ± 8^{a}	366 ± 8^{b}	399 ± 9^{c}	406 ± 14^{c}	352
Motility (%)	1075	75.4^{a}	78.6^{a}	77.3^{a}	76.4^{a}	76.9
	408	78.7^{a}	79.9^{a}	79.4^{a}	77.8^{a}	78.9
	402	78.8^{a}	80.4^{a}	80.3^{a}	79.0^{a}	79.4
Concentration (millions/mL)	1075	351 ± 8^{a}	372 ± 9^{b}	420 ± 8^{a}	346 ± 9^{c}	372
	408	286 ± 14^{a}	298 ± 8^{acd}	320 ± 9^{bc}	305 ± 13^{d}	302
	402	292 ± 13^{a}	307 ± 7^{b}	331 ± 7^{c}	316 ± 13^{b}	312
Sperm per ejaculate (×10^{9})	1075	94 ± 12	119 ± 14	143 ± 11	107 ± 12	116
	408	61 ± 12	110 ± 12	123 ± 11	124 ± 13	105
	402	70 ± 10	116 ± 12	129 ± 12	128 ± 11	111
N° doses/ejaculate	1075	17 ± 6	21 ± 9	24 ± 8	20 ± 7	21
	408	12 ± 5	21 ± 6	23 ± 6	23 ± 6	20
	402	16 ± 6	20 ± 8	25 ± 7	23 ± 7	21

[1] Semen was collected at 5-d intervals; [2] Five boars per genetic line.

[a,b,c,d] Means with different superscripts are statistically different ($P < 0.05$).

months old and between 304 mL and 406 mL at 37–42 months old. Significant differences were apparent between both boars and age periods. For example, an upward trend with age of animal was observed in all three lines, and the maximum volume of semen was produced between 25–36 months by PIC 1075 boars and after three years by those from the PIC 402 and PIC 408 line. The finding of lowest values in all three lines at the beginning of reproduction activity is in accordance with the values presented in the literature, and the fact that the function of male genital organs and age of breeding stock are closely inter-related (Feredean, 1974; Bogdan et al., 1999; Nacu, 2005). Considering the whole reproductive life, best results were obtained in the PIC 402 bloodline. The difference between PIC 402 (352 mL) and PIC 1075 (294 mL) was 16.5%, but relative to PIC 408 the difference was only 2.8%.

Motility is also an important quality characetristic of semen, but significant differences were not recorded between the three PIC boar lines or with age, although highest motility was recorded at 13–24 months of age. Expressed in relative values, the differences between the average level observed in PIC 402 line (79.4%) and those found in the other lines, were very minor (3% and 0.6% respectively for the PIC 1075 and PIC 408 lines). The values for spermatozoal motility registered in the boars studied here are similar to those recorded earlier from different synthetic lines and pure breeds (Thibault and Levasseur, 1991; Watson and Behan, 2002).

The concentration of spermatozoa in semen is the main parameter used to dilute semen for insemination. Spermatozoal concentrations varied with age in the three PIC lines, with lowest concentrations being recorded at 8–12 months and highest between the ages of 25 and 36 months. This was probably a reflection of the intensification of spermatogenesis from the onset of sexual maturity associated with appropriate feeding and husbandry conditions, while the decrease noted at 37–42 months was the result of the slowing down of spermatogenesis function after the age of three years. Comparing the values recorded in each boar line, the differences between PIC 1075 (372 × 10^{6} spermatozoa/mL) and PIC 402 (312 × 10^{6} spermatozoa/mL) and PIC 408 (302 × 10^{6} spermatozoa/mL), were 16% and 19% respectively.

Although the conditions of husbandry and semen collection were similar during the whole exploitation period, the concentration of spermatozoa in ejaculations fluctuated greatly from one collection to another, with differences occurring between periods and lines being significant or highly significant. For example, the maximum number of spermatozoa from an ejaculate (143 × 10^{9}) was registered between 25–36 months of age, and the minimum (94 × 10^{9}) from the age of 8 months - 1 year. In the PIC 408 boars, the average ejaculate contained 105 × 10^{9} spermatozoa, with the maximum (124 × 10^{9}) being registered during the 36–42 months age period, and the minimum (66.1 × 10^{9}) from the age of 8–12 months.

Earlier publications quote lower concentrations i.e. between 211 and 315 million spermatozoa/mL (Popovici et al., 1980; Bogdan et al., 1999), than reported more recently e.g. 480–690 million spermatozoa/mL (Kunk et al, 2001; Stoica, 2003; Sgura et al., 2008). However, in the PIC 1075 boars the average number of spermatozoa/ejaculate was 116 × 10^{9} i.e. much higher than any of the above. Such differences may have arisen because the harvesting frequency in the present study was one collection followed by 5 d of rest.

The number of doses issued from each ejaculate was calculated on the basis of a minimum 75% spermatozoal motility and to provide four billion spermatozoa/insemination dose. For the genetic lines studied here, the maximum number of doses/ejaculate was achieved in all three PIC bloodlines during the period when the boars were 25–36 months of age, while the lowest number of doses was obtained during the onset of reproductive activity (i.e. at 8–12 months of age). The highest number of doses (21) was produced by the PIC 402 and 1075 lines, but differences between lines were not significant.

CONCLUSIONS

Analysis of quantitative and qualitative variables relating to the semen of three lines of PIC breeding boars showed that sperm production both quantitatively and qualitatively was in accordance data presented in the literature. However, values for the main indices fluctuated with both age and the line of boar, indicating that opportunities exist for improving boar management to improve productivity.

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Progesterone Levels in the Ovarian, Uterine, and Systemic Venous Blood in Alpacas with Embryo Mortality

L. Echevarría[1]*, G. Mendoza[2], A. Delgado[3] & R. Evaristo[3]

ABSTRACT

Embryo mortality was studied in a group of 20 pregnant alpacas. The reproductive organs of the females were monitored by ultrasound examination to determine signs of sustained pregnancy or embryo mortality. Blood samples were collected from the jugular vein twice weekly from mating to determine progesterone levels through gestation or until the occurrence of embryo mortality. Ovarian hysterectomy was conducted in four animals at day nine post-mating, in three animals at the time of embryo mortality detection and in two others at day 73 of gestation. Blood samples from the ovarian and uterine veins were collected during the surgery and prior to hysterectomy for progesterone determination. The remnant of embryo membranes and the uterus and ovarian structures were macroscopically examined after surgery. The three cases of embryo mortality occurred at days 19, 40 and 69 of gestation. Progesterone levels were high during the process of embryo mortality.

Key words: *alpaca, embryo mortality, progesterone, ultrasound, ovarian veins, gestation.*

INTRODUCTION

The harsh environmental conditions of the highlands of Peru and Bolivia limit agricultural activities, including livestock production. The South American camelids, especially the domestic species of alpaca and llama are suitable options for large commercial farmers, community farmers, and peasants. Fibre and meat are the main animal products, but manure is used for heating and cooking, and llamas are used to carry products from and to markets. Conception rate is adequate in all camelid species during the 3–4 month breeding season, but unfortunately embryo mortality can be as high as 50% (Fernandez Baca, 1970).

Several studies have attempted to identify the main factors involved in embryo mortality but most have focussed on independent factors without much success. Studies on possible relationships between age, pathogenic agents, and genital tract alterations in relation to embryo mortality are scarce and unreliable. Embryo losses are affecting genetic programmes and breeding systems as overall productive performance cannot meet the expected goals.

Embryo mortality in the alpaca outside the Andean region is much lower, but still, New Zealand reported 24% embryo mortality between 21 d and 30 d of gestation some years ago (Ridland et al., 1993). It may be that specific factors related to the high altitude, nutritional deficiencies, and local pathogens are affecting these indigenous animals. Nutritional restrictions decrease growth rate and follicular size affecting the ovulation of the single dominant follicle (Mackey 1999), and this would be related to leptin release as this indirectly regulates gonadotropin-releasing hormone neuronal function (Quennell et al., 2009). Also, it has been reported that cows fed with diets rich in energy produced smaller but better quality follicles than cows fed with low energy diets (Boland et al., 2001).

Several pathogens have been reported to cause embryo mortality, among them being *Toxoplasma gondii* (Gorman et al., 1999), *Neospora* (Serrano-Martínez et al., 2007), bovine viral diarrhoea virus (Carman et al., 2005), and bacteria involved in uterine infections as consequence of retained placenta, dystocia, and vaginal or uterine prolapse (Tibary, 2006).

Oestradiol has been associated with maternal recognition in alpacas (Chipayo, 2003). Females that received estradiol on d 9 and 11 after ovulation had corpora lutea with extended lifespan and showed increased serum progesterone levels (Powell, 2007). It is also known that progesterone increase in the late luteal phase is associated with smaller and less viable embryo in ewes (Mann et al., 1996). According to Boland et al. (2001), there is no relation between peripheral serum levels and ovary-uterus circulation levels of progesterone; meaning that embryo survival in camelids would be more related to progesterone levels in the ovarian and uterine veins than variations of progesterone levels in peripheral blood.

The objective of the present study was to relate progesterone levels in the uterine-ovary circulation and in peripheral circulation in pregnant alpacas that have maintained the gestation or lost the embryo.

MATERIALS AND METHODS

Twenty non-pregnant multiparous female alpacas without calf at foot were selected for this study. The animals were kept in corrals at the Veterinary Faculty of Cayetano Heredia University in Lima, at sea level. They were fed with alfalfa hay and sustained a body condition score of 3 (Australian Alpaca Association, 2001). Two adult and fertile males were used for natural mating.

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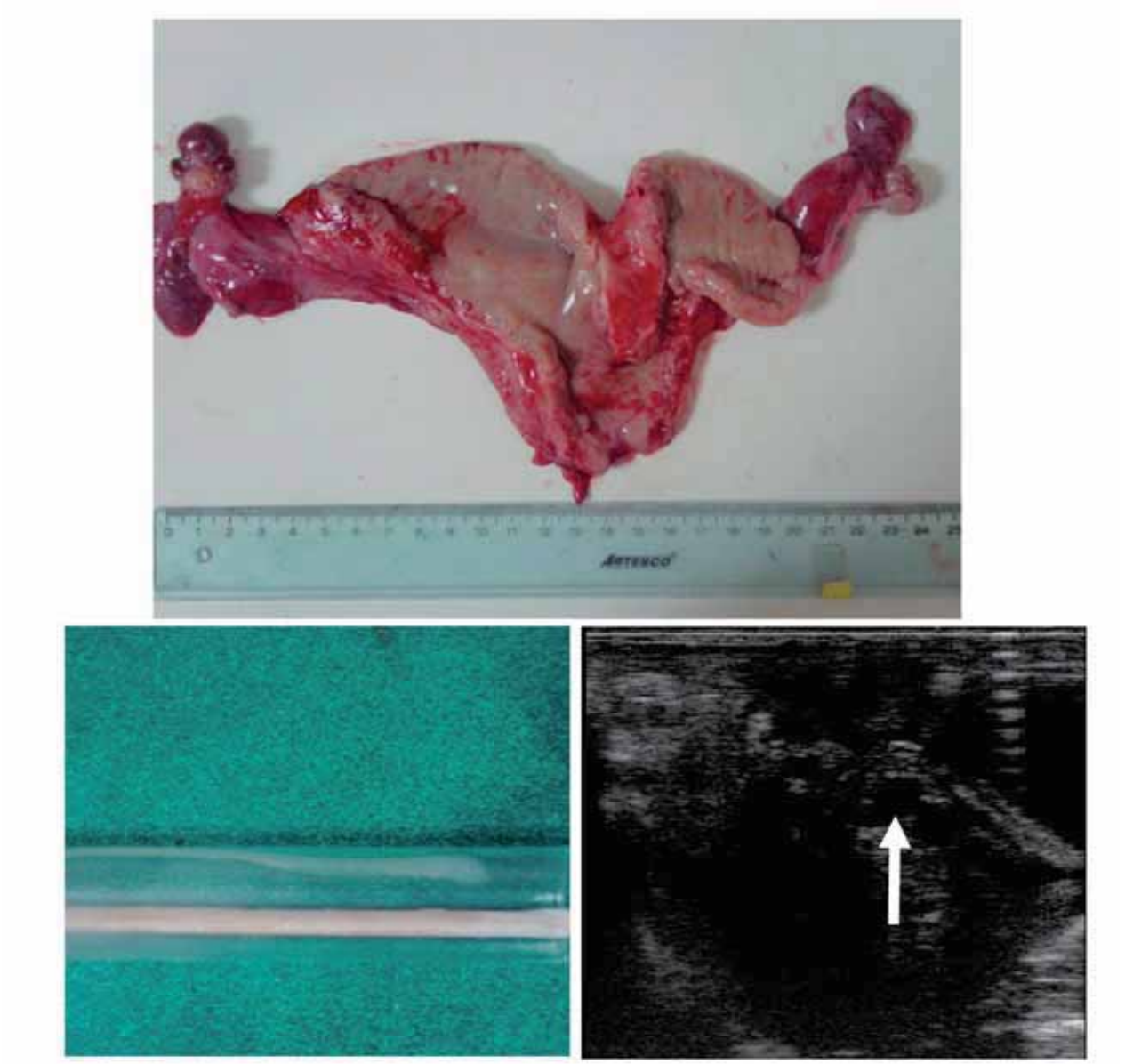

Figure 1. First case of embryo mortality. The upper picture shows the uterus and ovaries. The picture on the left shows the chorionic membranes, and on the right the echographic image of the uterus is displayed. The presence of anechoic content in the uterus five d prior to the embryo loss is shown by the arrow.

Table 1. Serum progesterone levels in alpacas of various reproductive statuses.

Reproductive status	Days post breeding	Progesterone (ng/mL)		
		Jugular vein	Ovarian vein	Uterine vein
Dioestrus	9	2.2±0.4	67.5±12.1	5.9±0.5
Normal gestation	73	4	68.3±8.9	4.7
Embryo mortality (Case 1)	19	3.4	65.3	4.2
Embryo mortality (Case 2)	40	0.4	8.7	1.4
Embryo mortality (Case 3)	69	0.5	4.8	1.1

Copulation was allowed when females showed sexual receptivity and had an 8 mm follicle in any of the ovaries based on ultrasound examination (Bravo, 1991; Vaughan, 2004). Female sexual behaviour was evaluated 13 d after mating and receptive females were mated again.

Pregnancy was monitored by ultrasound examination every other d in 16 animals starting on d 15 until signs of embryo mortality occurred or pregnancy continued until d 90. Embryo mortality was considered to have occurred when embryo cardiac beat decreased, embryo motility was lost, or suspended particles appeared in foetal fluids (Ginther, 1985; Adams, 1989; Parraguez, 1997).

Ovarian hysterectomy was performed in animals as soon as they showed signs of embryo mortality, in four animals chosen at random on d 9 post-mating and in two animals at d 73 of gestation. The anaesthetic protocol included ketamin 10%, tramadol 0.1%, xilacine 20% and atropine 0.03% (Hinostroza, 2010). Exposure and resection of uterus and ovaries were by laparoscopy with a 10 cm skin incision cranial to the mammary gland in the ventral midline (Mendoza et al., 2007). Uterus and ovaries were evaluated macroscopically.

Blood samples for progesterone determination (5 mL) were taken from the jugular vein twice weekly from the d of mating until surgery or 90 d after mating. Also, blood samples were collected prior to hysterectomy from both uterine and ovarian veins (left and right). Blood samples were centrifuged at 3 000 rpm for 10 min and then, serum was harvested and kept at –20 °C until analysis.

Progesterone concentration in serum samples were measured by radioimmunoassay (Coat-A-Count Progesterone In-vitro Diagnostic Test Kit). The standards were 0, 0.1, 0.5, 2, 10, 20, 40 ng/mL, and the coefficient of variation was 5.5% and 1.5% for the high and the low control sample respectively. Progesterone values from the uterus and ovarian vein of hysterectomised animals on d 9 were used as dioestrus values and those at the end of the trial in pregnant animals as normal gestation values.

RESULTS

Three cases of embryo mortality were found during the study. In the first case, embryo mortality occurred on d 19 of gestation (**Figure 1**). Macroscopically, chorionic membranes were observed in the left

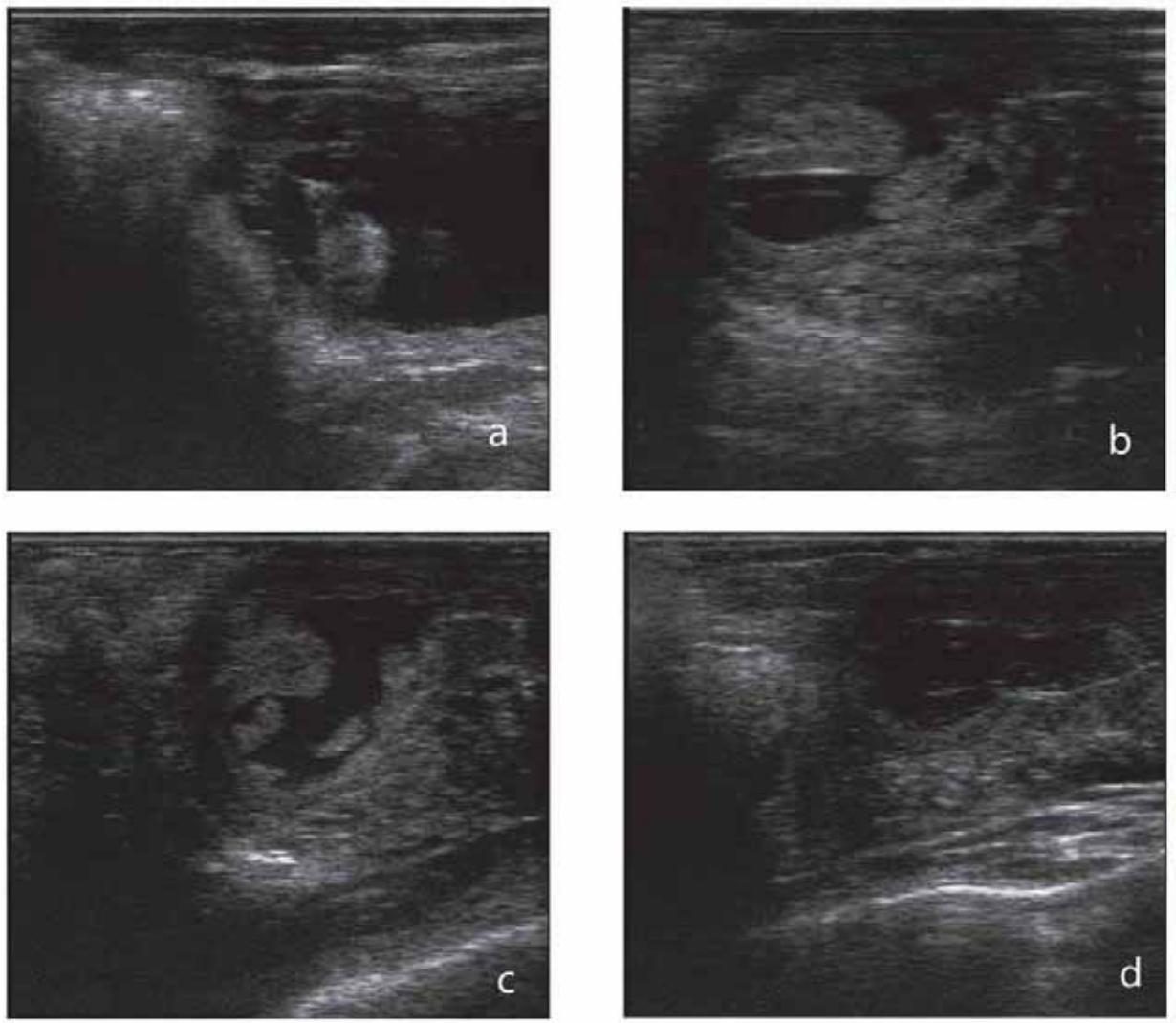

Figure 2. Echographic images of embryo mortality in a female alpaca. a — normal embryo; b — loss of shape of the embryo sac; c — partial loss of embryonic structure; d — complete loss of embryonic structure.

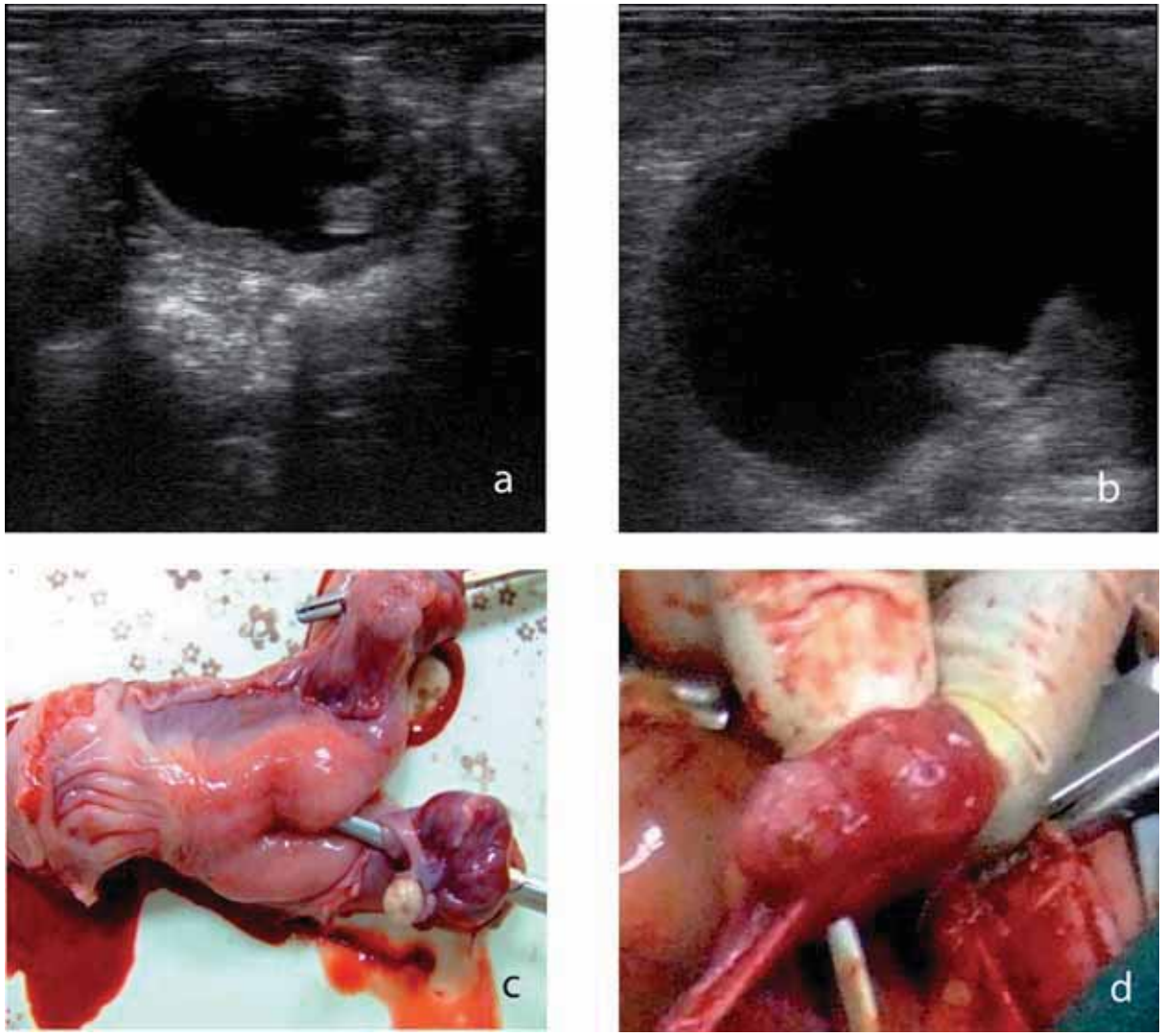

Figure 3. Reproductive organs and echographic images of an embryo death at day 69 of gestation in an alpaca
a — normal pregnancy, b — particles in suspension in foetal fluids, c — view of the uterus and ovaries immediately after ovarian hysterectomy showing the corpus luteum in the right ovary, d — view of the left ovary with presence of a large follicle.

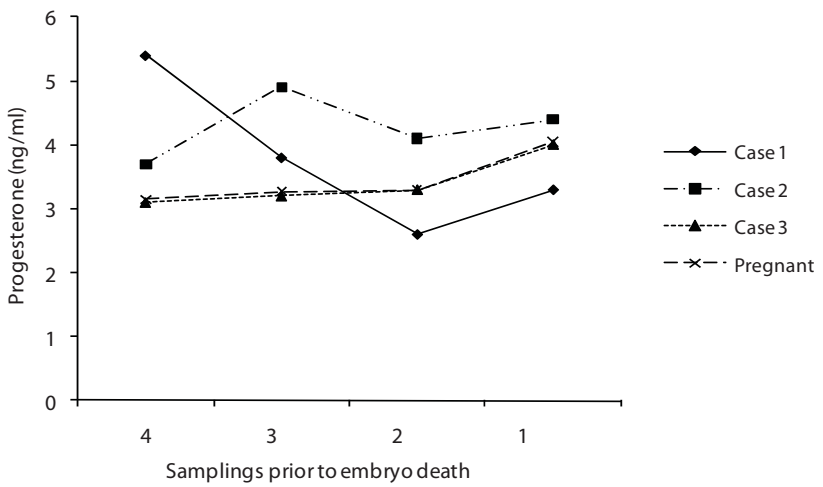

Figure 4. Progesterone levels in systemic venous blood prior to embryonic death in samples collected twice a week in three alpacas as compared with progesterone values in a pregnant alpaca in the second month of gestation.

horn, and a corpus luteum (CL) was found in each ovary. The systemic progesterone values were similar to those observed during the normal gestation and dioestrus periods (**Table 1**).

The second embryo mortality occurred on d 40 of gestation (**Figure 2**). Cheesy structures were observed in the left horn, both ovaries had a CL, and progesterone levels were reduced. Ultrasound images showed a gradual process of damaged embryonic structures. In the third case, mortality occurred around 69 d of gestation. Gestation took place in the left horn and a CL was present in the right ovary. Previous ultrasound images showed evidence of suspended particles in foetal fluid (**Figure 3**).

Serum progesterone levels were different between local (ovarian vein) and systemic circulations (jugular vein) in relation with reproductive status of animals (**Table 1**). Progesterone concentration in systemic blood was high during the four samplings prior to detection of embryonic deaths and similar to animals carrying viable embryos (**Figure 4**).

DISCUSSION

Three cases of embryonic death were observed among 16 pregnant alpacas during the first 90 d of gestation, representing a 28% embryo mortality rate. This number is lower than the 50% previously reported in the first 30 d of gestation in the classical study of Fernández-Baca et al. (1970). Only one embryo was lost in the first 30 d of gestation and the other two died in the second month of gestation (d 40 and 69). The late mortality, if usual in these animals, might result from a management problem as routine technical procedures indicated that female sexual receptivity should be teased with males two weeks and one month after mating to rebreed those accepting the males. The results indicate that females diagnosed as pregnant at the middle or final part of the breeding season will not deliver a calf as they will not have another opportunity to be bred.

The deleterious processes in the embryos are clearly shown by the ultrasound images. Autolysis in the embryo initiates after suspension of cardiac beats, which is followed by a gradual decrease of the volume of placental fluids, and finally, only membrane remnants can be seen (Ginther, 1985). Progesterone secretion by the CL was not involved in any of the embryo losses since in all three cases peripheral serum levels were higher than 1 ng/mL, and those values are considered from functional CLs (Stefanczyk-Krzymowska, 1998). Also, progesterone levels at the initial stage of gestation were similar to those reported in the literature (Aba et al., 1997; Raggi et al., 1999; Echevarría et al., 2007).

Surgery in the first case was performed during the process of embryo mortality and the progesterone concentration in the ovarian vein was similar to values obtained during the dioestrus period and those during normal gestation (**Table 1**), but quite different from the serum basal progesterone levels recorded on the d of mating and on d 13 post infertile mating (Raggi et al., 1999; Echevarría et al., 2007). Progesterone values in the ovarian vein in the other two cases were still high but much lower than in the first case as the process of embryo mortality was already complete. However, the data clearly showed that CLs were functional while embryos were dying.

Progesterone levels in the ovarian vein are directly related to ovarian progesterone secretion (Stefanczyk-Krzymowska et al., 1998), as this blood vessel originates in the ovary. On the other hand, progesterone levels in the uterine vein were similar to those in the jugular vein.

Embryo mortality is a multifactor process that needs to be elucidated in alpaca. Results from this study indicate that the CL is functional during the process of embryonic death and therefore, other factors probably related to the quality of the embryo may intervene and have to be elucidated in further studies.

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Establishment of Multiple Ovulation and Embryo Transfer (MOET) Technology for Goats in Sri Lanka

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ABSTRACT

This study was conducted to determine a suitable follicular stimulating hormone (FSH) preparation for superovulation in goats, establish techniques for embryo production and transfer in goats, and to examine the feasibility of applying such techniques in Sri Lanka. Two groups of genetically superior does were inserted with progesterone releasing intravaginal pessaries (45 mg Cronolone) on d 1 of the programme. On d 8, the does in Group 1 (n = 3) and Group 2 (n = 4) were given 2.5 mL injections of pure porcine FSH (pFSH, 20 mg/mL) or pure ovine FSH (oFSH, 0.88 mg/mL), respectively. On the same day, all animals were injected with 300 IU pregnant mare serum gonadotropin (PMSG, 500 µg/mL). Subsequent injections of 1.25 mL pFSH or oFSH were given in the morning and evening on d 9 and 10. Does were injected with 197 µg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 263 µg/mL) in the morning of d 9 and vaginal pessaries were removed on the evening of d 10. On d 11, 1.25 mL of pFSH or oFSH and 1 mL of luteinising hormone releasing hormone (LHRH, 50 µg/mL) injections were given in the morning and evening, respectively. On the same day, does in oestrus were bred to two Jamnapari bucks. Seven d post-oestrus, embryos were collected surgically, using embryo flushing medium. The quality of the embryos was assessed and the recovered embryos were transplanted surgically to oestrus synchronised goat recipients (n = 4/group) at 7 d post-oestrus. Following embryo transplantation, four does (Group 1, n = 1, Group 2, n = 3) were found to be pregnant by ultrasound scanning at 35 d into pregnancy. One healthy female offspring (Peradeniya Kumari) was born to Group 1. Another four goat kids were born to Group 2, while one kid died. In the same group, one abortion was reported. The results suggest that oFSH is better than pFSH for the superovulation of goats and that embryo transfer technology can be used in goats in Sri Lanka.

Key words: *goats, hormones, oestrus synchronisation, multiple ovulation, embryo transfer.*

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INTRODUCTION

Reproductive biotechnologies play a key role in improving animal reproduction. Multiple ovulation and embryo transfer (MOET) is a technique in which embryos are collected from a genetically superior (donor) animal and transplanted into another animal (recipient) for the remainder of development until term (Betteridge, 1981). It can be used to multiply genetically superior females by increasing reproductive efficiency, as an easy method to transport genetic material across the world at low cost and with minimum risk of spreading diseases, together with embryo sexing to get more female offspring from a genetically superior animal (Abeygunawardena, 2002; Cognie et al., 2003; Gonzalez-Bulnes et al., 2004). It can also be used to conserve endangered species (Senger, 1999). With the combination of embryo splitting, the multiplication rate of the donor can be further accelerated and identical twins can be made according to the aims of the research. Embryos can be produced even from animals which have conception failures or are unable to have normal pregnancies (Noakes, 1986).

Several key steps are involved in the MOET process including synchronisation of the oestrous cycles of donor and recipient animals, superovulation of donor animals, artificial or natural insemination of the embryo donor, recovery of embryos from the donor animal, *in vitro* maintenance of quality embryos until transfer, and transfer of embryos to recipient animals.

Usually embryo transfer (ET) in goats and sheep is performed through a laparotomy under general anaesthesia and two to four embryos are simultaneously transferred to a recipient (Alexander, 2005). For successful embryo transfer, both donor and recipient animals should be in the same stage of the oestrous cycle (Senger, 1999).

There have been no studies in Sri Lanka to investigate the feasibility of ET in goats. Therefore this study was carried out with the objectives of comparing the efficacy of using pFSH and oFSH for superovulation of goats and establishing the techniques for embryo production and transfer in goats in Sri Lanka.

MATERIALS AND METHODS

Selection and Preparation of Goats for ET

Genetically superior Jamnapari donor goats were selected on the basis of their production and reproductive performance and their pedigree records. They were 3–5 y old and between 3 and 6 parities. Donors were 2–3 months from their last weaning and 5–6

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months from their last kidding. Their health status was examined and anthelmintics and multivitamin injections were given. Four d after the injections 1 mL of tetanus toxoid was given to all selected goats. The animals were provided with *ad libitum* forage, 650 g of concentrates and 30 g of mineral mixture daily for two months.

Embryo recipient goats were selected on the basis of their reproductive performance and health status over the previous three years. They were treated with anthelmintics and given multivitamin injections. Four d later, 0.5 mL of tetanus toxoid was given to all recipient animals. These animals were provided with *ad libitum* forage, 400 g of concentrates and 15 g of mineral mixture daily for two months.

Two genetically superior Jamnapari bucks raised at the Veterinary Teaching Farm were selected as studs. Although semen quality was not studied, they had demonstrated good serving capacity and had successfully bred females over the previous 2.5 y. They were given forage *ad libitum*, 650 g of concentrates and 30 g of mineral mixture daily.

Superovulation of Embryo Donors

The selected does were inserted with progesterone releasing intravaginal pessaries (45 mg Cronolone, Intervet) on the morning of d 1 of the superovulation schedule. Does were divided into two groups. Does belonging to Group 1 (n = 3) and Group 2 (n = 4) were injected intramuscularly with 2.5 mL of pure pFSH; (Folltropin-V, 20 mg/mL NIH-FSH-P1, BIONICHE, Canada) and pure oFSH; (Ovagen™, 0.88 mg/mL NIADDK-oFSH-17-Standard, ICPbio Limited, New Zealand) respectively, on d 8 of the programme. In addition, 300 IU PMSG, (Folligon, Intervet International BV, Boxmeer-Holland) was given to all does on the evening of d 8. Folliculogenesis and maturation were further supported with subsequent injections of 1.25 mL pFSH or oFSH in the morning and evening of d 9 and 10 (Gonzalez-Bulnes et al., 2004). Does were injected with 197 µg of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, cloprostenol sodium; PGF Veyx fort, Veyx Pharma, Schwarzenborn) in the morning of d 9 and vaginal pessaries were removed in the evening of d 10. On d 11, 1.25 mL of pFSH or oFSH and 1 mL of LHRH (50 µl/mL, Depherelin Veyx Pharma, Schwarzenborn) were injected in the morning and evening, respectively. Immediately after the LHRH injection does in each group were kept separately for 48 h with a genetically superior Jamnapari buck for natural breeding. At 12-h intervals bucks were exchanged between the two groups.

Synchronisation of Embryo Recipients

On the morning of d 1 intravaginal progestegen-impregnated pessaries (Chrono-gest, which contained 45 mg flugestone acetate; Intervet), were inserted into all recipients, (eight crossbred does), and 125 µg of $PGF_{2\alpha}$ (Veyx® fort, Veyx-Pharma GmbH, Schwarzenborn) was administered intramuscularly to each animal. On d 17, vaginal pessaries were removed, and 400 IU of PMSG; (Folligon, Intervet) were given to each doe by intramuscular injection. The animals were observed for visible signs of oestrus on the following day.

Surgical Embryo Collection

Seven d after mating, embryos were collected as follows from three and four donors in Groups 1 and 2 respectively.

Intramuscular (IM) injections of xylazine 2% (0.2 mg/kg BWt) were given intramuscularly to sedate embryo donors (Bishop, 2001), and ketamine hydrochloride 10% (22 mg/kg BWt), was injected 20 min later. Once the donors were anaesthetised, they were kept on a surgical cradle in a dorsal recumbent posture. After shaving of the ventral abdominal region of the animal, the surgical site was scrubbed alternatively with 70% isopropyl alcohol and povidone iodine solution three times each. The uterus and both ovaries were exteriorised through mid ventral laparotomy (7 cm).

After measuring the length, width and thickness of each ovary, the number of corpora lutea in each ovary were counted. The uterotubal junction of the right uterine horn was pierced with a blunt ended 18 G hypodermic needle and the tip of an embryo flushing catheter (Tom cat catheter; 3 ½ FR, 14 cm; Sovereign™, Mexico) was inserted and pushed towards the uterine horn. The same uterine horn was pierced with a small artery forcep at the level of the bifurcation and a two-way pediatric silicon elastomer coated Foley catheter (8 Ch/Fr, 3/5 mL/cc; Unomedical, Malaysia) was inserted. After inflating the cuff, the stylet of the Foley catheter was removed. Fifty mL (10 mL × 5) of embryo flushing medium (lactated Ringer's solution with one percent bovine serum albumin) was passed through the flushing catheter and with gentle tapping on the uterine horn, fluid was collected into a 100 mL beaker. The same procedure was repeated for the left horn. After removal of both catheters the incision on the uterus was sutured with 3/0 cat gut (Ethicon). After applying hydrocortisone cream the reproductive tract was repositioned in the abdominal cavity.

Depending on BWt, the required doses of intra-abdominal and intra-muscular penicillin streptomycin were administered. The peritoneum and muscle layers of the incision line were sutured using 1 USP chromic cat gut and a simple interrupted suture pattern, while the skin incision was sutured with 0.45.G nylon using a simple interrupted suture pattern. Coumaphos, propoxur and sulfanilamide containing powder (Negasunt, Bayer Polychem, India) and povidone iodine solution were applied to the site after suturing.

Evaluation of Embryos

Embryos were separated from the flushing medium just prior to the evaluation process using a wire trawl. Their quality was assessed as excellent, good and poor (IETS, 2008).

Surgical Embryo Transfer

Eight crossbred recipient does were divided into two groups and subjected to a 12 h withholding period of feed and water. Blastocyst or morulae stage embryos were transferred on the same day as described below, to four recipients in each group.

Recipient does were placed on a surgical cradle in dorsal recumbency. The lower abdominal area was shaved and disinfected using povidone iodine and 70% isopropyl alcohol. The cradle was tilted 60^0 with the head facing down. An incision of approximately 1.5 cm was made in the skin about 3 cm to the left of the midline and about 5 cm from the udder, using a no. 23 scalpel blade. A trochar and cannula were inserted into the abdominal cavity through the incision made on the skin. The trochar was removed and a laparoscope (6.5 mm diameter) was carefully inserted through the cannula into the abdomen. Another incision was made on the right side in the same manner and a Babcock forcep was introduced. The uterine horns were visualised using the laparoscope and the ovaries were carefully inspected to find the ovary with large corpora lutea (CLs).The tip of the respective horn was grasped and three–four cm exteriorised using the Babcock forcep. At the same time the embryos were loaded into the tip of a 3.5 Fr tom cat catheter with the help of an insulin syringe.

In each transfer, three embryos of excellent and good quality were loaded onto the tip of the catheter as follows: First, a small amount of embryo holding medium (Vigro holding plus, AB Technology) was aspirated into the tip of the catheter followed by an air bubble and then the embryos with medium and finally another air

bubble. The uterine horn was punctured (very close to the utero-tubular junction) using a blunt 18 G needle and the tip of the catheter was inserted into the lumen of the uterine horn and the embryos expelled. Before releasing the horn into the abdomen, the tom cat catheter was examined to confirm that all the embryos were expelled successfully. This was carried out by washing and pipetting the catheter with a small amount of embryo holding medium in a 35 mm dish under the microscope. The abdominal incisions were sutured using 1USP-chromic catgut with a far-near-near-far suture pattern. A long-acting penicillin streptomycin injection was administered. The does were monitored post-operatively for several h and released to the shed.

Pregnancy Diagnosis

Pregnancy confirmation was carried out at 35 d post ET using an ultrasound scanner attached to a 7.5 MHz linear type per rectal probe.

Data Analysis

Mean values for the parameters measured during the superovulation and embryo transfer processes were compared between groups using the Student's t-test. Differences were considered significant at P values < 0.05.

RESULTS

Donor and recipient animals showed oestrus 24–36 h after removal of sponges. The common signs of oestrus were swollen hyperemic vagina, clear colourless vaginal mucus discharge, frequent wagging of the tail and restlessness (Abeygunawardena, 2002), but the animals did not exhibit all signs at any given time.

Values for the parameters measured during the superovulation and ET processes are given in **Table 1**, while the number and quality of the embryos recovered from each group are shown in **Table 2**. Following embryo transplantation, four does were found to be pregnant. One healthy female goat kid was born to a doe in Group 1with a birth weight of 3.6 kg at full term. Another four kids with birth weights of 3.2 kg (♀), 1.8 kg (♀), 1.6 kg (♂) and 1.2 kg (♂) were born at full term to does in Group 2. The third kid of the last three kids born as a triplet died shortly after birth. In Group 2 there was one abortion.

During the first six weeks weight gains of the first kid born to Groups 1 and Group 2 were 152.3 g/d and 149.2g/d respectively.

DISCUSSION

The study described here resulted in healthy live offspring, claiming the first five kids born through ET technology in Sri Lanka.

Signs of oestrus are reported to depend on a number of factors, such as the health status of the animal, nutrition, environment, breed etc (Stephen, 1971a).

The flushed embryos were in different developmental stages such as compacted morulae, blastocyst stage and expanded blastocyst stages. This could be due to the asynchrony of ovulation and fertilisation of oocytes (Cognie et al., 2004). A few unfertilised and degenerating embryos were also found.

The responses of embryo donors to the superovulatory treatment differed — a finding explained by the fact that responses to exogenous hormones depend on several factors such as the level of nutrition, age, breed and reproductive status of the embryo donors (Gonzalez-Bulnes et al., 2004). In the present experiment the number of embryos recovered ranged from zero to nine and the number of transferable embryos varied from zero to seven. Use of recently improved gonadotrophin preparations and programmed insemination protocols in this experiment could not avoid this variability.

Low oestrogen levels produced by the granulosa cells of developing follicles exert a negative feedback on the secretion of gonadotrophin. Similarly, inhibin secreted by developing follicles also exerts a selective inhibitory action on the secretion of FSH (Greenwald and Terranova, 1988). The number of ovulations and transferable embryos following administration of commercial FSH preparations depends on the number of small antral follicles (2–3 mm) present in the ovaries. Similarly the presence of large follicles (>6 mm) at the onset of

Table 1. Size of the ovaries, number of *corpora lutea* and number of embryos produced in two groups of donors.

Parameters	Group 1	Group 2
Mean size of the ovary (cm) ± SEM		
Length	2.4 ± 0.1	2.5 ± 0.2
Width	1.2 ± 0.1	1.7 ± 0.1
Thickness	1.1 ± 0.1	1.5 ± 0.2
Number of *corpora lutea* (range)	6–10	11–17
Mean number of *corpora lutea*	7.6 ± 1.2	14.25 ± 1.2
Mean number of embryos per animal	4.3 ± 2	4.25 ± 2

Table 2. The number and quality of embryos recovered.

Animal group	Hormone used	Number of embryos produced of given quality		
		Excellent	Good	Poor
Group 1	pFSH	2	8	3
Group 2	oFSH	4	11	2
Total		6	19	5

the superovulatory treatment exerts an inhibitory effect on the final number of transferable embryos recovered (Cognie et al., 2003).

In cycling animals, the size of ovaries correlated with the number of developed and developing follicles present. In the present study, the mean size of ovaries in Group 2 was comparatively higher than that in Group 1. Ovulations after pFSH and oFSH stimulations were 7.6 and 14.25 in Groups 1 and 2 respectively. Thus oFSH had a superior superovulatory effect in goats. However, in this study there was no correlation between the number of ovulations and number of embryos recovered, especially in Group 2. In this group there was one blind fallopian tube in the tract of two animals; but they were having more ovulations. This could be the reason for the lower embryo recovery in such animals.

The exchange of bucks between the groups provided equal opportunities to animals in both groups and also increases the fertility of embryo donors.

One abortion was reported in this study. Viability of the foetus or the embryo depends on many factors including a functional corpus luteum, alterations in the preovulatory follicles and level of ovarian abnormalities (Gonzalez-Bulnes et al., 2004). From the five kids that resulted from the experiment, one died shortly (within 20 min) after parturition. The cause of the death may have been hypoxia, due to placental detachment. Delayed kidding may be due to foetal mal positioning, calcium deficiency, uterine inertia, abdominal muscle fatigue and nutritional deficiencies (Stephen, 1971b).

The last triplet of kids had relatively low birth weights, likely due to the high nutritional demand of all three foetuses from the same maternal tissues throughout their gestation period.

In this study all procedures were conducted under conditions similar to those in field situations using portable instruments, so that they could be repeated under farm conditions.

At present there is good potential for using MOET to establish elite herds in selected goat breeding farms. This would enable them to provide improved breeding animals, which are currently in short supply, to smallholder farmers.

CONCLUSIONS

Compared with pFSH, oFSH was found to be better for superovulation in goats. It is feasible to produce viable offspring of goats using ET in Sri Lanka, but further studies are needed to optimise the procedures and reduce the costs.

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Is Embryo Transfer a Useful Technique for Small Community Farmers?

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ABSTRACT

Four main aspects of embryo technology are dealt with in this paper. The first analyses the reasons for the poor selection of recipients for embryo transfer, the second relates to inaccurate evaluation of embryos at least under tropical conditions, the third proposes alternative methods to evaluate embryos for selection and freezing, and the fourth analyses the feasibility of establishing this technique as a biotechnology approach for improving production in small community tropical farms.

Key words: *embryo transfer, embryo assessment, freezing, small farmers, economics.*

INTRODUCTION

Several researchers have provided sufficient evidence that the best crossbreeding programme to produce milk in the tropics is the direct cross between *Bos taurus* and *Bos indicus* (F1). The problem arises when the farmer faces the challenge of breeding the crossbred animal. If the choice is to cross with *Bos taurus* the resulting product is quite vulnerable to the harsh environmental conditions in the tropics. If, on the other hand, the selection is to sire with *Bos indicus*, then the offspring will be deficient in milk production (Madalena, 1993). Another alternative is to transfer F1 embryos to F1 dams, thereby avoiding the hazards of crossbreeding (Cunningham, 1989). Although the technique of embryo transfer (ET) has been available for many years, at least under tropical conditions there are several pitfalls such as the inadequate selection of recipients, the production and evaluation of embryos and finally, the economic feasibility of the technique itself.

Selection of Recipients for Embryo Transfer

These are usually animals displaying spontaneous oestrus or treated with hormones to synchronise this event. The shortcomings of both these methods have been described by Montiel et al. (2006). In short, the use of spontaneous oestrus is time-consuming and inaccurate (for review see Galina and Orihuela, 2007). On the other hand, when using synchronised oestrus the response with an ensuing ovulation can fail in as many as 30% of cases if the animals selected are not in reasonable body condition (Diaz et al., 2002). Moreover, if the drug used for oestrous synchronisation contains oestrogens, the response of animals displaying overt signs of oestrus without an ovulation can increase by almost two-fold (Solano et al., 2000; Velásquez, 2004). Hence, the selection of recipients displaying oestrus but with the adequate formation of a corpus luteum can be time consuming and at times frustrating (Montiel et al., 2006).

Due to the above, embryo transfer programmes in small community farms can be tricky because the selection of recipients is restricted to a few animals in the herd and the distance between farms can pose a serious threat to success. Thus, just because of this constraint, government programmes have ceased to function as the resources necessary to visit farms distant apart are limited.

Embryo Assessment

The main components of successful embryo production are: the quality of the superovulatory response in the donor cow, the ability of the individual to recover as many embryos as possible, and the skills of the technician in judging the quality of the embryos destined for freezing. In relation to the first, figures for embryo production can vary enormously, although some groups demand that the number of good quality embryos cannot be less than eight (Baruselli et al., 2006). However, others have not been so successful (Barros and Nogueira, 2001; Chebel et al., 2008). In general, the superovulatory response can be directly related to the follicular dynamics at the moment of treatment (Bo et al., 2003). Few studies have addressed this issue although it has become apparent that animals undernourished do produce follicles of lesser dimensions and compromised fertility when compared with their well fed peers (Oliveira et al., 2002). In studies where follicular dynamics were characterised in postpartum and barren cows, it was evident that the diameter of the largest follicle can be affected by the stage of the postpartum period or the time of the year when the study was undertaken (Molina, 2000). In various experiments (Molina, 2000; Montiel et al., 2006; Alarcón 2008), the superovulatory response judged by the number of corpora lutea formed was always above nine, but the number of good quality embryos hardly surpassed five.

The recovery of embryos can be difficult especially as it has been reported that almost 30% of the donor cows have curved cervices increasing the difficulties in negotiating the catheter to flush the uterus properly (González et al.,1983). Hernández (1988) showed that even when cervices were dissected, the degree of torsion was so great that it became virtually impossible to pass the catheter even under postmortem conditions.

Another issue demanding attention for future research is the ability of the clinician to accurately determine the health of the divided embryo by light microscopy in *Bos indicus* cattle. In an early study, Aguilar et al. (2002) showed that inaccuracies in the judgment of embryos can be as high as 30%. This observation was confirmed using other diagnostic criteria (López-Damián et al., 2008; Gutiérrez, 2009; Godínez, 2009). Moreover, Marquez et al. (2005) showed, also

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N.E. Odongo, M. Garcia & G.J. Viljoen (eds), Sustainable Improvement of Animal Production and Health. Food and Agriculture Organization of the United Nations, Rome, 2010: **219–221**

in Bos indicus females, that the number of healthy embryos evaluated by their resistance to freezing and their degree of apoptosis, was affected if the embryos were produced in the spring when compared with the autumn even using the same donor cows.

Methods to Evaluate Embryos for Selection and Freezing

Marquez-Alvaredo et al. (2004) studied embryos kept frozen for various periods of time and reported that these structures with shorter storage time, presented a lower number of dead cells evaluated by apoptosis compared with embryos with a longer storage. The authors concluded that embryos produced on an industrial scale have the potential disadvantage of being subjected to a variety of assessment criteria when they are selected for freezing. This observation merits further research.

There is agreement between investigators that embryo assessment is an important source of error (Lindner and Wright, 1983). This observation has been recently confirmed (Gutierrez, 2009). In fact using invasive methods to judge embryo soundness, these researchers demonstrated that cells classified by experienced clinicians as viable, are in fact defective. Another potential source of error in ET relates to embryos which are routinely classified by stereoscopic microscopy before freezing and the quality of the embryo is no longer reassessed. Considering that the most important point in the ET is the grading of the embryos, this practice can lead to placement of cells in cows with little possibility of rendering a pregnancy as they were of mediocre quality from the start. It is also important to consider the handling and care of the embryos when they are stored, because both are critical for their viability. In an effort to reduce this shortcoming, Contreras et al. (2008) found that after culturing fresh embryos for up to 8 h, good and fair quality embryos did not undergo major detrimental changes in development even after 7 h of incubation, whereas poor quality embryos experienced changes as early as 2 h. Good quality embryos invariably had fewer numbers of apoptotic cells than those of fair and poor quality suggesting that embryo culture can be a useful method to assess viability and to confirm the quality of recently collected embryos. These results were recently confirmed by Godínez (2009) in fresh embryos, but not in cells previously frozen. Her results suggest that the culture medium can be toxic to embryos expanding after thawing. Further research is required to elucidate the reasons for these differences.

Economical Feasibility of ET among Small Community Farmers

Government organisations in developing countries have launched initiatives to popularise the evident benefits of ET, particularly in enterprises not bigger than 50 cows. These programmes have experienced a high degree of acceptance, especially those with a substantial subsidy. However, when the programme terminates, it will invariably have proven not to be sustainable for the farmers themselves; thus disappointment is the natural outcome (Molina, 2003; Chávez, 2008). In a recent study (Alarcón, 2008) estimated the cost of preparing the donor and recovering embryos was about US$600. The average number of embryos recovered was 3.8. Taking into consideration the cost of gestation, calculated as the percentage of animals pregnant (27%), the cost for preparing the donor, the technique of embryo transfer and the cost of producing the embryo itself, the overall cost per gestation was US$1320. Considering a 50:50 ratio of males:females born, the cost for a replacement heifer was US$2 640 — surpassing by far the commercial cost of a crossbred heifer (approximately US$900 dollars).

CONCLUSIONS

Considering the difficulties in distributing F1 embryos among farmers in small enterprises, the cost of production and the low success rate found in terms of fertility, for the time being it does not seem profitable for farmers themselves to sustain the costs of an ET programme. Government organisations would therefore need to play a more active and systematic role to ensure that the costs inherent in ET technology are reduced.

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The Effect of Management Practices on Prevalence of Mastitis in Large Scale Dairy Farms

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ABSTRACT

A study was conducted to investigate the impact of management practices on the udder health status of dairy cows in Thuringia-Germany. Forty-eight dairy farms were randomly selected and 64 542 milk samples from 10 741 dairy cows were collected and subjected to bacteriological investigation. The prevalence of the infection was 27.57% of the quarters, and 49.66% of the composite milk samples. *Staphylococcus aureus* and coagulase-negative staphylococci (CNS) were the most frequently isolated contagious pathogens with udder and quarter prevalences of 28.7% and 35.5% and 26.6% and 32.7% respectively. On the other hand, *Streptococcus daysgalactia* and esculin-positive streptococci (environmental pathogens), had prevalences in udder and quarter samples of 12.9% and 13.9% and 9% and 10.6% respectively. Incidence rates were 32.8% in small herds and 31% in large ones. Housing and milking systems, feeding and udder cleaning methods significantly influenced the mean incidence rate of mastitis. Ignorance of inter-milking sanitisation resulted in a higher incidence rate (33.5%), which was lowered by practising sanitary measures (31.5%). Application of teat dipping reduced the incidence of mastitis to 32.3%, whereas, the non-use of teat dipping resulted in an incidence rate of 33%.

Key words: *dairy farms, mastitis, management, prevalence, sanitisation.*

INTRODUCTION

In recent years the demand for liquid milk has increased tremendously worldwide due to increased population growth and incomes. In most countries, dairy cattle breeding programmes are directed toward milk production traits. Although these traits are of primary economic importance, functional traits such as longevity, fertility and udder health are of increasing interest to producers to improve herd profitability. Mastitis is defined as an infection of the udder, caused by bacteria entering the quarter through the teat end (Rodenburg, 1990). Several researchers (Wendt et al., 1994; Smith and Hogan 1995; Kalmus et al., 2006) concluded that mastitis-causing organisms can be classified into two main groups: contagious pathogens which spread by means of hands and milking units and include *S. aureus, St. agalactiae,* and *Mycoplasma*; and environmental organisms which live in the cow's environment and are always present and include *E. coli, St. dysgalactiae, St. ubris.* Hogan and Smith (1987) stated that the percentage of quarters infected with environmental streptococci is low and seldom exceeds 10% of quarters. Smith et al. (2000) stated that small herds reported more cows being removed for mastitis than high medium and low medium herd size. The National Mastitis Council's fact sheet (1997) states that housed cows are at greater risk from environmental mastitis than cows on pasture. Also, post milking teat barrier dips reduce new coliform intra-mammary infection but their efficacy against the environmental streptococci and contagious pathogens appears to be lower than that of germicidal preparations. It has been also shown that back flushing of the milking unit does not control environmental mastitis. Additionally, malfunctioning milking machines which result in frequent liner slips and teat impacts can increase cases of environmental mastitis. Washburn et al. (2002) compared seasonally calved Holstein and Jersey cows in confinement and on pasture systems and found that cows in confinement had 1.8 times more cases of clinical mastitis and eight times the culling rate for mastitis than did cows on pasture. Radostits et al. (1994) summarised the control measures of mastitis among which pre-milking udder hygiene, post-milking teat dipping and environmental control during the dry and calving periods are most important.

Each of these control measures is aimed at the management of specific pathogen types. Pankey (1989), Boddie et al. (1993) and Malinowski (2000) concluded that pre-milking udder hygiene and teat dipping are aimed at reducing infections mainly caused by contagious pathogens and preventing new infections and to a lesser extent at preventing infections that might be caused by environmental pathogens. Sargeant et al. (2001) claimed that producing high quality milk requires effective udder health programmes at the herd level. Management practices at the time of dry-off and during the dry period are essential in this respect. Peeler et al. (2000) found that the incidence of mastitis increased when milking cows were housed in a straw yard, while Oliver et al. (2001) demonstrated that pre- and post-milking teat disinfections with phenolic combination were significantly more effective in preventing new intra-mammary infection than was post-milking teat disinfections only. They also added that pre-milking teat disinfections with phenolic combination in association with good udder preparation and post-milking teat disinfections can further reduce the occurrence of new intra-mammary infection by numerous mastitis pathogens during lactation. Saloniemi and Kulkas (2001) in describing mastitis control in Finland, recommended post-milking teat dipping as a control tool in herds with contagious udder pathogen problem.

MATERIALS AND METHODS

Milk samples from 10 742 dairy cows in 48 large scale dairy farms in the state of Thuringia-Germany that were calving between June

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N.E. Odongo, M. Garcia & G.J. Viljoen (eds), Sustainable Improvement of Animal Production and Health. Food and Agriculture Organization of the United Nations, Rome, 2010: **223–226**

1998 and April 2000 were used in the study. Milk samples were subjected to bacteriological investigation and a questionnaire for the collection of management data was prepared which included housing system, milking system, udder cleaning methods, inter-milking sanitation of the milking units and post-milking teat dipping. Bacteriological and questionnaire data were merged into one data set by means of a statistical program using the SAS package (SAS, 1996). Data were analysed using the frequency procedure of SAS (SAS, 1996) and results were presented as contingency tables.

RESULTS

Figure 1 displays the frequencies of the bacterial types that were found in the udder quarter and composite milk samples. The total positive findings were estimated to be 15 701 and 3 765 respectively, which represented 27.6% and 49.7% of the total samples collected from each site. *Staphylococcus aureus* and CNS were the most frequently isolated pathogens from the udder quarter samples and composite milk samples (35.5% and 28.7% and 32.7% and 26.6% respectively). However, *Streptococcus daysgalactiae* and EPS infections were more frequent in the composite milk samples than in the udder quarter samples (13.9% and 12.9% vs. 10.6% and 9.0% respectively).

The study also indicated that the infection rate was influenced by the herd size (**Table 1**), farms with large herd size having a significantly lower infection rate (31%) compared with those having small herd size (33%). It was also found that infection rate decreased steadily as the number of animals increased.

Infection rate was found to vary between housing systems. Animals housed in either slat or plain floor loose housing barns had higher infection rates (32% and 31.8% respectively) than animals kept in barns other than loose housing (30.5%). Milking systems also influenced mean infection rate, being significantly higher (33.5%) in animals milked in pipe system units than those milked in either carrousel or milking parlour units (32.5% and 32.3% respectively).

Results revealed a significant statistical effect of feeding method on infection rate, with rates being higher in farms using both mobile and stationary methods of feeding (32.5%) compared with farms using stationary method of feeding (31%).

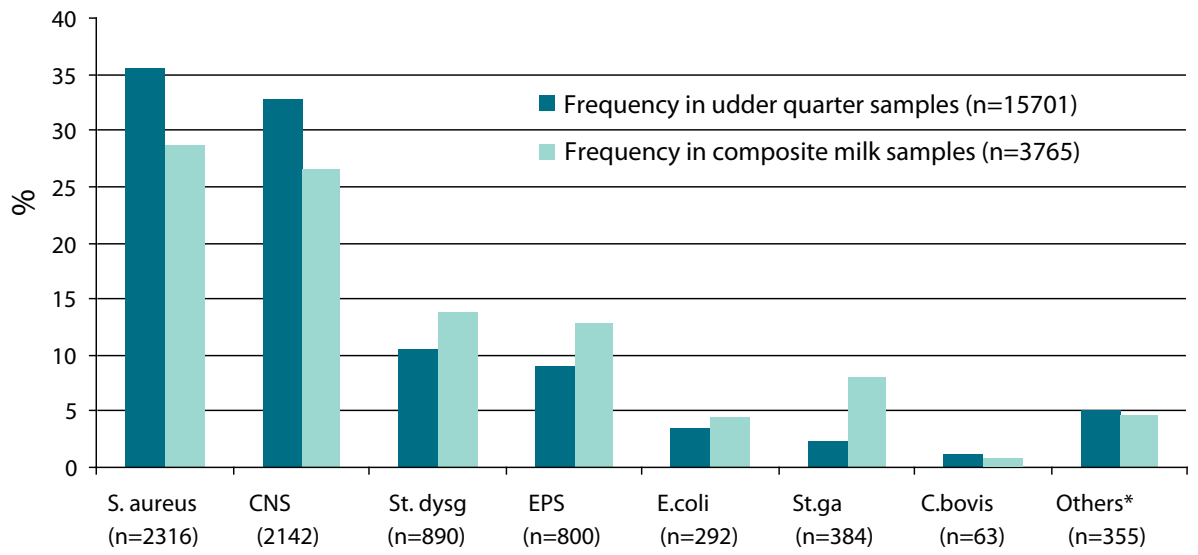
Methods of udder cleaning before milking had significant effects on infection rate. Farms which used moist udder cleaning had slightly higher mean infection rate (32.5%) than those that were practising dry udder cleaning (32.3%). **Table 2** shows the effect of the sanitisation methods used in the milking units on infection rate. Infection rate was highest in farms that ignored sanitisation between milking (33.5%); this was nearly the same as in the farms that were

Table 1. Infection rate of mastitis as influenced by herd size.

Class of the herd size	Number of cows	Infection rate (%)
Small	< 200	33.0
Medium small	200–400	32.0
Medium	401–600	31.8
Medium large	601–800	31.3
Large	> 800	31.0

Table 2. Infection rate of mastitis as influenced by inter-milking sanitisation method.

Sanitisation methods between milking	Infection rate (%)
Back flushing	33.3
Air wash	32.0
Bath (Tub)	31.8
Spraying	32.0
Other	31.5
Not used	33.5



* — *Pseudomonas aeruginosa, Actinomyces pyogenes,* spore forming bacteria, yeast etc.

Figure 1. Frequency of individual bacterial types in udder quarter and composite milk samples.

using back flushing (33.3%). The use of a combination of methods reduced the infection rate to 31.5%; this is significantly different from the aforementioned means. The use of air wash and spraying resulted in infection rates that did not differ (32%), whereas the use of the bath lead to an intermediate infection rate (31.8%).

Teat disinfection also influenced the degree of the infection, teat dipping reducing the mean infection rate to 32.3% compared with 33% when no teat dipping was employed.

DISCUSSION

Mastitis control is a never-ending battle for dairymen with many individual and interacting factors involved in causing problems. The present study was based on 64 542 randomly collected foremilk sample (56 960 were samples from quarters and 7 582 were composite milk samples). The most frequently isolated pathogens from both types of samples were *Staphylococcus aureus* and CNS — a finding consistent with those of Trinidad et al. (1990), Nickerson et al. (1995) and Waage et al. (1999). In small farms, infection rate was highest, and decreased gradually to reach the lowest value in the large farms. These results are in agreement with Wilesmith et al. (1986), who showed that the incidence of mastitis declined with increasing herd size. Dego and Tareke (2003) found that the prevalence of mastitis was significantly higher in Holstein-Friesian than in indigenous Zebu cows and in non-lactating than in lactating cows.

Farm management and hygienic factors are considered to be among the main risk factors, as they predispose the animal to intramammary infection. The study investigated the influences on intramammary infection of housing systems, milking techniques, feeding methods, udder cleaning methods before milking, inter-milking sanitisation methods as well as post-milking teat disinfection. Cows housed in muddy lots or pastures are obviously at a high risk for pathogen contact via organic bedding materials or dirty stalls. Mean infection rate was significantly higher in animals kept on plain floors and in loose stalls, followed by animals kept in slat floored loose housing where animals had the same infection rate as those kept in other stall types. Among the other housing types is the tie-stall barn in which animals are always under threat from pathogens as the stanchion limits animal movement and subjects the teat to injury. In loose barns, there is also the problem of lying on rubber floors or straw bedding. Well maintained and loose bedded stalls and well drained dry lots minimise possible contamination of the teat ends from inter-mammary infection causing pathogens compared with animals managed in pasture. This conclusion is supported by Peeler et al. (2000) who found that the incidence of mastitis increased in milking cows housed in straw yards, as well as those standing in a yard after milking. Also by Rodenburg (1990) who showed that stalls that were too small subjected animals to injury; in free-stall barns cows are less likely to lie in dirty and such barns are always of adequate size.

Milking techniques are also considered as factors that can affect the udder health status of the cow. Milking units are the primary means of transferring contagious bacteria from cow to cow. In infected herds there will be relocation of bacteria from infected cows to non-infected cows by the milking unit and this allows mastitis spread. Pipeline milked animals had a significantly higher frequency of pathogens (35.5%) and consequently higher mean infection rate. If pipelines are not correctly and regularly cleaned and rinsed with plenty of water this will lead to bacterial lodgment and raise the problem of inter-pipe pathogen transmission. Animals milked in carrousel units had higher mean infection rates than those milked in milking parlor. Better cleaning and disinfection of the milking unit always leads to reduce the effect of the pathogens. This difference is again of a managerial nature as superior management of the milking unit is assumed to improve udder health status of milking herds. On the other hand, faulty management (besides sampling error that should always be taken into consideration), will exacerbate the condition.

Farms using both mobile and stationary methods of feeding were found to have a higher frequency of pathogens than those using only a stationary method or mobile method of feeding. These consequences are believed to be slightly dependant on the kind of feeding system, but to a great extent on the nature of the feeding and feeding equipment. This is in addition to how well such equipment is cleaned after feeding in order to prevent carry-over of contaminants whether contagious that can be transmitted through hands or environmental which live in a suitable environment created by faulty management processes.

To achieve an optimum level and quality of production, it is of paramount importance to clean the udder of the cow before commencing the milking process. In this study two types of udder cleaning were routinely performed, moist and dry. The mean infection rate in animals whose udders were cleaned by moist means was significantly higher than in animals whose udders were cleaned by dry cleaning. This difference could be attributed to the fact that moist cleaning can predispose the animal to intra-mammary infection, and since the intra-mammary infection causes pathogens to enter the udder through the teat opening, milking wet teats increases considerably the chance of forcing bacteria into the quarter. Also when a disposable towel is used to dry the teat of more than a single cow, this will overwhelm the condition and allow bacteria to be transmitted between cows. Higher frequencies of pathogens resulted in higher mean infection rates of the animals milked in milking units that were not subjected to sanitisation compared with in farms practising inter-milking sanitisation. These findings emphasise the importance of sanitisation as a routine management practice to control or reduce intra-mammary infection.

From analysis of the sanitisation methods examined, it can be concluded that a combination of one or more methods were effective in reducing the mean infection rate. Spraying was otherwise the most effective method in reducing contagious pathogens, while bathing was effective in reducing environmental pathogens. Back flushing was not effective in reducing infection rate with either contagious or environmental pathogens, a finding that was also reported by the US-National Mastitis Council's fact sheet (1997). Of the other hygienic measures adopted by the dairy farms inspected, teat dipping reduced the frequency of pathogens (37.3% infection in farms practising dipping compared with 40% in those that did not). Natzke (1981), Pankey (1989), Boddie et al. (1993), Radostits et al. (1994), and Malinowski (2000) all concluded that teat dipping reduced infections mainly caused by contagious pathogens but also prevented new infections and to a lesser extent infections caused by environmental pathogens. Oliver et al. (2001) demonstrated that pre-and post-milking teat disinfection with phenolic combination was significantly more effective in preventing new intra-mammary infections than post-milking teat disinfections only.

CONCLUSIONS

The most frequently isolated pathogens were *Staphylococcus aureus* and CNS, which led to significantly higher infection rates in the farms studied. Herd size affected the degree of infection in that small herd sizes were more at risk from udder infections than large herds. The use of hygienic measures are of utmost importance in reducing infection rates.

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Foundations, Fallacies, and Assumptions of Science for Livestock in Development

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ABSTRACT

Molecular genetics is a new scientific discipline offering the technology to transfer exotic genes into livestock species. Scientific and business interests aim to apply this technology in the near future to make genetically modified (GM) livestock for the food chain. In Europe there is a strong move by citizens against milk and meat products from GM livestock. The possibility of using the technology on livestock in the developing world is under consideration as advocates claim that it would be a major contributor to world food security. This paper presents the opposite view. There are several sets of reasons against using this new technology at this time that are explored here. First, scientific knowledge of the mammalian genome is inadequate and a vast amount of research is needed before success will be ensured without negative consequences for humans and animals. Second, livestock are an essential resource for survival of billions of rural poor in the developing world and they should not be exposed to risk. Third, ethical considerations are not evident but are essential because the plans are so radical and affect public interest at many levels. Scientists today show lack of wisdom in failing to see the consequences of using their limited knowledge. Reasons for this absence of wisdom are explored in a brief review of the historic development of science. Livestock scientists need to learn lessons from the sagas of GM crops and mad cow disease (BSE). Other ways to empower the poor to increase food security are described. Scientists are urged to continue research and to seek a moratorium against GM livestock being used for food until objective and tested results enable stakeholders to decide.

INTRODUCTION

This paper covers the subject matter by dealing with four inter-related topics: the role of scientists in society, the present world situation, genetics and revisionist geneticists, and molecular biology, livestock and the poor.

THE ROLE OF SCIENTISTS IN SOCIETY

The West versus the Rest

Measured by scientific, economic and military power, the West is the most advanced society in human history. In these terms, other societies in the world today have less clout; but they are nevertheless long-established and sustainable with their own cultures, lifestyles, values and world views. In these economically simpler rural communities several billion people are still dependent upon livestock as a primary resource for life - just as in the West not so long ago. The agenda of this Symposium examines the transfer of molecular biology technology and products to those societies for use on the genomes of livestock. While offering total support to the objectives of improved livestock production and health, enhanced food security and alleviation of poverty, this paper raises some serious reservations on scientific, socio-economic and ethical grounds about using molecular biology techniques for genetic manipulation of livestock in developing countries at this early stage of knowledge. The paper advocates caution alongside the exciting potentials most of which remain exploratory, untested and speculative. Real uncertainty lies in contemplating action programmes in developing countries for these novel molecular techniques which are not accepted by many in the West. For ten years, more than 60% of EU citizens have consistently rejected GM foods (EuroBarometer website) and, in 2008 through their parliamentary representatives, EU citizens overwhelmingly rejected milk and meat products from cloned livestock. These reservations call for deeper examination of using such technologies and their products in rural societies where livestock are the major foundation of life and of society.

There is a vast difference between research and application of molecular biology. This paper fully supports research and affirms that this Symposium will advance knowledge of the livestock genomes thereby contributing positively to scientific research. But, logically, there is something incongruous about importing unproven technology to change extensive livestock systems of Africa, Asia and Latin America when intensive farming systems are themselves proving unsustainable in the developed West. Wisdom demands answers to some deep questions in these circumstances. The question is not about researching the potentials; it is about use in the foreseeable future. Responsible use must be built upon sound scientific knowledge which, at present, is sparse on the molecular universe of the mammalian genome.

Effective use of scientific knowledge must be accompanied by a deep understanding of human values upon which civilisation has been built. This duet of scientific knowledge and social wisdom goes beyond the protocols needed in a research laboratory. Human societies in rural settings, though simple and based upon livestock, have proved to be sustainable over millennia and deserve respect. Scientific knowledge and social wisdom must proceed together to understand the local and global matrices of human affairs. This has always been the posture of good science — until recently. Today the newly emerged worldview of many scientists sets caution aside and moves into immediate use of the new molecular genetic techniques in food. Scientists who question this position are often seen by their peers as heretics.

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Why Address Genetic Manipulation of Livestock at this Time?

This paper addresses the foundations, fallacies and assumptions of science in the context of genetic manipulation of the livestock genome to produce transgenic animals. Why? The reason is that society today is poised on the brink of using these techniques to produce human food. So far as publicly known, no milk and meat products from cloned or transgenic animals have yet reached the market. But the scene is being actively prepared, as shown by the following facts:

- clones and transgenic animals have been produced in all the major livestock species since Dolly, the cloned sheep, was born in 1997;
- in 2006, the USA Food and Drug Administration (FDA) approved milk and meat from cloned cattle, pigs and goats for the human food chain without labelling;
- large biotechnology companies are already engaged in promoting transgenic livestock as a means of improving animals and their products for human consumption (Biotechnology Industry Organization, 2008);
- some of these companies already hold patents on transgenic livestock in the USA and the EU;
  further, this International Symposium raises the possibility of using these techniques in developing countries.

This paper opposes the use of transgenic livestock in practical farming and in the food chain because release of such animals would irreversibly change the agro-bioresources and the environment; also these manipulated resources cannot easily later be withdrawn. These changes would be particularly hazardous in developing countries. This paper does not oppose the use of molecular biology in the field of animal health where new products for old animal disease problems are being generated for diagnosis, prevention and treatment. These biotechnology products can be researched and produced within controlled conditions. The products come from genetically modified organisms (GMOs) but normally do not include tissues containing DNA itself. Further, use is targeted and limited to specific animals and the choice rests with the owner of the livestock.

Wisdom and Knowledge

As scientists we are tempted to think that the scientific method has such a firm foundation that it can answer all the issues of life. After all, science has yielded phenomenal progress in understanding the material world and by improving the quality of life. We must beware of confusing the objective scientific method with scientists who are human and fallible. In 2008, at a European conference, a top animal scientist was describing and advocating immediate use of cloned livestock for food; when asked about consumer resistance he said it was not an issue as "scientists think: others feel". That is a dangerous and arrogant world view that elevates scientists to an elite position as decision-makers for society because they 'know'. Doubtless within a narrow field, one can be rational, objective and super-intelligent in processing knowledge in the abstract. Reality, however, is much more complex. The application of knowledge requires wisdom, balanced judgement and concern for the larger consequences on the whole community of life today and in the future. The 2008 global banking and financial collapse was brought about by a small group of professionals who used highly rational and thought-intensive processes to create complex financial instruments — but the values they used were those of self-interest. This scenario clearly demonstrates the dangers of the concentration of power in the absence of basic human values. Those who neglect the values that build community lack wisdom. They cut off the branch that supports them. We violate community at our peril.

Scientists have an important role in society: basically, to serve the interests of all. Unfortunately some scientists now misunderstand their role. Rationality and reductionism are key intellectual components of the scientific method for successful study of the material universe. But the new knowledge they yield must be linked with wisdom on how such knowledge will be used. To apply a narrow scientific view to life as a whole raises the question of how knowledge differs from wisdom. Has humanity the ability and willingness to use its great intellectual resources to sustain life? This question lies close to the heart of whether emerging molecular technologies that are poorly understood should be used on livestock in simpler cultures with historically different values and traditions from the West. What is the difference between knowledge and wisdom? The simple yet profound answer is this:

Knowledge = power; knowledge + wisdom = sustainable life.

The difference between knowledge and wisdom is also highlighted by the two possible responses of scientists to any new discovery: "We can do it — so let's do it now"; or "We can do it — why do it now?"

The question of knowledge and wisdom is illuminated by a brief look at the origins of Western science that emphasises their different but complementary roles in civilisation. Modern science grew from the 15th century onwards shaped by the great European movements of the Renaissance, Reformation and Enlightenment. In the preceding European Ages, the common world view included positive as well as negative elements. Negatively, society in general was ignorant of the composition and function of much of the material world beyond the practical needs of living. Life contained much mystery. Lack of knowledge fostered superstition, for example, by attaching supernatural powers to material objects like trees, rocks, rivers, wind, fire and some animals, etc. Nevertheless, those simpler societies had many positive aspects — similar to many rural societies in the developing world today.

People living in poorer periods and countries know that community is essential for survival and for hope of a better life. In Early Medieval Europe prior to the Enlightenment, as in all sustainable societies, positive values and standards of social behaviour were basic with sanctions against anti-social conduct. Such communities lived within the boundaries set not only by natural resources and seasons, but they also cherished values that protect and sustain life. Individuality was valued, even encouraged, but was subject to the overall priority of living together. Thus, although simpler societies lacked the fuller knowledge of the physical universe brought later by science, they practised wisdom by sharing values that went deeper than material prosperity and individual success.

Before the Enlightenment, most people held a single unified worldview that included two distinct components:

- knowledge: facts about the material world based upon life experiences such as farming, weaving, building, cooking, health, etc;
- wisdom: using knowledge and resources for a sustainable life; being conscious of right and wrong and good and evil; treasuring human values that define good behaviour; being committed to quality of life for all in society over successive generations.

In that earlier society, values were learned by cultural osmosis while skills were acquired by apprenticeship working alongside experienced workers. The Enlightenment ushered in reductionism and education for professional disciplines in special institutions separate from the workplace. In the Pre-Enlightenment Age, the common worldview was illuminated by Christianity which was the historic

base of European beliefs, morals and values. The moral foundation of this world view, not always practised but accepted throughout society, was the teaching of Jesus to treat others as you wish them to treat you and not doing to others what you wish them not to do to you. The teaching style of Jesus emphasises the unity of knowledge and wisdom in that worldview, for his profound moral teaching was not abstract philosophy but was built upon practical examples from the routines of life that everyone knew.

The Enlightenment and the Birth of Modern Science

The Enlightenment challenged the Medieval unified worldview. A major change was formulated by René Descartes (1596–1650) that is particularly significant for scientists today, namely 'dualism'. In simple terms dualism means separation of what science can know from other ways of knowing. Science focuses upon the material world that is susceptible to analysis and scrutiny by objective debate. Descartes put all other components of knowing into a second category that he said is unquantifiable, subjective, and conjectural, based upon belief and sometimes invalid in some components. This dualist view of reality was new. Before Descartes, people saw a unity of the material and the spiritual. Knowledge, wisdom and superstition were mixed. But dualism separated knowledge of the physical world from the transcendent - and modern science was born.

Science slowly revealed the facts about the material world, showed how things worked and how they could be changed to improve life. This process eliminated superstition about matters that had not previously been understood. Nevertheless people and communities continued to hold and practise values and beliefs. The scientific revolution is often considered to have started formally with Sir Francis Bacon (1561–1626) who was also the inspiration for the founding of the Royal Society in England. He was an outstanding scientist and philosopher who emphasised the inductive method and recognised both categories of knowing; but he commented that "scientists have nothing to say about values". In the early centuries after the birth of modern science, wisdom about life and scientific knowledge were identified as separate but real partners in the common worldview. Unfortunately in recent decades there has been a tendency to elevate scientific knowledge above wisdom, giving rise to a posture which considers that only scientific facts matter while values can be discarded along with superstition. Some scientists today feel that scientific knowledge is the only type of information that counts. This attitude can lead to the arrogance that scientists 'think' whereas others only 'feel', thus equating feelings with superstition. This posture is both new and dangerous. E. O. Wilson (1998), the distinguished contemporary Harvard biologist described the position well: "We are drowning in information while starving for wisdom".

The early European scientists made two major systemic contributions to civilisation. They defined the foundations of the scientific method which include reductionism, hypothesis, testing, and replication. These bulwarks of modern scientific knowledge were predicated upon the assumptions that everything has been made by God who is neither chaotic nor capricious and that the material world reflects his consistent character. The second major contribution of the early scientists was wisdom to use science in a moral and ethical way for the welfare of all society. In general, they were men and women of integrity, transparency, honesty, modesty and objectivity. They replaced superstition with knowledge and retained wisdom on their agendas for use of the new scientific knowledge. These two postures formed the basis of the new science.

For several centuries, scientists were heroes as they uncovered facts and enlarged knowledge of the material world, thereby facilitating an improved quality of life. But it is now becoming clear that more recently many scientists have jettisoned much of the ancient wisdom, values and community identity that enabled European civilisation to grow and flourish. Today our advanced Western civilisation is dominated by human activity devoted only to economic efficiency, much of it led by science combined with business. The superb levels of scientific knowledge revealed by rationalism and reductionism are often applied without wisdom and are challenging the foundations of the society that gave them birth. Climate change is a high profile example. Subjection of the food chain to science and global business is another area that threatens the future of humanity. That challenge comes not primarily from increasing world population, but from excessive consumption by the rich developed West combined with absence of wisdom in using knowledge and resources in farming and the food chain.

The wisdom, values and sense of community that brought Europe out of the Dark Ages into the present advanced civilisation have been rooted in Judeo-Christian traditions. The key positive life precept expressed succinctly by Jesus is to do good to others. Over the centuries, this principle has been the accepted standard that guided good judges, good lawyers, good governments, good business leaders, good educators — and good scientists.

Founders and Successors of Modern Science

Some scientists today assume that the worldview of scientists has always consisted only of knowledge. However, we have already seen that science was birthed by both knowledge and wisdom. The neglect of wisdom is of recent origin starting at the end of the 19th century and accelerating towards the end of the 20th century. This movement has been greatly influenced by genetics and the new concept of the selfish gene leading to a value system emphasising individual competitive success in which community does not matter. As we look back to the scientists on whose shoulders we stand and to whom we owe our privileged opportunities, we see that for centuries many of them were gentlemen-amateurs like Darwin and Mendel or they were individuals working in an academic environment supported by funding that did not press them for economically important results. The older universities were not founded to increase gross national product (GNP) but as centres of learning and teaching to improve the quality of life over the centuries. Many of the early and great scientists, if not the majority, saw their discoveries in the context of the whole of life. Their wish was to explore God's creation and to do good.

Some historians consider that modern science was born out of the Renaissance, Reformation and Enlightenment because the new scientists gave up superstition and believed the physical world had been made by the God of the Bible who is rational and consistent. They brought into science the values of European society that were based upon Judeo-Christian teaching enlightened by the Reformation. For them knowledge gained by science and wisdom derived from the transcendent view of life were part of the same world view. Doubtless some were cultural Christians, but many of the most famous scientists were active in their Christian life and stated that commitment in their writings. Each of these famous scientists were practising Christians: Galileo, Priestly, Newton, Pasteur, Lavoisier, Kepler, Faraday, Copernicus, Maxwell, Pauling, Planck, Fermi, Gauss, Dalton, Linneaus, Pascal, Boyle, Hertz, Marconi, Kelvin, Mendel, Dobzhansky. Exemplifying their world view Joseph Priestly, the 18th century scientist who discovered oxygen, wrote: "The contemplation of the works of God should give a sublimity to the scientist's virtue, expanding his benevolence, extinguishing everything mean, base and selfish in his nature".

If today we argue that the transcendent world view of these leading scientists was simply a part of the culture for their day and age, then we need to think seriously about how much our own scientific view reflects the materialism of the 21st century that makes no place for transcendent values. Unless we consciously consider our values and beliefs we inevitably become participants of the current dominant world view that directs resources and makes decisions only on the basis of economic and biological efficiency. Interestingly one of our fellow biological scientists, Francis Collins, who was for many years Director of the Human Genome Project, writes (Collins, 2006) that as a scientist he was a committed materialist until he realised in mature life that he had never looked at the evidence and data for transcendence and God. His examination of the data convinced him intellectually, partly through the writings of C.S. Lewis (1898–1965), that he must take the Creator into his world view.

Today, scientists face a new and deeper problem. Scientists used to be regarded as trustworthy. Not today. As a profession we are seen by many as fallible humans who have sold ourselves to business and who thereby have lost interest in serving society. This is an especially critical problem for scientists working in agriculture and food who earlier had been highly successful in averting the famine predicted by Malthus (1798). Today, scientists working in the food chain have lost their heroic status and even their credibility. The false assurances and scandals over food safety and negative effects arising from intensive farming systems have raised public suspicion. The Enlightenment opened the door not only to modern science and wisdom but also to capitalism and democracy. Sadly, science in the food chain has become a bed-fellow with elite capitalism while democracy and wisdom are ignored and sometimes abused.

The ancient values of goodness and concern for others maintained community that was itself the infrastructure for all activities. Goodness is now a neglected word, replaced by efficiency and profit. Western civilisation has degenerated into a singular focus on self interest. Few look after the values of community. The public square is largely devoid of goodness. Looking back we can see that modern Western society has used the dualism of Descartes not only to reject superstition but also to throw out values of community, belief and goodness. Today, materialism rules. We are paying the price.

THE PRESENT WORLD SITUATION

Although transcendent values have been sidelined while materialism, financial profit and consumerism dominate the scene, occasionally religious leaders have spoken about the terminal nature of this world view. The more sensible spokesmen among them have called for inclusion of transcendent values in the daily routines of life, to build positive human communities and to restrain human brutalities based only upon self-interest. Under economic prosperity, most people have turned a deaf ear to such views. Now an extraordinary and radical change has taken place among the prophets. Today secular leaders proclaim the end of civilisation as we know it unless we change. Many now take up the theme of the religious leaders in calling for ethical behaviour based upon transcendent values in the public place and in the market and for government regulations based upon these ancient human values. We frequently hear from secular leaders in all areas of life including a few top scientists, for example, astrophysicist Professor Martin Rees, President of the Royal Society and Master of Trinity College Cambridge, that Payback Time has already begun and it may be too late to change (Rees, 2003). We are on a course of self-destruction. The threats we create come from human actions motivated by greed and short-term benefits that erode sustainability in human institutions, in the environment and in the community of life. Most people agree intellectually but do little to change.

Thirty years ago, one of the earliest secular leaders to foresee this sorry state of affairs was Alexander Solzhenitsyn, who, speaking at the 1978 Harvard University Commencement Ceremony, predicted the end of Western civilisation. He had confronted the evil Soviet system and after suffering in the Gulag had been expelled from his own country and was living at the time in exile in the USA. His view was that our worldview of materialistic consumerism would fail. He noted that the West was already committed to humanism and had rejected the transcendent values of historic Christianity that had guided Europe and its cultural colonies for nearly two millennia. We now know he was right. Thoughtful leaders in the 21st century are asking for those transcendent values to be brought again into the fabric of life and commerce. Scientists have a special role in re-introducing ethics in the food chain which in recent decades has become simply another global business.

Molecular biology and genetic manipulation are currently the frontier topics of science especially in the food chain. How do they and the scientists working in this field fit into the present world view that has been described? Two characteristics are central. First, molecular biology is highly complex and knowledge of it is minimal. We are dipping our toes into an ocean of integrated systems that will take decades to measure and to understand. Second, this universe of molecular biology lies at the heart of life. Everything we change, or even try to change, in the plant and animal agro-bioresources of the planet carries implications for unknown effects on life in its many forms. We are at the frontier of what it means to be human and civilised. That is why we need to stop and carry out 'due process' in the scientific realm before seeking to modify the genomes of livestock. Our knowledge of the inner universe of the mammalian genomes is primitive. Accumulated human wisdom should warn us that tinkering with the genomes of other mammalian species so close to our own is fraught with danger. It is time to question the Enlightenment motto that "Man is the measure of all things". As scientists in the food chain, we can look back to a success story of increasingly cheap and surplus food in the West since 1945. That transformation involved substantial inputs from governments and societies in addition to science and market forces. Now we must realistically address the needs of the billions of poor and hungry at a higher moral level than simply viewing them as a market from which we can make money. We need to return to the couplet of doing good science and also doing good.

GENETICS AND REVISIONIST GENETICISTS

Where did Genetics come from?

Genetics is relatively new. Before 1850 heredity was an open field of speculation. The mechanics of heredity were not understood, but from the time of settled agriculture, about 10 000 BC, farmers started to domesticate plants and animals which they slowly improved by phenotypic selection over many millennia. Charles Darwin (1859) worked only with phenotypic observations and comparative biology but was able to conceptualise the process of natural selection without knowledge of the genome. Gregor Mendel (1866) broke new ground by using phenotypic measurements to realise that discrete units of genetic material pass from one generation to the next, but he did not know anything about their structure. From Darwin and Mendel knowledge of genetics grew though the 20th century into the Neo-Darwinian synthesis. But it was nearly 100 years after Darwin that Watson and Crick (1953) discovered the architecture of DNA revealing it as the common molecular language of all life forms separated by species boundaries. Crick hypothesised the existence of a 'messenger' molecule which carries the genetic information from

the gene and facilitates assembly of specific proteins. This assumption was affirmed by the discovery of messenger RNA (mRNA).

In 1958 Crick announced the 'Central Dogma of Molecular Biology' and later wrote of it in Nature (Crick, 1970): genetic information flows from DNA to RNA to proteins with the possibility of some flow from RNA to DNA. But the Dogma asserted that information never flows from proteins to nucleic acids. The generalised model was described as replication, transcription and translation with all the interest focussed upon DNA as the controlling molecule of the genome. The discovery of reverse transcription RNA in retroviruses like HIV was a challenge that was given inadequate attention at the time. Some scientists questioned the Central Dogma. But mainstream thinking extended the Crick model by linear thinking to incorporate the slogan "one gene = one protein". Hence, in the search for means to transfer genetic traits from one species to another, the gene has been the supreme target. This ability to move DNA artificially was developed in the 1970s and was first called recombinant DNA.

World-class scientists were so awed by this new technology that in 1974 they met and agreed in the Asilomar Moratorium to suspend further work on gene transfer until the process could be better understood. Today, safety standards in most research laboratories are maintained to prevent escape of GM micro-organisms. But global release of GM crops for food was launched in the 1990s and continues. Approval by national regulatory authorities is required for these releases. The primary government authority is the US Food and Drug Administration (FDA), which normally makes its decision using only data supplied by the corporation seeking approval for its GM product. This regulatory process has been criticised for its lack of independent verification and longer-term testing — an issue that is discussed later in the context of GM crops which, having been released, have failed to meet performance claims.

DNA molecules lie at the root of the integrated genomic and proteomic systems that define form and function in all living organisms from viruses to man. This awesome power of DNA derives from the way it is assembled, making it analogous to a language that can be marshalled into words, sentences, paragraphs, chapters and an infinite number of books. That analogy has been well made by Collins (2006) in "The Language of God". Thus, the 50 years since the structure of DNA was clarified have seen astonishing progress. However, recent research reveals that gene expression involves much more than DNA acting unilaterally. The genome is an integrated system with many levels of control.

Scientists now understand the basic structure of DNA and have the ability physically to invade inner molecular space and to manipulate the genome leading to changes in gene expression though not yet in predictable ways. But we lack adequate knowledge of how life functions at the molecular level. In our enthusiasm for using the limited knowledge, we construct models of molecular function and use them for genetic modification. But our models are inadequate and the process of making a functioning GMO results in many failures, details of which have rarely been published in the scientific literature and sometimes concealed. In New Zealand in 2009, for example, failure to publish details of abnormal animals resulting from transgenic research is being contested in the courts. Considering that the stability of the mammalian genome is the result of millions of years of trial and error with many discarded non-functioning individual organisms, these negative cases of genetic modification by human intervention are not surprising.

Revisionist Genetics in the 21st Century

The story of scientists is not always splendid. While the scientific method is objective and amoral, scientists are human and susceptible to all the failures and foibles of mankind. Scientists have sometimes advocated use of incomplete or wrong models, explanations and world views that later they have had to correct. That is part of the scientific process. Models are invaluable for research but need to be thoroughly tested and proven in controlled conditions before public and widespread projects are built upon them. Thus wisdom often calls for more knowledge before action. A tragic case of basing public policy on limited knowledge concerns mad cow disease or bovine spongiform encephalopathy (BSE). Top UK scientists later had to change their model and then retract their earlier assurances to the public that beef from affected cows was safe to eat and that mad cow disease does not affect humans. Many people have died. Only 50 years ago among cosmologists seeking to understand the origin of the universe, the idea of a Big Bang was a joke, far less popular than the hypothesis of Continuous Creation. Similarly over the last 100 years, genetic models to explain inner molecular space have changed. Blending has been discarded along with Lamarckism and Soviet genetics that emphasised the inheritance of acquired characteristics. Society has also had to contend with the abusive social constructs of eugenics and racism advocated by a few high profile scientists and backed by some politicians, social reformers and philanthropists. The realisation that DNA is the universal material of heredity tempted mainstream geneticists to presume that Crick's Dogma was the final model. But recently, in the early years of the 21st century, research has shown fundamental flaws in this model revealing a far more complex genome. Following the familiar path of science we must now engage in revisionist genetics for the 21st century.

It would be folly to release GM livestock into the poor areas of the developing world where livestock is one of the key resources of the people. Our models are inadequate and we know so little. It is like launching a rocket to take people to the moon before the co-ordinates of its motion have been comprehensively measured and understood.

Crick's contribution on the structure of DNA and his prediction of mRNA were brilliant, but his Central Dogma was misleading and became the working model for many scientists until recently. Forty years after Crick announced his Central Dogma a major shock came from the Human Genome Project (HGP). The one gene = one protein model and the sheer quantity of human DNA had fostered an expectation that the hundreds of thousands of known mammalian proteins were associated with up to half a million human genes. In fact the HGP found only about 23 000 genes, little more than the nematode *Caenorhabditis elegans*!! The discovery of the low number of human genes added credibility for a time to the earlier concept: 'Junk DNA'. That model claimed to explain the large quantity of human DNA by postulating that much non-coding DNA was an inert residue from evolutionary development – useful at one time but now junk. However, high profile researchers in different centres now assert that since coding genes represent only about five percent of DNA it was a wild statement to assert the rest was junk. We now know that at least 50% of DNA has specific functions though most of the functions are still not documented. Transposons and jumping genes, whose roles are often unclear, also occur frequently. We do know that much DNA, formerly called junk, plays a key part in gene expression. Mattick (2005), from the Institute of Molecular Bioscience, University of Queensland, challenges the orthodoxy that 95% of DNA is evolutionary 'junk' as follows: "Most of this DNA is transcribed into non-coding RNA and consists of a hidden layer of

gene regulation that directs the development of complex organisms. Expression depends on which tissue the genome is directing".

So in the early years of the 21st century, three assumed models — the Central Dogma, one gene = one protein, and Junk DNA — have all been buried. Today our understanding of mammalian genes has changed. We now see that:

- genes multitask;
- genes are interdependent;
- genes overlap in function;
- information flows both to and from genes;
- switches can modify gene expression;
- the genome is highly integrated, compact and efficient.

Some of these characteristics of genes are explained by alternative splicing. In forming mRNA and proteins, some genes contribute information from only part of their DNA which is joined to limited sections of DNA from other genes, thus opening the door to numbers of proteins disproportionately greater than the number of genes. All this new information is significant for genetic modification of livestock because it will be difficult to anticipate all the effects of transgenes.

Analysis of the Human Genome – ENCODE Project

A further important contribution to the revision of genetics has been published. The ENCODE (2007) project involved 80 scientific teams in 11 countries over five years and cost $42 million. This mammoth study was a further step beyond the HGP analysis of base pairs. The ENCODE project analysed 1% of the Human Genome in detail and concluded, *inter alia*, that: "the genome is pervasively transcribed, such that the majority of its bases can be found in primary transcripts, including non-protein-coding transcripts, and those that extensively overlap one another". Further, the ENCODE scientists say that: "integration of the new sources of information, in particular with respect to mammalian evolution based on inter- and intra-species sequence comparisons, has yielded new mechanistic and evolutionary insights concerning the functional landscape of the human genome". They conclude that RNA has astonishing tasks, even controlling genes. The mammalian genome can no longer be viewed simply in terms of autonomous genes, since it consists of a complex integrated community of molecules. For example mRNA not only comes from protein coding genes but also from many other parts of DNA whose function is, as yet, unknown (Check, 2007). These coding sequences appear to be widely scattered throughout the genome (Callinan and Batzer, 2006), probably as protection against transposons and retroviruses being randomly inserted with disruptive effects upon the code. Greally (2007) in an Editorial in Nature on the ENCODE Project argues that we must go back to the beginning of molecular genetics. The Editorial reaffirms the point made by Mattick (2005) that certain regulatory processes are specific to cell type and further research into the functioning of the human genome will have difficult choices to make on which cell types to study. The realisation that gene expression is dependent upon cell type, as described by Mattick and the ENCODE project, effectively places a time bomb under the assumption that genetic modification of livestock by transgenes and cloning will be easy, predictable and safe.

The *Bos taurus* bovine genome was recently analysed for the first time (Elsik et al., 2009) and was found to have a minimum of 22 000 genes, not much different from the human genome. The study facilitates comparison between the two genomes to identify highly conserved DNA, specific break points in the evolution of cattle, frequency of repetitive elements, etc. However exciting these comparisons may be for research, the similarities are also warning signals against premature release of modified bovine genomes into food production, especially in developing countries where livestock are herded on wild herbage and their milk and meat is consumed by humans. The BSE saga speaks into this situation especially as genes involved in metabolism are highly conserved.

Other current areas of genetic research contribute to the call for caution and more knowledge as they identify further factors likely to complicate the way in which the mammalian genome functions; for example, stress proteins that act as chaperones to the DNA against heat and other environmental stress (Calderwood, 2007) and endocrine disrupters that affect gene expression (Kortenkamp, 2003). Another major factor is the realisation that epigenetic effects are more common than earlier thought. They include not only methylation, a well-known and common cause of modifying gene expression, but also discoveries of flows of information from other sources that arise in widely separated areas of the genome. For example, feedback information has been discovered from cell tissue to RNA to DNA that affects the way a gene operates. The detection of many forms of RNA with differing abilities to affect gene expression, described by the Nobel Laureate Cech (2004), is a revolutionary finding when viewed against the former model of one gene = one protein; earlier models must now be regarded as simplistic. Further challenges to understanding the genome are emerging from clues that information flows from the external environment to cell tissue and thence to possible modification of DNA expression. This possibility raises important issues concerning livestock adaptation that would be relevant if GM livestock were placed in the tropics.

MOLECULAR BIOLOGY, LIVESTOCK AND THE POOR

Mixing Advanced Science and Subsistence Farming

What do genetic manipulation of livestock and the poor have in common? They belong to completely separate worlds. Molecular genetics is at the cutting edge of Western science where it is buttressed by the resources, facilities and infrastructures of the most advanced civilisation in the history of the world. Its practitioners are highly educated scientists working in controlled laboratory conditions with access to finance and equipment. They are beneficiaries of a high standard of living and quality of life in an urban society where the system works, food is plentiful and cheap, life expectancy is high, employment opportunities good, government stable and the vagaries of the natural environment and the weather are largely irrelevant.

There are about three billion poor in Africa, Asia and Latin America whose lives are totally different. They live in simple conditions on $2 a day or less often in remote locations where they are exposed to the uncertainties of the natural world with little opportunity for employment, health care, formal education or prospects of change. Their worldview is more similar to that of pre-Enlightenment Europe than to that of the West today. Land and livestock, including poultry, are their major resources for security of life and food. Those who do not own land or animals are nevertheless dependent upon a rural community where these resources support the local economy. Livestock are wealth and insurance against an unknown future where government assistance is absent or very limited.

How can these two worlds be brought together? Should the attempt be made to unite them? One is strong in science and the other strong in community values and practical knowledge of how to survive in a hostile environment. Have the poor been asked if and how they want their livestock changed? It would be very difficult for the West to ensure Prior Informed Consent because of the paucity of knowledge of the genomes of GM livestock and the unknown

consequences. Ethics should be a major issue in the genetic modification of the livestock of the poor.

Consumers in Europe made strong statements against the GM crops when they first appeared in 1999 and labelling is now mandatory in the EU for the few GM foods allowed. More recently, some EU governments have stated their opposition to GM crops being planted experimentally. The EU parliament debated cloned livestock for food production in September 2008 and the vote was overwhelmingly against milk and meat products from cloned animals entering the food chain — 622 against, 32 for with 25 abstentions. It is rare to have such a strong vote on any issue. Should the view of European consumers affect the issue of GM livestock being introduced to the developing world? There is a frightening gap between the negative view of the European population and the posture of many European scientists who are confident that these new molecular techniques will bring increases in milk and meat production. Research directed to this end is in progress in both private and public institutions with the aim of using transgenic livestock in the food chain as soon as possible.

Proponents of transgenic livestock have serenely announced already that the technique will produce animals with superior qualities (Biotechnology Industry Organization, 2008). This organisation represents the companies and their scientists working in this field and speaks of enhancements to almost all aspects of livestock production: milk and growth, carcase composition, animal health and welfare, nutrition and public health, environment, hair and fibre - all are listed heralding a new era in animal science. These changes are described as though they are guaranteed rather than simply potentials visualised by scientific visionaries and speculative business interests.

Because of the difficulty of producing a GM animal, one plan is to use somatic clone nuclear technology (SCNT) to multiply superior GM individuals as a means of spreading the transgenes throughout the commercial livestock population using enhanced reproductive techniques such as artificial insemination and embryo transfer. This strategy for transgenic livestock opens a vast minefield of uncertainty that would need extensive and time-consuming research over several generations. The effects of producing successive generations of GM livestock by techniques that by-pass the filtering effects of gene segregation must be researched before commercial populations are committed to this new policy. Some surmise that gametogenesis will be an effective filter against abnormalities. We simply do not know. Such ignorance is dangerous. Some scientists argue that these dangers are greatly exaggerated because GM animals simply have their DNA coded differently and people have been eating DNA since the beginning of time. The crucial point is that risks emerge not from eating DNA *per se*, but from the harmful proteins that transgenic DNA may produce in a recipient organism — and these are unknown. Thus, the hazards are more likely to appear in the proteomics not the genomics. Most research is directed to the latter and not to proteomics which is a very new discipline.

The approval by the US Food and Drug Administration in 2006 of clones of cattle, pigs and goats for production of food products opens the way for SCNT clones to be used to propagate GM livestock. The complexities of using these technologies in commercial livestock and the human food chain raise a variety of concerns that have not yet been adequately researched. For example, genes from animals produced by SCNT have already taken part in cell differentiation during which specific genes are switched on and off. The importance of cell type in gene expression raises deep questions since an SCNT genome derives from an arbitrary choice of a somatic cell type that is then triggered artificially to start again as an embryo. The activity within a cell involving genes, DNA, RNA and proteins is affected by cell type. This fact raises a fundamentally important question of which somatic cell type and therefore which specific complex of molecules should be chosen to form the genetic profile of the SCNT clone and of the ensuing livestock population. This substantial question has not been tackled by planned experiment, but has been partially addressed by default as large numbers of failures occur before a live and apparently healthy SCNT animal is achieved. Dolly resulted from the 276th attempt. Mattick (2005) and the ENCODE (2007) project anticipated the problem in general without reference to SCNT when commenting upon the huge research task that lies ahead — to examine the expression of the genome of each cell-type. Use with livestock at this stage is risky. The unknown hazards of the type mentioned have been recognised as a possibility by the US Department of Agriculture which has taken the realistic step of wanting to compile a record of cloned livestock on farms - no doubt to facilitate tracing in the event of problems arising.

The Scientific Imperative or Empowerment of the Poor

Scientists working in this field appear to operate under a scientific imperative. A novel technique has been discovered enabling scientists to manipulate the genomes of livestock species. The methods are far from perfect, animals suffer in the process and knowledge of the molecular micro-universe is scanty. European citizens have expressed their wish to keep their food chain free from such products. Nevertheless scientists speak confidently about increasing the world food supply of meat and milk with these techniques. In their thinking the research stage has been passed. The moral high ground is invoked by claims that this new technology will help feed the world and is the legitimate scientific successor to the Green Revolution. Much more research is needed leading to comprehensive independent evaluation followed by consultation among the many stakeholders. Decision-making is then rightly in the hands of those who will benefit and carry any risks. A broader point currently ignored by the scientific imperative is the possibility that GM livestock will turn important segments of the consuming public against meat and milk. There seems little doubt that the growing demand for organic products in the more affluent food markets has been helped by the appearance of GM crops. The perception of spoiling your own market for animal products requires wisdom which, surprisingly, seems absent from the scientific imperative.

Assessment of Risk

The field of GM livestock faces Black Swan Events — defined as "unknowns that have a large impact but which are hard to predict". Mad Cow Disease (BSE) was a Black Swan event in the production of food from livestock. The defence against Black Swan events is not to be found in conventional use of probability statistics which are commonly used in biology. Even one Black Swan is one too many. The defence against such events is wisdom. This means looking at the scenario from every possible angle and then making a prudent decision based upon the likely scale and reversibility of any negative consequences if such were to appear.

Lessons for GM Livestock from the GM Crop Saga

Livestock scientists can benefit by the lessons arising from GM food products that have been on the market in the USA without labelling since 1998. Although accepted in the USA, they were initially rejected by consumers in the EU and approved in 2004 only with labelling. A widespread public controversy still over-shadows GM

crops with opposition from environmentalists, from farmers serving the growing organic market whose crops are sometimes polluted by GM crops growing nearby, from consumers who fear for their health, and from some independent scientists. An independent assessment of the first ten years of GM crops from 1998–2008 is now available (International Assessment of Agricultural Knowledge, Science and Technology, IAAKSTD, 2009), This study was made by 400 researchers worldwide working under the independent umbrella of the World Bank and multiple UN Agencies and was led by Dr. Robert Watson who is a high profile and experienced international scientist. The authors exhaustively examined the peer-reviewed publications that could contribute to an assessment of science and technology used in agricultural production including both developed and developing regions. The report concludes that GM crops have not, on average, increased crop yields. In some cases output was transiently increased and in others it was reduced, but this global study found no sustained increase in yield levels from GM crops. Farmers using GM seeds of corn, canola and soya are mainly large-scale even in developing countries such as Argentina, Brazil and Mexico. They use GM seeds to reduce their costs of spraying as most GM crops have transgenes resistant to more concentrated chemicals. The study found that reduction in costs and transient increases in production depend upon local conditions and crop management. The IAAKSTD study shows that GM crops to date cannot be regarded as a 'silver bullet' whose use will automatically increase food production.

GM crops were introduced to the market ten years ago with the promise of increased food production but without either adequate research or public debate. As a consequence a promising new technology has received a negative public image. The new science has been used almost entirely upon the four staple crops which are the most profitable markets in large-scale farming. The technology was promoted with the claim to feed the world better by producing crops that are able to grow in harsh and unfavourable locations. This has not been achieved to date and some critics see this claim largely as propaganda to promote the image of the four staple GM crops. The story revealed by the IAAKSTD study does not enhance the image of scientists involved with GM crops whether they work in the laboratory or as scientists advising governments on regulations and approval.

Consequences and Regulation of GM Crops

GM cotton (Bt cotton) is a non-food crop which Monsanto promoted among both large scale cotton producers and small scale peasant farmers especially in India. Proponents have considered it a GM success story while there have been anecdotal accounts of negative results and consequences. High levels of suicide among small scale Indian farmers using GM cotton have been recorded that allegedly were especially associated with credit taken to buy GM seeds and the subsequent inability to use harvested GM seed. The International Food Policy Research Institute examined this situation (IFPRI, 2008) and concluded that the documented increase in suicides could not be linked directly with the use of GM cotton and was due to institutional, climatic and economic constraints among many factors. Some farmers' associations in India have complained of livestock deaths after ruminants have foraged on the GM cotton vegetation in the fields following harvest. The Indian government extension services have undertaken to carry out controlled experiments on this issue. Monsanto has now disclosed that the cotton pest, pink bollworm, has developed resistance to Bt cotton in several regions of India (Monsanto, 2010). This response of naturally occurring organisms to GM crops was one of the dangers predicted by some scientists who, from the early days of GM crops, considered that it was premature and dangerous to release transgenes in domestic crops into farming, the environment and the food chain. Understandably, large segments of the public have an image of scientists who have sacrificed objectivity and normal scientific protocols of transparency, failed to publish negative research results, and should have sought wider public debate before the products of the new technology were used extensively. One such scientist is Schubert (2002) a cell biologist at the Salk Institute and earlier a colleague of Francis Crick. It is difficult to avoid the conclusion that some scientists have become self-serving and that consequently society as a whole has not benefited from the new knowledge and technology.

A further important issue for scientists is the need to remain in touch with farmers, consumers and citizens on issues of GM food. If scientists become isolated from common people they are in danger of making decisions on the basis of the latest technology results and of pressures that commercial corporations place on governments for swift regulatory approval. For example, the Indian government in 2009 decided to give approval to GM Bt brinjal (aubergine) which is a widely used indigenous food source in India. Farmers and people from all walks of life protested and called a fast. Several leading Indian scientists, including Dr. M. S. Swaminathan of international stature as a plant breeder, spoke against the release of GM brinjal. The government changed its decision and GM brinjal will not be released. Dr. Swaminathan (2010) took the opportunity to repeat his view that GM crops should be subject to testing in an internationally qualified and independent laboratory as privately generated data are inadequate; further he considers there is a risk that untested Bt brinjal may be like tobacco and lead to chronic dosage problems; he also thinks that introducing GM brinjal will destroy indigenous varieties. Another highly placed scientist in India, Dr. P. Bhargava (2010) who serves on the national GM regulatory body has called for a freeze on GM crops on biosafety and health grounds before release.

The IAAKSTD study and the recent experiences with Bt cotton and Bt brinjal show how vital it is for scientists to combine wisdom with the new-found knowledge in molecular biology. These mistakes must not be repeated with livestock. The risks that may arise are too great for GM livestock to be put into the public domain in the developing world. The hazards include the failure rate such as abortions and birth of abnormal animals which could be catastrophic in the small herds and flocks of poor livestock keepers.

Lessons for GM Livestock from the BSE & vCJD Saga

The aims of those advocating transgenic livestock include substantial modifications to the life processes. Such changes clearly target key proteins — a scenario that opens the door to dangers from the unknown and unexpected proteins that will be produced and eaten by humans in milk and meat from transgenic livestock. The plan is to use gene transfer on livestock to change growth and lactation physiology, metabolism, endocrine systems and reproductive hormones of livestock. To be safe, such radical interventions will require decades of research to ensure that no unwanted proteins are produced somewhere in the mammalian body.

Transgenes are chosen because they are known to produce a desired trait in the donor species, but in the recipient species there is no guarantee that they are located correctly or supported by the appropriate RNA and protein components, enabling them to function as predicted in each cell type. Like natural mutants, transgenes are not inserted precisely and therefore experience a similar high rejection rate, already confirmed by experience. In the case of livestock the high failure rate highlights animal welfare issues. Many attempts are needed to produce one designer organism, indicating that most (alien mutant) transgenes are tested and rejected by

recipient species that have been on the evolutionary test bench for millions of years and have enduring homeostasis. The question must also be asked about generational stability in transgenic organisms. The process of meiosis is a powerful filter that any mutant must survive. Reversion to the wild type would undermine claims for increased food production and negate the purpose of the whole enterprise. By any standards of logic, the rushed ambition to sell products from transgenic livestock is courting a large harvest of abnormal animals and the possibility of economic failure for herd owners or misfortune among consumers before untarnished success is achieved. There is also a deep question of how a GM animal should be tested for normality before release for use.

The lesson of BSE (mad cow disease) and its human variant Creutzfeldt Jacob Disease (vCJD) speaks into the issue of transgenic livestock. These conditions are caused, so far as is known, by an aberrant protein (prion). The economic losses and increased costs were enormous; hundreds of thousands of cattle were killed; and 177 people died by 2009 from vCJD. The genetic aspects of this scientific study have been given too little serious attention in the plans to produce GM livestock. BSE was spread from cow to cow though eating the prion present in offal used as a feed supplement. People were affected by vCJD through eating the prion in beef from cattle with the condition. But not everyone who ate the prion died. Most of those who died from vCJD carried a specific allele combination at one codon in their genome. Other people with a different allele did not get the condition even though eating the prion. This fact points strongly to a genetic linkage between the bovine and human genomes. In view of the very close matching of human and bovine DNA arising from evolutionary history, this finding is not surprising. But it is a serious indicator of other unfortunate and as yet unknown linkages that may appear when humans eat meat from transgenic cattle. The lethal agent is not the DNA but the protein. The unknown BSE-type dangers that may arise from GM livestock are unlikely to be caused by animals or people eating transgene DNA. The risks are associated with the expressions of the transgene as unexpected proteins, either alone or more likely in combination with existing genes in the recipient organism with which the transgene interacts. A further complication is the possible effect upon wild mammalian predators that may eat GM livestock. The scenario of unforeseen transfers of harmful proteins from GM livestock to humans, to other livestock and to wildlife opens the door to Black Swan events too serious to be ignored. Everything points to the need for long and intensive research over many years and generations before GM livestock are released and used for food.

GM Livestock, Adaptation and Empowering the Poor

The idea of inserting genes into livestock by radical and untried methods for use in the developing world raises a host of questions about adaptation to local environments, feed resources, animal health, disease and parasite resistance. Any genetic modification has to focus upon a limited group of traits like growth, milk, etc. The effect of such genetic modification on adaptive traits will be a huge research project, for genotype-environmental interactions are well known to be difficult to measure and to interpret. Adaptive traits suited to local conditions that have been developed by natural selection over long time periods are key to the value of livestock to billions of poor in the developing world. Any deliberate or unintended modification in adaptation could have dire consequences for the herd owners. The IAAKSTD (2009) report addresses the issue of farming among poor small scale farmers in the developing world and comments that genetic modification of agro-bioresources is a feature of Western intensive farming that requires the support of infrastructures and capital that are absent in many rural areas of Africa, Asia and Latin America. In general the authors concluded that intensification of farming by technology transfer from the West has very limited value among the poor. This is a stark conclusion from an authoritative and independent body of researchers. The Assessment was also critical of the environmental impact of intensified Western agriculture and found it unsustainable. After the IAAKSTD Report, a pan-African study was published by UNEP-UNCTAD (2008) showing that agro-ecological agriculture, when properly applied in Africa, out-produced other technologies. The IAAKSTD Report advocates that research should be re-directed towards improving locally sustainable and high yield methods instead of technology transfer.

CONCLUSIONS

The molecular structure of life is complex and highly organised. It has been shaped by billions of years of mutation, testing, rejection and adaptation: an extraordinary process with remarkable results. A key feature is the astonishing way in which information is stored, accessed, transmitted and used for life processes by a remarkable integration of DNA, RNA, proteins and other molecules that is also open to information causing epigenetic effects. The enthusiasm for genetic modification of livestock needs to be tempered by the evident current lack of understanding of this highly integrated process. These facts merit serious reflection as scientists of this generation attempt to improve upon the natural product.

Enormous periods of evolutionary time were essential for producing the integrated species genomes that show enduring stability, economy of structure and efficiency of function. This process does not fit the pulse of science and business in the 21st century. Wisdom would suggest that the dash for swift returns on investment is highly unlikely to yield livestock genomes that are durable, functional and safe.

Scandals like BSE and its human form (vCJD), toxins in animal products, unlabelled GM food, foot-and-mouth disease epidemics, avian and, so-called, swine flu, have diminished the reputation of animal scientists in the eyes of the public, many of whom now see scientists as agents of big business. The historic image of objectivity and service to the public good has been reduced. Animal scientists must now, more than ever, take seriously their obligation to balance scientific knowledge with social wisdom in evaluating molecular technology in livestock production — especially in the rural areas of Africa, Asia and Latin America where populations are more vulnerable to change.

Citizens in Europe feel precarious and at risk from the activities of a combination of elite scientists and business interests whose self-appointed mandate to manipulate food has not been put through the democratic process and, in the USA, due to the government decision no labelling is required. Absence of labelling to identify GM products contravenes the principles of the market economy. The molecular biologist today is well equipped as a technologist with specialist knowledge. But the extreme reductionism of scientific education, research and practice combined with the priority given to mission-oriented research leaves many scientists deficient in two areas:

- absence of the grand vision of science held by many great scientists of earlier generations;
- lack of wisdom and understanding of the deep complexity of human life.

These two deficiencies are particularly relevant to the issue of using transgene technology and products to change livestock.

SUMMARY

Gene transfer for livestock may be evaluated in terms of Foundations, Fallacies and Assumptions.

Foundations

Knowledge has lost the underpinning of wisdom that is essential if science is to contribute to improved quality of life for society as a whole.

Fallacies

Some biological scientists have embraced an erroneous belief they can quickly produce 'better' species than evolution and millennia of domestication and selection.

Assumptions

The expectation that livestock owners want transgenic livestock and that consumers will accept milk and meat products from them is unethical and probably wrong.

Proposal for a Moratorium on the Use of GM Livestock for Food.

The author asks fellow scientists in the field of animal science and molecular genetics to consider making an independent statement of their commitment to the well-being of society at large. They should consider following the initiative of their predecessors who agreed the Asilomar Moratorium when they realised they had a new technology with great potential for good or bad consequences. Scientists should call for a Global Moratorium banning the use of genetically manipulated livestock for food. Research should continue until knowledge is sufficiently advanced to preclude negative effects when consumers can indicate in a referendum if they want milk and meat from GM livestock.

Postscript

Some great thinkers and people of action in the past have recognised that scientists are easily tempted by power through specialist knowledge.

- Michael Polyani: There is a danger of scientists coercing society because of specialist knowledge. They must not be allowed to seize levers of power and must work through democracy.
- Winston Churchill: Scientists should be on tap and not on top.
- Albert Einstein, Bertrand Russell, Linus Pauling, Andrej Sarhkarov: All warned of the dangers of scientific anarchy.

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Merino Breeding Program Improves Wool Production in Western US Range Sheep Flocks

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ABSTRACT

A Merino breeding resource flock was established at Rafter 7 Ranch, Yerington, Nevada. Initially, 500 Rambouillet ewes were purchased from two established breeders in 1990. These ewes were bred naturally or by artificial insemination (AI) to imported Merino rams from Australia and to crossbred rams selected within the flock. The flocks were expanded to 1300 ewes and bred in 30 single-sire mating groups as of the 2006 breeding season. Flock management is in two breeding lines, one as a registered Rafter 7 Merino flock (n = 650) and the other (Merino × Rambouillet) as Rafter 7 Line (n = 650). The spring lambing flock winters on desert rangelands, is grazed on irrigated pasture from shearing through lambing and early weaning. Compared with the original base ewe flock, Merino and Merino crossbred ewes produced higher clean wool yields, longer staple lengths, and higher grease fleece weights. The body weight and greasy fleece weight showed a significant ($P < 0.05$) difference between two flocks whereas no differences were observed for wool fibre diameter, length and comfort factor in most recent analysis. However, fibre diameter variation was significantly different ($P < 0.05$) between the two flocks for age groups and birth years. Body weight, fleece weight and fibre diameter showed significant ($P < 0.05$) but low to moderate correlations. Approximately 1000 breeding rams and 500 replacement ewes were distributed to commercial range flocks in the western states. The dissemination of introduced Merino genetics in the western range sheep flocks is expected to enhance wool quality and wool profits in the western region of the USA.

Key words: *Merino, breeding, genetics, wool, fibre diameter, rangelands.*

INTRODUCTION

In the United States, sheep and lambs are raised primarily in small farm flocks in the Midwest and the East, and on large ranching operations in the West (NRC, 2008; www.nap.edu). The first domesticated sheep were brought to the United States in 1493 with the second voyage of Columbus. With growing importation of Spanish Merino sheep in the late 1700s and early 1800s, the U.S. wool clip began to grow substantially. As the industry moved to western states, wool production from the French Rambouillet, originally developed from Spanish Merino genetics, expanded, and by 1870, about 80% of all USA sheep were of Merino origin (ASI, 2002). However, the USA sheep inventories declined steady from 56 million head at peak of 1942 to the present day seven million head. Nevertheless, sheep grazing in the western rangelands can be profitable and environmentally sustainable (Glimp and Swanson, 1994). Most of the sheep inventory of the country is in wool-meat dual-purpose sheep and the majority of flocks produce medium to strong wool of 23.5–26.4 micron (spinning count in 58–60s). In 2006, the 14 western range and intermountain states accounted for 72% sheep and lamb inventories but produced 77% of the USA wool clip and received 88% of income from wool sales (USDA, 2007). The USA clean wool sale price analysis indicated that every one micron decrease in fibre diameter of fleece (for example 22 micron to 21 micron) increased by 10% the market value of USA produced wools (Anderson et al., 2007). Over the last two decades, the fine wool clip has become progressively finer while textile technology has improved for processing superfine wool types. The consumer demand of a light weight, next to skin comfort wear, and fashion trends drive the world apparel wool markets. Consequently, the international raw wool trade premium prices set for fine (19–21 micron) and superfine wool (16–19 micron) categories have increased significantly over the other type of wools during 1990s (Land, 1990; Purvis, 1995; Wuliji et al., 1999; Wuliji et al., 2001), which facilitated the Merino breeding projects in some wool growing regions. While the size of the USA sheep industry is expected to be stable with possible slow growth in future, in order to be profitable, sheep producers should take advantage of both domestic and international fine wool niche markets, and the biological ability of the sheep to control weeds and thrive in suboptimal ecosystems (Lupton, 2008). This paper describes a wool sheep selection programme at the Rafter 7 Ranch and the impacts of Merino genetics dissemination into western USA range sheep flocks. Animal performance and wool characteristics were analysed and are presented for two selection flocks, namely, Rafter 7 Merino and Rafter 7 Line.

MATERIALS AND METHODS

Flock Establishment

Two decades ago, the University of Nevada-Reno and Rafter 7 Ranch established a Merino breeding programme at the Rafter 7 Ranch near Yerington, Nevada to introduce superior fine-wool Merino genetics from Australia to provide genetically improved and adapted breeding rams and ewes for the U.S. western range regions. The sheep flock at the Rafter 7 Ranch has been managed as a quarantine flock since its establishment. The only live animal introductions to the flock have included seven Merino rams imported from Australia in 1990 and ten Merino rams imported in 1997, which were quarantined and tested in Australia for 90 d and further quarantined and tested in the USA for 30 d to meet USDA–APHIS importation requirements. Frozen

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N.E. Odongo, M. Garcia & G.J. Viljoen (eds), Sustainable Improvement of Animal Production and Health. Food and Agriculture Organization of the United Nations, Rome, 2010: **237–241**

semen, collected from quarantined rams in Australia to meet USDA–APHIS requirements, has been imported from another 32 rams. The flock met the USDA–APHIS Certified Scrapie Free certification requirements by 2005. The average fibre diameter (AFD) of the original imported rams was in range of 17.5–19.5 microns according to the stud book information, while mixed age Rambouillet ewe flock fleece were estimated at 58–60s spinning counts (objective test was not available at the time) during the initial crossbreeding phase. All animals born on the ranch are provided an individual metal ear tag at birth and an electronic ear tag number incorporated into their scrapie tag at weaning at approximately 90–120 d of age. Sheep numbers have steadily increased to meet the needs for breeding, replacements, and distribution of breeding stock to sheep producers. The flock is physically inventoried at breeding (approx. Nov. 20), shearing (approx. March 20) and lamb weaning and ewe culling (approx. Aug. 15), and inventory changes due to sales and animal death losses are recorded.

Range and Pasture

A flock of 1 300 breeding ewes and 35 stud rams are maintained at the Rafter 7 Ranch, a University of Nevada-Reno (UNR) cooperative sheep station owned by the Edwin L. Wiegand Trust. The ranch includes 1 400 ha of private land and grazing permits on 40 500 ha of Bureau of Land Management Lands, and 1800 ha of USDA forest land. The flat pasture elevation is at 1200 to 1 500 m, and high desert range elevation is up to 3000 m. The annual precipitation within the area of perimeter is less than 200 mm, mostly as winter snowfall with unpredictable frosts and wind patterns. Desert shrubs include black greasewood, basin big sagebrush, black sagebrush, bud-sage, white sage, and ephedra. Grass species include Indian ricegrass, bottlebrush squirreltail, and cheatgrass. The established pastures were primarily tall fescue, over-seeded with Ladino clover. Improved irrigated pastures include a mix of tetraploid perennial ryegrass, improved fescue cultivars, a grazing variety of alfalfa and Ladino clover. An additional 50 ha of irrigated land is used for alfalfa hay production and aftermath grazing. Irrigated pastures, 35 pastures at 2–6 hectares, are set stocked during breeding and lambing, with an intensive rotation the rest of the grazing season.

Animal Breeding and Distribution

Natural mating and AI were used alternatively during the upgrading phases. A computerised record and data base program that includes individual animal pedigree, sex, birth date, birth and rearing rank, weaning and yearling performance record file is maintained on the ranch. Two seasonal lambing managerial options were adopted since 2006 although the majority of lambs are scheduled to be born during spring lambing. Animal selection was made each year prior to the breeding season on a multi-trait Performance Index in conjunction with 'independent culling' for undesirable traits such as poor conformation and structure, wool face cover, jaws, infertility, and coloured fibres. Animal selection was based on objective wool measurements as well as subjective assessment, growth rate, and reproductive performance traits.

There are five wool breed societies established in the USA including American Cormo, Boroola Merino, Rambouillet, Debouillet, and Delaine Merino (ASI, www.sheepusa.org). The Rafter 7 Ranch Merino flock was fully inspected, pedigreed and registered with the Delaine Merino Breed Registry. Flock management is in two breeding lines, one as a registered Rafter 7 Merino flock (n = 650) and the other (Merino × Rambouillet) as Rafter 7 Merino Line (n = 650), both of which are selected for high fleece weight and quality, twinning, and growth traits. The spring lambing flock was wintered on desert rangelands, and grazed on irrigated pasture from shearing through lambing and early weaning. Lambs were subjected to pre-selection culling at weaning and final selection based on yearling performance including body and fleece weight, and wool characteristics. A final selection performance index was derived by various adjustment weightings to birth and rearing ranks, age, body weight, weight gain, fleece weight, fibre diameter and length. Ram distribution catalogues for selected rams and ewes with a comparative Performance Index were posted to the potential sheep producers/clients 2–4 weeks before the ram sale field day on the ranch. Selected rams and ewes were presented with IDs, pedigree and yearling performance data sheet, and health certificate in subdivided pens on the field day. Regularly, about 70–100 producers participated in an annual ram sale and several dozen ranchers purchased their choices of breeding rams and ewes by an open bidding at the field day auction. Genetic distribution and impacts on range wool sheep production were monitored on a number of ranches who consistently used Rafter 7 Ranch rams. Four of these associated ranches located in Reno, Ely, Fernley and Rafter 7 of Nevada were surveyed for their superfine category (<19 micron) wool lot weight ratios in the clips using the wool warehouse records and public auction information from 2004 to 2009 wool sale catalogues.

Wool Production and Clip Preparation

Individual fleece weight and wool characteristics were recorded for the life time of breeding ewes and rams. Pre-shearing midside wool staples were collected from each sheep and a set of programmed wool tests for wool characteristics, including average fibre diameter (AFD), fibre diameter variation coefficient (FDcv), average staple fibre length (ASL), and estimated comfort factor (CF) were measured using an OFDA 2000 instrument (IWG Pty Ltd, Australia). Shearing was scheduled at least four weeks prior to lambing. Fleeces were classed according to the pre-shear test classification with some subjective alternatives, such as short, discoloration or tender strength. Wool clip volumes and sale values were recorded and presented for last five years. At the 2009 spring shearing, approximately 3000 fleeces at Rafter 7 Ranch were pre-tested, shorn and classed into five category lots, namely, ultrafine, superfine, fine, medium and coarse lines, which were baled and transported to the wool warehouse that provided a sale lot test certification.

Measurements and Statistics

Animals were recorded for selection flocks, post-shearing body weight (BWt), greasy fleece weight (GFWt) and wool characteristics including AFD, FDcv, CF and ASL using the OFDA 2000 instrument on pre-shearing midside staple samples. An annual wool fibre diameter measurements of 556 selected mixed ewes in two flocks, which were born over four birth years in 2001–2004, were monitored for five years of wool production (five shearings) respectively. Therefore, the changes in AFD were compared to every year from the first to the fifth shearing (Age I, II, III, IV and V) consecutively. Post-shearing BWt, GFWt and wool characteristics of two flocks (2009, observed n = 2 218) were analysed. The procedure of GLM, CORR, and GLIMIX of SAS (SAS Inst. Inc., Cary, NC) were followed for data analysis of BWt, GFWt and fibre characteristics, and fibre diameter variations in birth year and ages.

Table 1. Means of body weight (BWt), fleece weight (GFWt), fibre diameter (AFD) and fibre diameter variation (FDcv), staple length (ASL) and comfort factor (CF) in flocks (2009 shearing).

	No Obs.	BWt (kg)	GFWt (kg)	AFD (μm)	FDcv (%)	ASL (mm)	CF (%)
R7 Merino	1 291	66.5[b]	5.32[a]	19.4	17.2	86	99.0
R7 Line	1 947	72.2[a]	4.63[b]	19.5	17.4	82	98.9
SE		0.95	0.9	0.1	0.05	0.5	0.05

[a, b] Column means with different superscript letters are different ($P < 0.05$).

Table 2. Least squares means of average fibre diameter of Rafter 7 Ranch breeding ewes by flock and age group (n = 556).

Flock	Means /Flock	Means by Age Group					SE
		Age I	Age II	Age III	Age IV	Age V	
R7 Merino	20.5	18.4[e]	20.9[c]	20.7 [c]	21.4[a]	21.4 [a]	0.1
R7 Line	20.6	18.7[d]	21.1[b]	20.8 [c]	21.6 [a]	21.5 [a]	0.1

Means with a different superscript letter (a, b, c, d) differ significantly at $P < 0.05$ level within and between rows for age groups; there is no statistical difference between the pooled flock means.

Table 3. Pearson correlation coefficients (r values) of body weight (BWt), fleece weight (GFWt) and fibre diameter (AFD), staple length (ASL) and comfort factor (CF) characteristics.

	GFWt	FDcv	AFD	ASL	CF
BWt	0.20**	-0.06**	0.21**	-0.13**	-0.10**
GFWt		-0.20**	0.64**	0.45**	-0.36**
FDcv			-0.16**	-0.09**	-0.16**
AFD				0.31**	-0.70**
ASL					-0.13**

**All r values are significant ($P < 0.01$).

Table 4. Body weight (BWt) at weaning, yearling, 16-month old and fleece weight (GFWt) of the Rafter 7 Ranch sale rams (n = 120/y) (Wuliji et al., 2009).

Year of Birth	Flock ID	Weaning BWt kg	Yearling BWt kg	16 Month BWt kg	10 Month GFWt kg
2006	Merino	27.7	50.9	75.5	3.55
	R7 Line	28.6	52.7	78.6	3.64
2007	Merino	32.7	67.3	74.0	4.09
	R7 Line	37.3	72.7	79.1	4.09
Average/Flocks		31.4	61.4	75.0	3.63

RESULTS AND DISCUSSION

Body weight, GFWt and wool characteristics are shown in **Table 1**. There are significant ($P < 0.05$) differences between the two selection lines for BWt and GFWt. The pure Merinos produced more wool than the Rafter 7 Line even though their wool was slightly finer. The Rafter 7 Line sheep weighed significantly more ($P < 0.05$) than the Merino sheep. Over the years of upgrading, the Rafter 7 Line has closed the gap with the Merinos so far as average fibre diameter is concerned while still maintaining a significant advantage in BWt and other subjective traits, such as a larger body frame and structure. But no differences were present between AFD, FDcv, ASL and CF of the flocks. Changes in AFD were shown to exist among birth year (and age) ($P < 0.05$) (**Table 2**), which reflected the environmental variation, and interaction of birth year and age groups. Pearson correlation coefficients were calculated between BWt, GFWt and wool characteristics and are presented in **Table 3**. There is a significant ($P < 0.01$) high correlation between AFD and CF, and also high correlations among GFWt, AFD and ASL; but a low and moderate correlation exists between BWt and GFWt, whereas small or negative ($P < 0.05$) correlations were observed between FDcv and AFD, BWt, GFWt, AFD, ASL, and CF. Weaning weight, yearling weight and GFWt of the Rafter 7 Ranch sale rams have improved over years (**Table 4**). The BWt and GFWt for sale rams of birth year 2006 and 2007 did not differ between the two lines.

Approximately 1000 breeding rams and 500 replacement ewes were distributed to range flocks in the western states in the last decade, which made a notable improvement for GFWt, fibre diameter and yield in clients' flocks (Wuliji et al., 2009). Wool sales from Rafter 7 Ranch have increased significantly in volume and value (**Figure 1**). Sheep flock performance and wool sale information show consistent

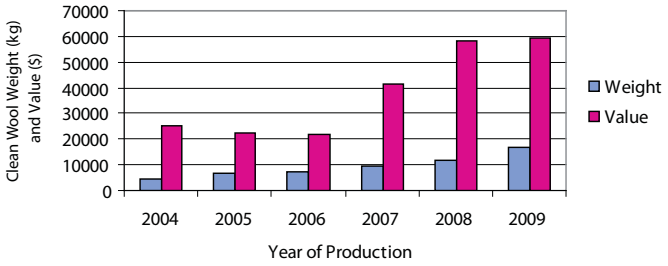

Figure 1. The Rafter 7 Ranch Wool Clip Volume and Value.

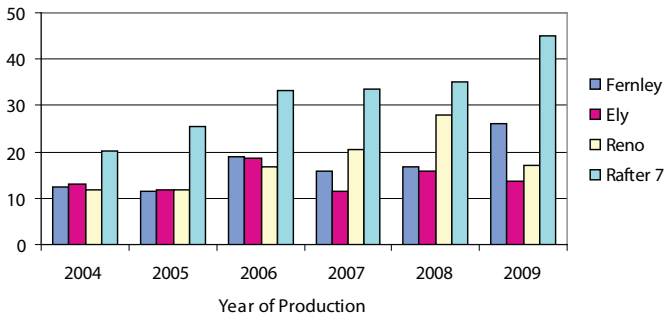

Figure 2. The Superfine Ratio (%) of Wool Clip.

improving trends within Rafter 7 flocks and associated clients' flocks. For the past eight years, the Rafter 7 Ranch wool clip has received the highest price of any wool grown in the USA. Sheep producers from 18 states, Mexico and Canada have purchased breeding rams and ewes from the Rafter 7 Ranch over the past 13 years. The dissemination of introduced Merino genetics of Rafter 7 Ranch into the western range sheep flocks has produced an improvement in wool quality by increasing the superfine wool (<19 microns) ratio of the clip in the associated sheep producer ranches (**Figure 2**). This trend is expected to strengthen a long-term competitive advantage for the western USA states' wool sheep enterprises.

The earlier analysis of Merino crossbred ewes in the flocks showed that clean wool yield, staple length, and GFWt were increased by 15% (67% vs. 52%), 2.5 cm (8.5 cm vs. 6.0 cm) and 1.14 kg/head shorn (5.3 kg vs. 4.2 kg), respectively (Glimp, 2006). Mean GFWt of both Rafter 7 Merino and Rafter 7 Line flocks were apparently higher than published data (Lee et al., 2000) of typical Rambouillet ewes, which were recorded for 4.16 kg/head mixed ages ewes but AFD were lower than the original Rambouillet foundation ewe flocks (25 micron). Body weight measures in sale rams showed a trend that two breeding flocks are converging in terms of for BWt and AFD, although the Merinos produce more and finer wool. The AFD changes by age groups were small to moderate, which is a similar pattern to increase in fleece weight, which showed a larger increase from the first shearing to the second, but small changes until four years of age. Such features of AFD and FDcv, and inter - trait correlations were also observed in ultrafine Merino flocks (Wuliji et al., 1999).

The sheep breeding, wool selection and genetic resource distribution of the Rafter 7 Ranch flock impacts and the breeding stock distribution for western range sheep flocks (Wuliji et al., 2007) and grazing efficiency of ewes on the range were discussed in detail elsewhere (Rauw et al., 2007a). The gradual and continual increase in superfine and fine wool ratios of the clip at Rafter 7 Ranch was consistent with the early within-flock analysis (Wuliji et al., 2008). Weaning weight of the Rafter 7 flocks were also analysed previously (Rauw et al., 2007b), which showed that ram lambs weighed heavier than ewe lambs, that single reared lambs were heavier than multi-litter lambs, and that lambs born from two-year-old ewes were lighter than from other age group ewes. The selection efficiency in premium wool characteristics and rapid genetic gain were reported for various wool breeding demonstration flocks (Wuliji et al., 1999 and 2001; Swan and Purvis, 2005; Brien et al., 2005). These characteristics showed moderate to high heritability (Atkins, 1997; Okut et al., 1999, Wuliji et al., 2001, Hanford et al., 2004). Therefore, we predict an increased likelihood of a higher rate of genetic dissemination into commercial sheep flocks followed by rapid genetic gains in wool quality traits.

CONCLUSIONS

The Rafter 7 Ranch Merino flocks have made significant progress in major selection traits including fleece weight and fibre diameter during the crossbreeding and upgrading phase. The Ranch is now disseminating elite genetics in many western range sheep flocks. The dissemination of introduced Merino genetics in the western range sheep flocks will improve wool quality and clip profits, thereby strengthening a long-term competitive advantage for the USA wool and sheep production sectors.

ACKNOWLEDGEMENTS

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Thai Indigenous Cattle Production Provide a Sustainable Alternative for the Benefit of Smallscale Farmers, Healthy Food, and the Environment

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ABSTRACT

In Thailand, there are 5.66 million Thai indigenous cattle and their crossbred derivatives (1.76 million cows). The Department of Livestock Development (DLD) has a policy to conserve and use Thai indigenous cattle as the genetic base for establishing and developing new breeds of beef cattle. The objectives of this study were to study the production performance, carcass quality, healthy food production, economic potential, and environmental impacts of four breeds of Thai indigenous cattle (Kow-Lamphun, Kho-Esarn, Kho-Lan, and Kho-Chon cattle). Data were collected from two studies: i) 1 220 cattle from an experimental trial in DLD part, and ii) 390 cattle kept by smallholders in Northern, Northeastern, Central, and Southern parts of Thailand between October 2004 and September 2008. Data were adjusted by group, location, month, and year to analyse for the above parameters. Kho-Lan and Kho-Esarn cattle had the highest weaning weight and preweaning daily weight gains while Kow-Lamphun cattle had the highest Omega 3 (8.98%) and conjugated linoleic acid (CLA) levels in their meat (0.02%), and produced the highest net incomes (306 915.80 Baht/y). Through the conservation and use policies of the DLD, Thai indigenous cattle provided various advantages for farmers, consumers, and environment.

Key words: *Thai, indigenous cattle, weaning weight, carcass, Omega fatty acids, conjugated linoleic acids.*

INTRODUCTION

In Thailand, there are around 5.66 million Thai indigenous cattle and their crossbred derivatives (1.76 million cows) (DLD, 2004). Thai indigenous cattle are classified as *Bos indicus*. Their characteristics are similar to other indigenous cattle in Southeast Asia. The four main breeds of Thai indigenous cattle are Kow-Lamphun, Kho-Esarn, Kho-Lan and Kho-Chon cattle in the northern, northeastern, central and southern parts of Thailand respectively. They are easily raised, selected by natural selection, have good reproductive performance, can provide a calf every year, and are resistant to diseases and parasites. Their meat texture is fine and firm and optimised for cooking Thai food. Thai indigenous meat is very tasty and has more specific nutrients that are useful for consumers, such as Omega 3, Omega 6, and CLAs (Boonyanuwat, 2009).

Farmers raise these cattle integrated with other agricultural products, such as rice, para rubber tree, corn, sugar cane, and fish. Their manure is used as fertiliser for crops and producing plankton for fish, and for producing biogas and electric power for household use. In addition, Thai indigenous cattle are used as draught animals. Thai indigenous cattle have various skin and hair colour such as red, light brown, black, piebald, but the Kow-Lamphun cattle in the northern part of Thailand have an orange-pink skin and white hair colour. They are small, heat tolerant, disease resistant, and have high fertility (Boonyanuwat, 2008).

The objectives of this study were to study the production performance, economic potential and impacts on the environment of Kow-Lamphun, Kho-Esarn, Kho-Lan, and Kho-Chon cattle.

MATERIALS AND METHODS

Animals

Four breeds of Thai indigenous cattle were used in this study:

- Four hundred and eighty eight Kow-Lamphun cattle (370 cattle of DLD, 118 cattle of kept on smallholder farms);
- Four hundred and eighty four Kho-Esarn cattle (367 cattle belonging to the DLD, 117 cattle on smallholder farms);
- One hundred Kho-Lan cattle (76 cattle from the DLD, 24 cattle on smallholder farms); and
- Five hundred and thirty eight Kho-Chon cattle (408 cattle from the DLD, 130 cattle on smallholder farms).

These four breeds were separated roughly into two groups (a cow-calf production group and a finishing group). They were fattened at DLD Research and Breeding Centres/Stations and on smallholder farms (Kow-Lamphun cattle in Lamphun, Chiangmai, Phayao, and Phrae provinces; Kho-Esarn cattle in Ubolrachathani and Chaiyaphum provinces, Kho-Lan cattle in Ratchaburi and Petchaburi provinces; and Kho-Chon cattle in Phatthalung, Trang, Songkla, and Yala provinces) between October 2004 and September 2008.

Measurements and Statistical Analysis

Data from the DLD groups were collected every six months while data from smallholder farms were collected every month. Data were collected on growth performance (bodyweight (BWt), average daily gain

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(ADG)), carcass performance (carcass %, loin eye area, shear force, fatty acid profile), economic performance (net income per head) and cattle manure production. Samples for shear force and fatty acid profile measurement were collected from the 7th–8th rib.

The samples and data for carcass performance were collected at the Research and Breeding Center/Station of the DLD, Kampangsaen slaughter house of Kasetsart University and the Central Laboratory of Chiangmai University. Data were adjusted for variations arising from group, location, month, and year and the adjusted data were analysed by ANOVA for growth, carcass and economic performances (Chantalakhana, 1991).

RESULTS AND DISCUSSION

Growth and Reproductive Performance

The average bodyweights of each breed were not significantly different. Kho-Lan cattle had the highest weaning weights (WWt) and ADG (103.18 kg and 0.421 kg) (**Table 1**). In the DLD group, they were fed with grass and legume. They were raised by grazing in pasture *(Bachiaria ruziziensis* and *Stylosanthes hamata*). In the farmers groups, they were fed with grass, or grass and legume, and supplemented with concentrates after calving. Kho-Chon cattle had the lowest WW. In southern Thailand, farmers prepare male calves for use as fighting bulls. There were no significant differences in reproductive performances. Thai indigenous cattle can give birth every year with calving intervals of 433 to 469 d.

Carcass Performances and Meat Quality

The meat texture of the four breeds was not significantly different. It is very firm (shear force = 5.45-5.56) (**Table 2**) and suitable for Thai food cooking. Meat from these cattle is used to make meat balls and Thai food.

Kow-Lamphun cattle had the highest Omega 3 fatty acids (8.98%). However, Omega 3 levels in all indigenous breeds were high (Kho-Lan = 6.25%, Kho-Chon = 6.26%, and Kho-Esarn = 2.37%) compared with Bos taurus (2.90% for grass fed and 0.64% for grain fed animals (Daley et al., 2009). Omega 3 beef was different from typical beef in that it was obtained by grass feeding while typical beef is most often obtained through grain feeding. Grass was higher in Omega 3 fatty acids while grains that were fed to animals were higher in Omega 6 (Helmet, 2009). Even so, Thai indigenous cattle that were grain fed had high Omega 3 levels (e.g. Kho-Esarn, grain fed and grass fed).

Native cattle had the highest Omega 3, Omega 6, and polyunsaturated fatty acid (PUFA) levels. Conversely, Kow-Lamphun cattle had the lowest Omega 6 levels (3.67%). Kho-Chon, Kho-Lan, and Kho-Esarn had higher Omega 6 (4.36, 5.48, and 10.14% respectively). Thus, the ratio of Omega 6:Omega 3 of Khow-Lamphun cattle was lowest, next to Kho-Chon, Kho-Lan, and Kho-Esarn. The recommended ratio of Omega 6 to Omega 3 fatty acids is 2:1 or better. Omega 3 beef is also loaded with natural vitamins and minerals. It is a great source of CLA (conjugated linoleic acid), a fat that is reputed to reduce the risk of obesity, cancer, diabetes, as well as some immune disorders. Beef in its natural state and grass fed, not grain fed, allows it to be categorised as a health food. This is a red meat that is actually good for consumers (Helmet, 2009).

Thai indigenous cattle meat was very rich in Omega 3 fatty acids as well. It is also free from hormones and antibiotics. A proper balance between Omega 3 and Omega 6 fatty acids helps to maintain and even to improve health. A healthy diet should consist of roughly one to four times more omega 6 fatty acids than omega 3 fatty acids (Daley et al., 2009).

Kow-Lamphun and Kho-Lan cattle had higher CLA levels than Kho-Chon and Kho-Esarn cattle, 0.02, 0.02, 0.01, and 0.01, respectively ($P<0.01$). CLAs are a group of polyunsaturated fatty acids found in beef, lamb, and dairy products consisting of a general mixture of positional and geometric conjugated isomers of linoleic acid (Sehat et al., 1999). They are produced in the rumen of cattle and other ruminants during microbial biohydrogenation of linoleic and linolenic acids by the anaerobic rumen bacterium *Butyrivibrio fibrisolvens* (Pariza et al., 2000). Over the past two decades numerous health benefits have been attributed to CLA in experimental animal models including actions to reduce carcinogenesis, atherosclerosis, the onset of diabetes, and fat body mass (Daley et al., 2009).

The anti-atherosclerotic evidence was first reported in CLA treated mice by Ip et al. (1994). Ip and co-workers showed that CLA levels as low as 0.05% of the diet can have a beneficial effect in mice. A level of 0.5% reduced the total number of mammary tumours by 32%. These results also demonstrated that CLA administered through a dietary route was effective in providing protection against cancer (Ip et al., 1994). However, there is high CLA in Thai indigenous cattle meat.

Although there were no significant differences between indigenous breeds in PFU levels, these levels were high compared with

Table 1. Growth and reproductive performances of Thai indigenous cattle.

Trait		Kow-Lamphun (n = 488)	Kho-Esarn (n = 484)	Kho-Lan (n = 100)	Kho-chon (n = 538)
BWt	(kg)	19.64 ± 2.86	18.38 ± 5.34	18.57 ± 2.58	19.91 ± 1.40
WWt**	(kg)	90.22^{b} ± 29.12	100.38^{a} ± 10.51	103.18^{a} ± 14.32	87.81^{b} ± 12.04
ADG**	(kg/day)	0.353^{b} ± 0.117	0.410^{a} ± 0.053	0.421^{a} ± 0.059	0.340^{b} ± 0.052
BWt 400**	(kg)	204.30^{b} ± 39.11	221.55^{a} ± 42.92	210.71^{a} ± 29.25	198.45^{b} ± 27.21
BWt 600*	(kg)	296.04^{b} ± 39.48	323.14^{a} ± 64.38	314.54^{a} ± 43.66	296.54^{b} ± 58.52
Age first calving	(month)	28.34 ± 4.43	26.47 ± 7.69	26.92 ± 3.74	28.67 ± 2.02
Calving interval	(d)	463.67 ± 117.24	433.00 ± 125.80	448.59 ± 62.27	469.04 ± 33.05

BW = birth weight; WW = weaning weight; ADG = preweaning ADG; BWt 400 = body weight at 400 d of age; BWt 600 = body weight at 600 d of age.

** Different letter in the same row means highly significant difference of means in each trait ($P<0.01$).

* Different letter in the same row means significant difference of means in each trait ($P<0.05$).

Table 2. Carcass performances and healthy food production of Thai indigenous cattle.

Trait	Kow-Lamphun (n = 34)	Kho-Esarn (n = 16)	Kho-Lan (n = 23)	Kho-chon (n = 36)
Final weight (kg)**	320.47^{a} ± 44.49	299.21^{b} ± 41.5	302.33^{b} ± 41.97	323.49^{a} ± 44.91
ADG (kg/d)	0.668 ± 0.79	0.624 ± 0.447	0.630 ± 0.452	0.674 ± 0.484
Carcass (%)	56.94 ± 10.03	54.11 ± 9.37	54.66 ± 9.46	58.49 ± 10.12
Loin eye area (cm^2) **	57.46^{a} ± 6.34	53.67^{b} ± 5.92	54.21^{b} ± 5.98	58.01^{a} ± 6.40
Shear force (kg) (21 d)	5.49 ± 0.57	5.50 ± 0.54	5.56 ± 0.54	5.45 ± 0.58
Omega 3 (% total fat)**	8.98^{a} ± 7.55	2.37^{c} ± 0.69	6.25^{b} ± 5.02	6.26^{b} ± 1.87
Omega 6 (% total fat)**	3.67^{c} ± 0.57	10.14^{a} ± 4.74	5.48^{b} ± 3.47	4.36^{b} ± 1.54
CLA (% total fat)**	0.02^{a} ± 0.02	0.01^{b} ± 0.01	0.02^{a} ± 0.01	0.01^{b} ± 0.01
SFA (% total fat)**	80.27^{a} ± 9.37	49.45^{c} ± 3.27	72.59^{b} ± 14.94	82.27^{a} ± 4.51
MUFA (% total fat)**	6.78^{c} ± 6.38	37.85^{a} ± 5.89	14.42^{b} ± 4.29	6.83^{c} ± 3.63
PUFA (% total fat)	12.94 ± 7.99	12.70 ± 5.45	12.00 ± 5.23	10.90 ± 2.04

CLA = conjugated linoleic acids; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

** Different letter in the same row means highly significant difference of means in each trait (P<0.01).

Table 3. Economic performances and impacts on environment.

Trait		Kow-Lamphun (n = 3)	Kho-Esarn (n = 15)	Kho-Lan (n = 3)	Kho-chon (n = 30)
Farm size	(No. cows)	18.00^{a} ± 5.18	3.80^{b} ± 1.86	19.30^{a} ± 7.81	2.94^{b} ± 1.07
Age of cow	(y)	4.42 ± 1.84	4.50 ± 1.87	4.88 ± 1.58	4.85 ± 1.25
Total cost	(Baht)	54 802^{b} ± 13 700	24 950^{d} ± 8912	12 6746^{a} ± 11318	26 768^{c} ± 13 859
Feeding cost	(Baht)	28 597^{b} ± 7149	17 217^{c} ± 4054	8 7464^{a} ± 5149	13 451^{c} ± 1 118
Total income	(Baht)	364 718^{a} ± 91 179	57 628^{c} ± 9616	29 2751^{b} ± 12213	23 161^{d} ± 18 253
Net income	(Baht)	915^{a} ± 76728	32 678^{c} ± 704	16 6006^{b} ± 3579	7 838^{d} ± 4 257
Fertiliser from manure	(ton)	75.88^{a} ± 1.04	16.02^{b} ± 1.49	81.36^{a} ± 3.78	12.39^{b} ± 0.86

** Different letter in the same row means highly significant difference of means in each trait (P<0.01).

other breeds and grain fed beef cattle (levels in Kow-Lamphun, Kho-Esarn, Kho-Lan, and Kho-Chon averaged 12.94, 12.70, 12.00, and 10.90%, respectively, **Table 2**). Nonetheless, intramuscular fat of other beef breed and grain fed typically consisted of approximately 47, 42 and 4% of total fatty acids as saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and PUFA, respectively. The PUFA in beef contains considerable amounts of *n*-3 PUFAs, particularly α-linolenic acid (C18:3*n-3*) and the longer chain PUFAs, eicosapentaenoic acid (EPA; C20:5*n-3*) and, docosahexaenoic acid (DHA; C22:6*n-3*) (Scollan, 2009).

Two important nutritional indices are used to describe the fatty acid composition of foods. The first is the ratio of PUFA:SFA (the P:S ratio), and the second the ratio of the *n*-6:*n*-3 fatty acids (usually expressed as the ratio of essential fatty acids C18:2*n*-6 (linoleic acid): C18:3*n*-3 (linolenic acid). The P:S ratio for beef is typically low at around 0.1, except for double muscled animals which are very lean (<1% intramuscular fat) where P:S ratios are typically 0.5-0.7. Results from EU Healthy Beef have demonstrated a strong relationship between total intramuscular fat content and P:S ratio (Scollan, 2009). The ratio of *n*-6:*n*-3 ratio for beef is beneficially low, typically less than 3 and the focus has been on methods of increasing the P:S ratio and lowering the *n*-6:*n*-3 ratio by increasing the content of beneficial *n*-3 PUFA (Scollan, 2009). Kow-Lamphun cattle had a high P:S ratio.

Economics and Environmental Impact

From the 51 smallholder farms used to collect data, the average herd sizes of Kow-Lamphun, Kho-Esarn, Kho-Lan, and Kho-Chon, were 18.00, 3.80, 19.30, and 2.94 cows respectively (**Table 3**). The data for Kow-Lamphun and Kho-Lan cattle were obtained only from conservation farms, while others were collected from general farm. On the conservation farms, farmers could earn higher net incomes. In these two farms, farmers could decide on the price for conservation and rare animals. These farms could produce fertiliser from manure to provide organic material for the land (**Table 3**). On the basis that there are 5.66 million Thai indigenous cattle, the data on manure production recorded here suggests that these animals could contribute 177 000 tons of dry manure. In this way, Thai indigenous cattle improve the structure of land and thereby enhance the environment.

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Community-Based Productivity Veterinary Service for Smallholder Dairy Farmers in Bangladesh

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ABSTRACT

The productivity veterinary services, which include disease control and management of reproduction, udder health and nutrition, are not practised in smallholder dairy farms although they are proven to increase milk production in large dairy herds. We introduced an on-farm service with the participation of farmer associations where individual veterinarians made a scheduled visit to perform preventive and emergency cattle health care, reproduction, and feed management. We examined 1 849 animals on 862 farms guided by specific forms, a breeding calendar and a herd summary generated from data of the initial visit by using a Microsoft Access based computer application. On average, 53% anoestrous heifers and 67% anoestrous cows resumed their oestrous cycle when treated with hormones, vitamin AD_3E or nutritional supplements. Forty percent of cows with uterine infections conceived when treated with intrauterine antibiotics or prostaglandin $F_2\alpha$ ($PGF_2\alpha$) was injected intramuscularly before artificial insemination (AI) was done. When GnRH was injected at the time of AI, 73% repeat breeder cows conceived. About 78% of cows recovered from mastitis and 88% of sick animals recovered when treatment was given based on clinical diagnosis. A database on common cattle diseases was established. More than 75% of farms that received the service had an income increase ranging from US$1 to US$40.7/month/cow. Productivity veterinary services can increase farmers' incomes and the number of cows available for breeding.

Key words: *productivity veterinary services, smallholders, health care, reproduction, feed management, anoestrous, uterine infections, artificial insemination, income increases.*

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INTRODUCTION

Bangladesh needs to improve the growth of its dairy industry from the current rate of two percent to at least six percent to meet half of the consumer demand for milk by the year 2025 against a population growth rate of 1.6%. Farmers' incomes would increase by between US$676.3–1 730.6/y if all of them operated their farms as efficiently as the 20% best farmers in the community concerning levels of milk production/cow/d, increasing lactation length, decreasing age to first calving, and decreasing calving interval (Shamsuddin et al., 2006). Farmers spend only a small amount of money on veterinary services (Shamsuddin et al., 2006), and the benefits of veterinary services on the productivity of animals have shown a poor return on investment. Veterinarians neither have information on the private job market nor do they have a model for delivering the service and recovering the cost. The biggest challenge is that farms are too small and farmers cannot buy the service individually. An alternative approach of working through farmers' associations would be useful to execute and finance a market-driven veterinary service. We report here a model of delivering productivity veterinary services to smallholder dairy farms through farmers' groups and associations which would substantially increase their income.

MATERIALS AND METHODS

Farms, Farm Sizes and Production Systems

In the popular areas for small scale dairy farming in the districts of Satkhira, Mymensingh, Chittagong and Sirajgonj, about 250 farms were selected in each and divided into groups of ten farms. One farmer in each group worked as the Group Leader. One veterinarian following a previously set schedule visited ten farms each d every month, with the Group Leader being kept informed. Thus during the 25 working d of a month, the veterinarian visited 250 farms. Twenty five Group Leaders made an association. Data reported here were from four of such associations constituting 1 000 farm families during the period March 2005 to June 2006.

The dairy production systems in these districts were published elsewhere (Shamsuddin et al., 2006 and 2007). Individual farms in Satkhira had an average of six cattle with 2.2 lactating cows. Individual farms in Mymensingh had 4.2 cattle with 1.4 lactating cows. The production system was crop-livestock mixed farming in Mymensingh and Satkhira. The dairy farmers of Sirajgonj had access to the Bangladesh Milk Producers' Cooperative Union Ltd. and they were heavily dependent on dairying for their livelihood. The average

N.E. Odongo, M. Garcia & G.J. Viljoen (eds), Sustainable Improvement of Animal Production and Health. Food and Agriculture Organization of the United Nations, Rome, 2010: **247–253**

number of cattle/farm was 10.5 with 3.4 lactating cows. Dairy farming is commercial and intensive in Chittagong. Here, the average numbers of total cattle and lactating cows/farm were respectively 8.0 and 2.8.

Collection of Information, and Provision of Veterinary Services and Follow-up

To guide delivery of the veterinary service and to follow up on its outcome and collect field data, we developed five forms (Shamsuddin et al., 2009). A calendar was developed to keep records on breeding, vaccination, de-worming and other health and production-related events in the farm.

Form 1

This form had four parts dealing with issues such as a farm inventory, preventive health management, feed management and a list of animals treated for sickness. Data on existing animals on the farm, animal types and their production were recorded. If any animal was sold, its type and price was recorded. If an animal died, its type was recorded. Information on de-worming, vaccination, teat dipping and examination of fore milk was recorded. The date of drug administration, its cost and administration fees were recorded with regard to the type of the animal. For feed management, at first the amount of feed given to animals was recorded together with the feed price. If necessary, changes in feed composition and amount were recommended and recorded in the form. The identity of individual animals treated and herd nutrition conditions were also recorded, the latter on the basis of a 1–5 scale (Nicholson and Butterworth, 1986).

Form 2

This form helped recording information on breeding cows and heifers, cost of breeding and pregnancy diagnosis. The form guided recording farmers' complaints and taking previous histories on e.g. parturition, puerperium, retained placenta, number of services used for last conception and milk production of cows with reproductive problems. Animals were examined and temperatures, heart rates, respiratory rates, rumen contractions-frequencies and strength and nutrition conditions were recorded. Genital tracts and ovarian cyclicity were evaluated by inspection and rectal examinations. In follow-up visits, results of treatment were determined and further necessary interventions adopted.

Form 3

This form allowed recording data on mastitis diagnosis and treatment. Farmers' complaints and animal history were recorded. In addition to physical examinations of cows and their udders, milk was examined by visual inspection and by the California Mastitis Test (CMT). Findings of the examinations were scored and recorded. Mastitis was diagnosed and its severity was graded as mild, moderate and severe based on scores given through examinations of the cow, udder and milk. The form had a guideline for the treatment or management of mastitis, and options for recording the outcomes of treatments at follow-up visits.

Form 4

This form was designed to guide examination of sick animals other than those with reproductive problems and mastitis. Farmers' complaints and animals' history of sickness were recorded. They were examined for heart rate, respiration rate, rectal temperature and rumen contraction-frequency and strength, consistency of faeces, hydration, appetite and nasal conditions for making a clinical diagnosis. Prescribed treatments and their costs were recorded in the form. The form had provision for recording the results of treatments at follow-up visits.

Form 5

This form was used to record all expenditures and incomes of the farm operation on a monthly basis. Incomes from milk and home use of milk, manure sales and/or home use, sales and slaughter of livestock and costs for feed purchase and freight, health care, labour and maintenance were recorded.

A Microsoft Access-based database application was customised, matching with the forms to record and analyse the data and to produce a herd summary. At farm visits, the veterinarian checked the results of earlier interventions and schedules of de-worming and vaccination. The veterinarian then checked the breeding calendar for reproductive events and especially examined cows bred 35 or more d earlier for pregnancy, cows that gave birth 60 or more d before for ovarian cyclicity, and cows that failed to conceive after three consecutive services. A clinical diagnosis was made and treatment and/or management changes were prescribed. Heifers that were more than two years old but had not shown oestrus were examined for ovarian cyclicity. The veterinarian also looked at the drying-off date of the cow, milking hygiene and post-milking teat dipping. Additionally, the farmer could call a veterinarian if any emergency or general cattle health care issue arose on the farm.

Data Management and Analysis

Data from the customised database application were exported to a Microsoft Excel Workbook 2002. Only farms that accepted the interventions were included. Descriptive statistics were computed and histograms drawn. Regression analysis was done to establish the relationship between the number of lactating cows on a farm and the net income from the operation. Farms from Satkhira and Mymensingh that had data from at least three visits were included for the analysis of economic impacts of interventions. Net income (gross income - gross cost) was calculated/cow/d for before and after interventions. The information obtained on the first visit was considered as pre-intervention data, while data from subsequent visits were averaged to calculate post-intervention values. The initial net income from individual farms was deducted from the post-intervention net income to determine changes in farmers' income from the service.

Considering the possible influence of flush and lean season on the economic impact of the service in Sirajganj, additional data on expenses and incomes from dairy operation were collected through one-day farm visits by using preset questionnaires from 204 member farms and 60 non-member farms. Non-member farms did not receive the service but were situated in the same locality where interventions were performed. Data were entered into Microsoft Excel Worksheet 2002, total incomes and expenditures/year/farm were estimated individually for intervened and control farms, returns calculated and histograms prepared. Differences in returns between intervened and control farms were tested for significance using the z-test.

RESULTS

We examined 1 849 animals on 862 farms. In follow-up examinations, 53% anoestrous heifers and 67% anoestrous cows resumed their oestrous cycle when treated with preparations of hormones, vitamin AD_3E or with nutritional supplementations, on average. Forty percent of cows with uterine infections conceived when antibiotic was given into the uterus or $PGF_2\alpha$ was injected intramuscularly

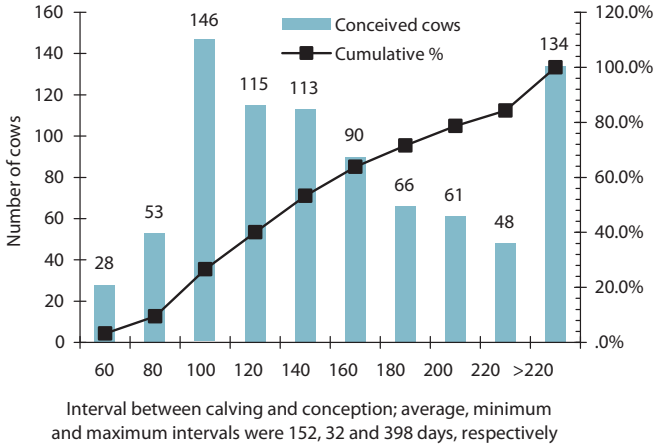

Figure 1. Number of cows and their cumulative percentages conceived at different days postpartum (number of cows = 854).

before AI was performed. When gonadotrophin-releasing hormone (GnRH) was injected at the time of AI, 73% of repeat breeder cows conceived. About 78% of cows with mastitis cows recovered after treatments and 88% of sick animals recovered when treatment was given based on clinical diagnosis.

Reproductive Management

In total, 914 cows and heifers were examined for pregnancy diagnosis by rectal palpation and 854 were pregnant (**Figure 1**). Sixty cows (6.6%) were found non-pregnant. Only 25% of cows conceived within 100 d of calving and 44% of cows took 160 to 398 d postpartum for conception. Farmers paid between US$0.6 and US$15.4 for breeding an animal to achieve a pregnancy.

Management of Anoestrous Cows and Heifers

In total, 389 cows and 279 heifers were diagnosed as anoestrus and prescribed treatments. Follow-up data were available on 93 cows and 101 heifers. On average, 71% of cows and 53% of heifers resumed oestrous cycles after treatment (**Table 1**). Farmers considered 35 cows (9%; n = 389) anoestrus but those had a corpus luteum at their ovaries (**Table 2**). These cows were treated with PGF2α with or without GnRH and inseminated either at detected oestrus or blindly at a fixed time i.e. 70 and 90 h after PGF2α injection. Eighty percent of cows were presumed pregnant (non-return by d 30 or diagnosed pregnant). The cost of treatment for individual animals varied from US$1.5–17.0. The overwhelming majority of animals (87%) were treated for US$1.5–3.7.

Management of Cows and Heifers that Failed to Conceive after Three Services

The veterinarian examined 118 cows with a history of conception failure after more than three services and prescribed treatments accordingly. Follow-up data were available on 56 cows. Forty animals were treated with GnRH preparations at the time of AI and 29 of those (73%) became pregnant. Sixteen animals were inseminated twice on the same oestrus at 12 h intervals; 12 of these conceived. The cost of treatment ranged from US$1.5–8.8 per cow.

Management of Cows with Infected Uterus

Fifty five cows with uterine infections of varying degrees — ranging from cloudy genital discharge to discharge of pus — were diagnosed and treated. The treatment was either intramascular injection of a prostaglandin F2α preparation or intrauterine application of antibiotics. Data on 30 cows were available on follow-up visits. Twelve (40%) of cows conceived, and the cost of treatment ranged from US$1.5 – US$4.4/cow.

Mastitis Cow Management

One hundred and fifty one cows with complaints of udder problems were examined and treatments were prescribed. Twenty eight of 36 cows showed normal udder and milk on follow-up visits (**Table 3**). The treatment cost for mastitis ranged from US$2.9– 8.8/cow.

General Cattle Disease Management

Three hundred and ninety five animals were examined and treatments were prescribed. Follow-up data were available on 187 animals. Most of the treated animals recovered from illness and only two animals died (Table 4). Frequency percentages for the different disease conditions are shown in Figure 2. The overwhelming majority of clinically diagnosed diseases were diarrhoea/dysentery (18.5%) and endoparasites (20.5%). The treatment cost for general disease

Table 1. Treatments with outcomes of anoestrus cows and heifers.

Treatment used	Number of animals treated		Number (%) of animals cycled	
	Cows	Heifers	Cows	Heifers
[1] GnRH (+ PG + AI + GnRH)	14	12	12 (86)	7 (58)
Anthelmintics + ADE	35	28	26 (74)	16 (57)
ADE	22	49	14 (64)	23 (47)
Nutrition supplement	22	12	14 (64)	8 (67)
Total	93	101	66 (71)	54 (53)

[1] Prostaglandin F2α was injected 12 d after GnRH injection and AI was done on oestrus with an additional GnRH injection at the time of AI; GnRH — commercially available synthetic gonadotrophin-releasing hormone preparations; PG = prostaglandin F2α preparations available commercially; AI — artificial insemination; ADE — an injectable preparation of vitamin A, D3 and E available commercially.

Table 2. Treatments with outcomes of cyclic cows that farmers considered anoestrous.

Treatment used	Number of cows treated	Number of cows non-returned or pregnant
PGx1+AI on oestrus	9	8
PGx2+2 times AI at 12 h interval	7	6
PGx2+2 times AI at 12 h interval + GnRH with first AI	10	7
GnRH (+PG+AI+ GnRH)	5	4
2 times AI at 12 h interval at detected oestrus	4	3
Total	35	28

PG — prostaglandin F2α preparations available commercially; GnRH — commercially available synthetic GnRH preparations; AI — artificial insemination.

Table 3. Treatments with outcomes of cows with mastitis.

Treatment used	No. treatments	No. positive responses
Intramammary antibiotics only	15	13
Intramammary antibiotics with parenteral administration of anti-inflammatory drugs	7	4
Intramammary antibiotic + anti-inflammatory drugs + antibiotics administered intramuscularly	12	10
Systemic antibiotic only	2	1
Total	36	28

Table 4. Number of animals examined, treated and followed up with results of treatments.

Parameters	Number	Percentage
Cattle examined and treatment given	395	
Cattle followed up	187	47.3
Animals completely cured	165	88.2
Animals showing some improvement	13	7.0
Animals showing no improvement	7	3.7
Animals that died	2	1.1

conditions ranged between US$0.7 and US$2.9. A database was made of cattle health problems (Figure 2).

Effects of Productivity Veterinary Service on Net Incomes of Farmers

Changes in farmers' net incomes due to the services delivered in Satkhira and Mymensingh are shown in **Figure 3**. In Satkhira, more than 75% of farms had an increase in net income, which ranged from US$1.0 – US$19.2/cow/month. In Mymensingh, more than 80% of farms had increased income due to the productivity veterinary services; increases ranging from US$1.0 – US$40.7. In Sirajganj, farmers that received the service had a higher ($P<0.05$) return than those who did not (**Figure 4**).

When data from all farms were pooled, a significant relationship appeared between the number of lactating cows on a farm and the net income (**Figure 5**; $P < 0.05$). Farms that incurred a loss had four or fewer lactating cows over the intervention period.

DISCUSSION

Community-based productivity veterinary services increased incomes in the majority of dairy farms. While the positive effects of herd health management or productivity veterinary services were demonstrated elsewhere (Dijkhuizen et al., 1984), reports on such programmes in smallholder dairy farms are scanty (Suriyasathaporn and Singhla, 2008).

Some farmers did not achieve income increases from our services. Firstly, one-year interventions on cattle health and reproduction may not necessarily produce an impact on farmers' incomes because in many cases the treated animals are not in production. Secondly, there were farms in this study where a lactating cow was not always present. Farmers with too few cows face difficulties in sustaining a return that is more than US$1.0 (**Figure 5**). Since milk is the major source of income on a dairy farm, small farms without a lactating cow generate no income but costs are incurred in feeding the animals. Providing veterinary services in such situations will not produce an immediate visible economic impact.

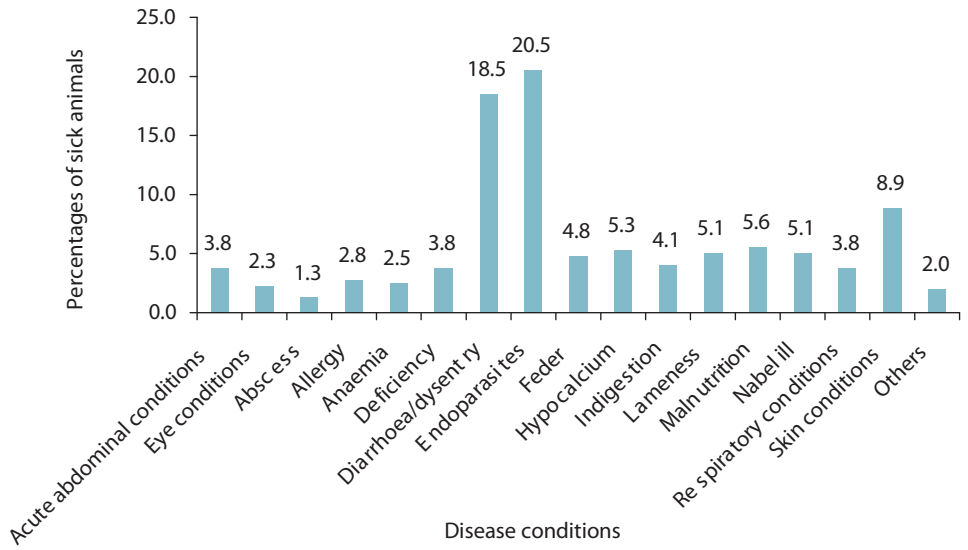

Figure 2. Frequency percentages for general disease conditions of cattle in Bangladesh (n = 395).

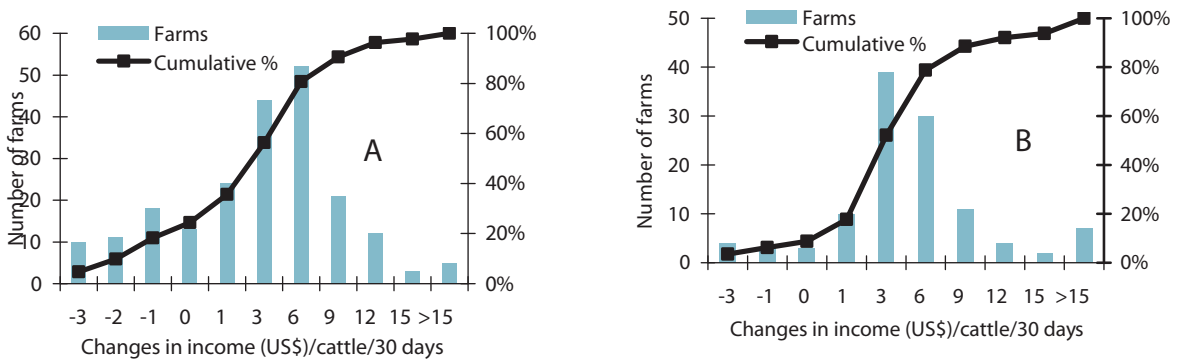

Figure 3. Effects of productivity veterinary services on farmers' net incomes. In Satkhira (A), minimum and maximum differences were US$ –8.0 and US$19.2 (number of farms = 213); in Mymensingh (B), the differences were US$ –8.4 and US$40.7 (number of farms = 114).

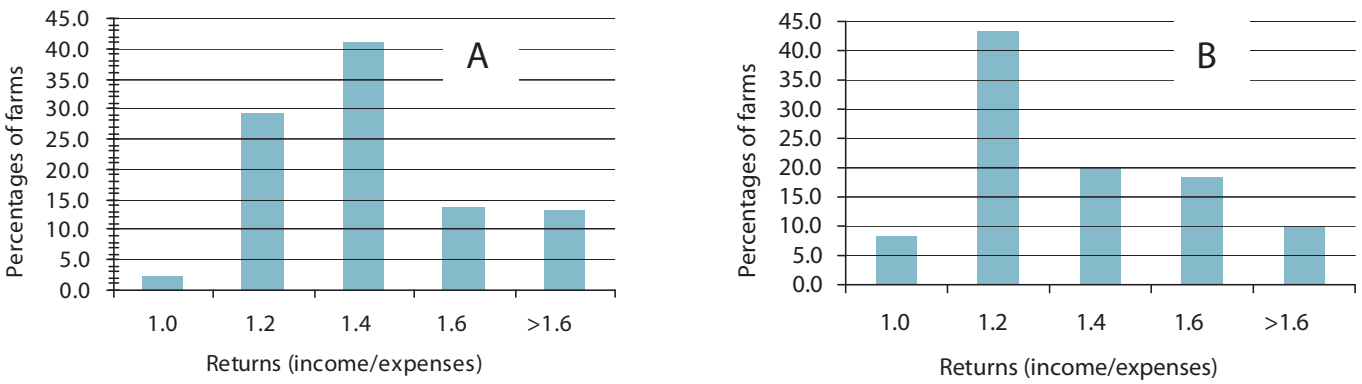

Figure 4. Distribution of returns. A = 204 farms that received the productivity veterinary service; B = 60 control farms.

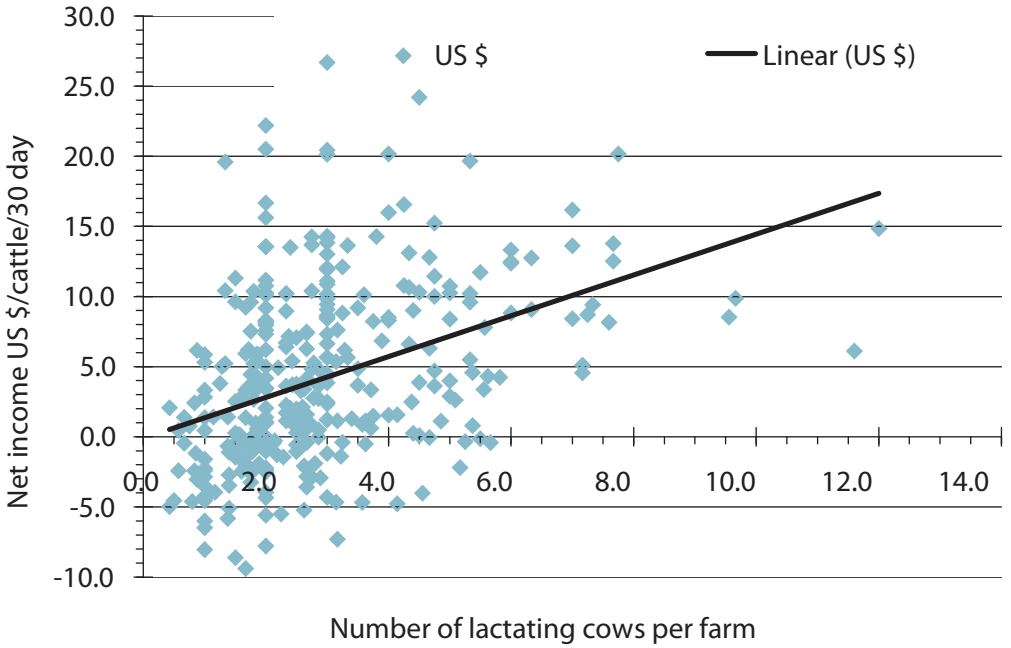

Figure 5. Relationship between number of lactating cows/farm and average daily net income/cow (number of farms = 332).

Thirdly, results of this study show that the method used for economic evaluation does matter in revealing the impacts of interventions in dairying. For example, Sirajganj has a clear flush and lean season for milk production (Shamsuddin et al., 2007). If the service starts at the flush season when the control data are taken and continues through the lean season when milk production is low, it is unlikely that the effect of intervention will be demonstrated.

The forms and calendar developed in this study proved useful in obtaining data consistently from smallholder dairy farm families. The database application was helpful for analysing the data collected from the field services and producing a herd summary for guiding the interventions in follow-up visits

Bangladesh and many other countries in South East Asia have farms with as few as one cow. Therefore, the herd health veterinary service delivery models available in countries with developed dairy industries may not be feasible for these situations. The challenges include the service-purchasing capacity of individual farmers, utilisation of veterinarian's time, time spent travelling from farm to farm, veterinarian's ability to adopt state-of-the-art technology and their unfamiliarity with such systems. The idea of delivering the service through farmers' associations proved useful in this pilot study. However, further studies are required to demonstrate the feasibility of replication and scaling-up.

Routine examination of cows by following a pre-scheduled farm visit identified cyclic cows which otherwise would have been considered non-cyclic by the farmers. Simple treatment with prostaglandin that incurs a reasonable cost allows breeding these cows with a good chance of resulting pregnancies.

A considerable proportion of cows and heifers that were identified anoestrous responded to treatment with GnRH or a mixed commercial preparation of vitamin A, D3 and E. Generally, the responses of anoestrous cows to GnRH treatment are poor since such treatment requires the presence of a functional ovarian dominant follicle in the ovary (McDougall et al., 1995). The good response in this study could be due to (a) the cows being quite late in their postpartum periods, (b) many of these cows could have been cyclic since a poor heat detection exists on the farm or (c) perhaps farmers paying more attention to the cows once diagnoses were made and costly hormone treatment was given. Whether rectal palpation of ovaries stimulate cyclicity in anoestrous cows and prepubertal heifers remains to be investigated. Sometimes postpartum anoestrous cows do not show oestrus within the expected period after GnRH administration but treated cows subsequently demonstrate a reduction in the postpartum anoestrous period (Khair, 2005).

In anoestrous heifers, the GnRH treatment did not add any benefit over de-worming, vitamin-mineral administration and/or nutritional supplementation. This again supports the beneficial effects of nutrition on reproduction. Whether any metabolite of gastrointestinal parasites has a negative effect on ovarian cyclity or the removal of parasites makes more nutrients available to animals needs to be examined.

In this study, clinical mastitis responded well to antibiotic treatment. However, more specific treatment guided by the results of milk culture for bacteria would hasten recovery of animals and reduce the risk of undue milk contamination with drug residues. The preventive programme for bovine mastitis in Bangladesh is quite new and currently we are running a project to support mastitis management based on information from milk culture for bacteria.

Common cattle health, reproduction and production-related problems were identified by this study, and the costs of different veterinary interventions are known. This will help in designing private, on-farm productivity veterinary services with the means of recovering costs to run the programme. A future opportunity would be a foundation of veterinarians that would work hand-in-hand with producers' associations for institutionalising a private veterinary service in Bangladesh.

In conclusion, the community-based productivity veterinary service increased the incomes of the majority farmers and the number of

cows breeding in farms; however, it needs to be further strengthened through consistent farm visits and institutionalising the service.

ACKNOWLEDGMENTS

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Managing Livestock in Degrading Environments

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ABSTRACT

Degraded environments are both widespread (being found on all continents on earth) and diverse. They have been broadly classified as: irrigated (and rain-fed) farmland with elevated water tables causing salinity; rain-fed farmland with soil erosion, loss of organic matter, nutrient depletion and weed invasion; and degraded rangeland. This review considers all these but with a focus on the first two, and particularly addresses options for simultaneous improvement in livestock production and landscape health. There is evidence that responsible grazing is consistent with ecosystem benefits and resilient land use systems; exclusion from grazing may reduce diversity and create management complexity. Responsible grazing however will only prevail if the land owner or user receives a financial benefit in the process. Solutions need to be profitable. In the development and management of grazing systems, expectations need to be realistic. The prescriptive approach to livestock feeding based on the selection and cultivation of a small range of improved plant species to meet predetermined energy, protein and mineral requirements is inappropriate. Degraded landscapes are often associated with a high edaphic and climatic variability that is best suited to a diverse range of plant species in an assembly that will fluctuate over time and space. This diversity means that under some circumstances degraded land may contribute to reduced risk within a whole farm business. Simultaneous objectives for livestock and landscape improvement may or may not contribute to the return of the landscape to its original state. In some cases stable vegetation that provides some of the functional benefits of the original landscape, such as improved biodiversity and soil health, combined with production benefits is the best option available. This provides an opportunity to establish a range of objectives in vegetation management and design. In Australia, such an approach is leading to the development of new farming systems that use salinised and degraded cropland for livestock. Livestock can cope with the diversity of vegetation that is suited to degraded landscapes; they have the ability to select a diet based on the minimisation of metabolic cost. They not only optimise energy and protein intake but select combinations that increase their ability to deal with toxins and parasites and to modify metabolic processes. This does not necessarily mean they will thrive; low biomass production cannot be overcome by increased choice alone, but it does mean we may need to learn from animal behaviour rather than endeavour to control it. With limiting biomass, complementary and supplementary feeds may still be required to improve the efficiency of use of grazed plants or to manipulate grazing where degradation is concentrated. There are also opportunities for strategic revegetation with plants selected for a range of nutritional, medicinal and ecosystem benefits. Just as plant species that have been bred for highly productive systems are usually inappropriate for degraded environments, so too are livestock. Traditional breeds may be better able to cope with the diverse feeding options, difficult terrain and variable climate and be more efficient in energy use. Animals bred for high production systems often partition a high proportion of available nutrients to production when feed supply is abundant but store less nutrients and are therefore less able to survive and reproduce during periods of low feed availability. Breeding within the relevant environment also exposes animals to stressors *in utero* and this may improve their ability to cope with these in later life. The concept of responsible management depends on available labour or technology for monitoring of both livestock and environment. Technology is now available or under development that will allow monitoring of livestock condition and detailed information on behaviours. These parameters are closely related to the condition of the grazing environment; the animal acts as a natural integrator of the information that describes the environment. This sensitive direct feedback mechanism is very powerful and offers new opportunities in the simultaneous management of livestock and the environment. In conclusion, degrading environments provide an opportunity for the profitable production of food. Livestock systems may be designed to retrieve or sustain landscape functionality. Livestock systems management within these environments requires an innovative approach that integrates the skills of animal physiology and behaviour, agronomy, plant ecophysiology, soil science and ecosystem ecology and management. This integration must operate outside the narrow perspectives that often characterise these disciplines.

Key words: *degraded environments, livestock productivity, landscape health, grazing management, plant diversity, genotype and phenotype selection, remote monitoring.*

INTRODUCTION

There is no shortage of publications on grazing livestock in degrading environments. Some of these address the landscape and how to protect it from livestock with limited consideration of the metabolic consequences for the grazing animal (Ash and McIvor, 2005). Others focus more specifically on the nutrition and feeding strategies of ruminants with less emphasis on the condition of the natural resource (Ben Salem and Smith, 2008). This paper primarily addresses options to improve both livestock and landscape health simultaneously. It is about how forage grown in degrading environments can supply the needs of grazing animals and, at the same time be managed to reduce or reverse degradation.

Kassas (1995) describes the three major land use types that are susceptible to degradation as:

- irrigated farmland with elevated water tables causing salinisation and soil damage;

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- rain-fed farmland with soil erosion, loss of organic matter and nutrient depletion and weed invasion; and
- rangeland degradation with a loss of biodiversity, invasion of non-palatable species and soil erosion.

This review considers all of these land uses but focuses on the first two with salinised land expanded to include both irrigated and rain-fed environments. The landscapes of relevance are those showing ecosystem degradation but not 'terminal illness' (Kristjanson and Hobbs, 2001). They are unlikely to recover to their natural state if abandoned (Curtin, 2002) but may still be transformed into a productive and sustainable condition. This new condition may or may not be the same as the historical state.

LIVESTOCK AND LAND DEGRADATION

While grazing is often blamed for environmental degradation, there is evidence that livestock are not inherently damaging to rangelands or farming landscapes, and, in fact, may be required for their sustained health and profitability. This is not unexpected as many natural and agricultural ecosystems have evolved under grazing by herbivores. Across environments, grazing is not consistently associated with a decline in above ground net primary production or root mass (Milchunas and Lauenroth, 1993) and may have little effect on native species richness, the spread of exotic species, soil texture or soil fertility (Stohlgren et al., 1999). Livestock have been used as ecosystem engineers to improve habitat for native grassland birds (Derner et al., 2009) and livestock systems were appropriate for maintaining biodiversity integrity in South African grasslands (O'Connor and Kuyler, 2009). Exclusion from grazing in a Mediterranean environment has caused a decline in species diversity as the most competitive plants dominate (Noy Meir et al., 1989); in effect grazing reduced the advantages of competitive plants, thus allowing more species to coexist. In sub-Saharan Africa, species richness in rangelands showed the ability to recover rapidly from grazing with a balance between grazing and recovery periods the preferred option for both livestock and species diversity (Yayneshet et al., 2009a). Moderate grazing has, in some cases created highly resilient and ecologically sound systems while undergrazing has resulted in dense woody growth and reduced species diversity — a situation that is both difficult to manage and a fire hazard (Perevolotsky and Seligman, 1998). Establishing annual plant-based agriculture in fragile land systems has resulted in salinisation and soil erosion over time (Hatton and Nulsen, 1999). Similarly, conversion of rangelands into intensive crop/fodder production has also led to progressive loss of diversity, species connectivity and ability to recover (Lambin et al., 2001). There is good evidence that well-managed livestock in either a grassland, shrubland or mixed crop/livestock system offer an efficient and sustainable method of increasing the production of high quality food with minimal impact on natural resources (Tilman et al., 2002).

Overgrazing however, has been shown to reduce species diversity, genetic variation within species and to deplete seed banks of annual species (Aarssen and Turkington, 1985; Osman and Cocks, 1992). It may also reduce soil microbial biomass (Holt, 1997) and damage soil structure in both extensive rangeland (du Toit, 2005) and crop dominant farming systems (Proffitt et al., 1993). A combination of inflexibility in stocking rates and a highly variable climate have also contributed to episodic periods of intense grazing pressure and land degradation (Illius and O'Connor, 1999; O'Reagain et al., 2009).

So, an ecological and agricultural case can be established for the responsible use of livestock in degraded landscapes, but the reality is that livestock will only be grazed responsibly if the owner or user receives a benefit in the process. By providing both a profitable and sustainable option, the responsible management or revegetation of degraded or partly degraded landscapes may take place through the expenditure of private rather than public funding. Given the vastness of the landscapes in question and the urban priorities for the expenditure of public funds, significant progress will rely on solutions that are profitable. The alternative is to become dependent on regulation and policy. The key role of profit, rather than subsidies in the adoption of improved agricultural practices on low potential lands has been reviewed and supported by others (Barbier, 1997) and provides the basis for the strategies outlined below.

MANAGING EXPECTATIONS

Expectations for degraded landscapes need to be realistic and the development of successful grazing systems requires innovative approaches. These must address long term sustainability of soil, plant and animal production systems. The prescriptive process to feeding livestock, developed by livestock farmers and scientists for landscapes with high production potential, is inappropriate. This approach involves the application of knowledge on the nutritive and feeding value of a plant, breeding, selecting and sowing a small number of these plants with highest perceived feeding value and then managing grazing to maximise pasture growth and persistence. Such feeding systems by definition rely on minimal plant diversity. They function effectively provided high inputs are supported by high levels of production and product prices. High yields often can only be sustained with increasing levels of inputs (e.g. herbicides and fertiliser) and, the effectiveness of these inputs may decline over time. Features of plants that confer agronomic value are rarely the same as the features that confer survival value (Donald, 1963). Whether these systems become 'stressed' in the long term through continued intensification with stagnant or declining output remains to be seen (Lambin et al., 2001); what is certain is that high input, high output systems are not appropriate for degraded land or land at risk of degradation.

Degraded landscapes are often associated with spatial variation in the edaphic environment and with low and/or variable rainfall, so they are rarely able to support high levels of management or production from monocultures of highly productive plants. Attempts to establish equilibrium systems based on average rainfall and biomass production are likely to fail (Oba et al., 2001a) and may create seasonal food shortages and induce desertification. This is not surprising given the large between-year variation in biomass production in arid, semi-arid and Mediterranean-type environments (Rossiter, 1966; Ash and McIvor, 2005). Heterogeneous landscapes are more likely to support a diverse species composition that will fluctuate over time. These mixed plant assemblages are not only better suited to spatial and temporal variation, they also have a reduced risk of total failure in response to environmental or biological stressors such as drought, diseases or pest infestations.

Appropriate plants are likely to consist of a mixture of native and introduced plant species that each occupy a niche within the micro-environment and fulfil much more than the role of a feed supply. There are complex management issues underlying the productivity and stability of any mixture. Hobbs and Morton (1999) emphasised that these agro-ecosystems are likely to be dynamic and Schulte et al. (2003) suggested spatial heterogeneity could stabilise ecosystems, even though the ecosystem may oscillate at the patch scale. Furthermore, they concluded that spatial heterogeneity could be maximised by increasing the incidence of small-scale disturbances and by minimising large-scale disturbances. In a practical grazing management sense, this suggests that set-stocking of large areas will lead to reduced stability of a plant mixture.

Saline landscapes provide a practical example of spatial heterogeneity. Norman et al. (2003) identified 35 different plant species growing on two highly saline revegetated sites. The high levels of plant diversity were related to spatial variability at the site and indicated that an individual species is unlikely to dominate or thrive in all functional niches. As well as occupying heterogeneous functional niches, the mixture of plants filled a range of roles; together contributing to the prevention of wind and water erosion, provision of shelter, fuel and habitat value and more efficient water use to an extent not possible with simpler plant communities.

Plant diversity also provides an opportunity to manage temporal variability. Improved pasture plants have been selected to perform with a regular and good supply of nutrients and water and, in degraded environments, usually lack tolerance to variable rainfall and are unable to respond to unpredictable and sporadic 'out-season' climatic events. This carries two penalties; one is the exacerbated risk of land degradation and the other is lost income to the landholder. An opportunity exists to revegetate and manage degraded land with a combination of plant types to actually reduce the risk to the whole-farm enterprise and the wider landscape. Niche differentiation (i.e. the use of different resources by individual species) can occur temporally as well as spatially. The concept of degraded land being managed differently to reduce overall risk is contrary to conventional thinking that sees this land as contributing to riskiness. Whilst it is degraded the risk of further damage remains, but careful intervention can have profound effects beyond the degraded land itself. For example, Monjardino et al. (2010) found that revegetating the most marginal soil types on a typical crop-livestock farm in southern Australia with perennial forage shrubs boosted whole-farm profit disproportionately; 10% of the farm area allocated to these plant types increased whole-farm profit by 20%. Thus, providing an improved seasonal distribution of feed through better management of degrading or low-productivity land classes can actually help to reduce costs e.g. through reduced reliance on expensive feed supplements (Richards et al., 1994) and increase flexibility in animal production systems. It may even help to improve animal productivity by the strategic use of quality forage to coincide with key stages of the life cycle of animals (e.g. ovulation, parturition, lactation, or weaning; see Martin et al. (2004) and Blache et al. (2008) and, in some cases, could allow producers to consider out-of-season production to attain price premiums for animal products.

The challenge is still how to achieve a predictable and profitable return from grazing such systems while at the same time providing ecosystem services that may or may not be of financial benefit to the landholder or land manager. Management that focuses on a prescriptive approach to livestock feeding is possible but risky, but one that considers plant diversity and grazing behaviours may offer better prospects.

FUNCTIONAL LANDSCAPES

Where the aim is to simultaneously create a sustainable landscape and a profitable livestock system, a primary requirement is to understand what function plants perform in both their original and modified assembly. A consideration is that livestock systems in degraded landscapes can be managed to be both productive and sustainable, but may or may not contribute to the return of the landscape to its original state. In some cases stable vegetation that provides some of the functional benefits of the original landscape, combined with the productive benefits of a profitable livestock system may be the best option available. This then provides an opportunity to design a landscape based on a range of predetermined objectives (**Table 1**). These objectives will include both profit and ecosystem services. For example, dryland salinity threatens significant areas of farmland in southern Australia where replacement of native perennial vegetation with annual crop and pasture species has resulted in water tables rising to the surface, bringing dissolved salts with them (Peck and Hurle, 1973). Salinisation threatens biodiversity and infrastructure on a regional scale. While revegetation with the original mixture of native perennials is an unrealistic goal, it is possible to restore the hydrological balance by strategic selection and cultivation of plants that will mimic the water use of natural ecosystems (Cocks, 2003). Plant species with deep roots and high canopy cover (green leaf area) will reduce recharge (Dunin et al., 1999; Ridley et al., 2001; White et al., 2003) while the overall composition of plant communities will influence the proportion of incident rainfall that is used, or lost through deep drainage, run-off or evaporation (Dunin et al., 1999; Ridley et al., 2001; White et al., 2003). In Western Australia, this strategy has resulted in the revegetation of 10% of the one Mha of saline land with halophytic shrubs and salt-tolerant forage. The growth and seasonal distribution of vegetation improves environmental health or 'sustainability' by reducing the recharge of saline groundwater and the run-off of both salt and sediment into waterways (Bathgate and Pannell, 2002; Turner and Ward, 2002; Peck and Hatton, 2003; Ridley et al., 2004; Norman et al., 2008a). It also provides valuable out of season feed for livestock from mixed-plant assemblies that include deep-rooted species with resilience to periods of drought and species that are able to respond quickly to rainfall by producing nutritious biomass.

In farming areas threatened by salinity but showing only early signs of degradation, planting deep-rooted perennial plants can also be used to manage the water table — these do not need to be salt tolerant but will almost certainly be forage plants rather than perennial crops (Masters et al., 2006).

Within the parts of the landscape that are already saline and those other parts at risk of salinity, there are also new and innovative farming systems under development. These involve the strategic planting of trees and/or shrubs in a wide alley configuration to provide forage and shade for livestock in the summer months while allowing inter-row cropping in winter (Lefroy and Scott, 1994). Perennial and deep-rooted woody plants contribute to the control of groundwater depth and in some cases provide biomass for power generation, activated charcoal and eucalyptus oil (Lefroy and Scott, 1994; Bell et al., 2001).

Salinity is not the only concern within the dryland cropping zones of Australia. The recent move away from livestock into more intensive cropping enterprises has produced some landscapes that display characteristics of degradation including both saline and acidic soils, wind and water erosion, loss of diversity (both natural and sown) and herbicide resistant weeds. The farming systems themselves, through a lack of enterprise diversity lack resilience. There are opportunities to redesign these systems through the use of perennial plants that are adapted for dry conditions but have the ability to respond quickly to infrequent and unpredictable heavy rainfall (Revell et al., 2008). This demonstrates a clear need for integrating evolutionary and community ecology concepts into agricultural planning and design.

Shrub systems have also been successfully established in deep sandy soils where cropping is uneconomic and traditional annual pasture plants leave the soil exposed to wind and water erosion for up to six months each year. These shrubs include *Acacia saligna*, *Atriplex spp.* and *Chamaecytisus palmensis* (Tagasaste). Tagasaste has high crude protein (>18%) and dry matter digestibility (> 65%) for at least part of the year (Borens and Poppi, 1990; Assefa, 1998), although livestock production potential is probably restricted through a high tannin and alkaloid content (Assefa et al., 2008).

While these examples focus on the specific and unique problems of degradation within Australian rain-fed farming systems,

Table 1. Objective based revegetation of degraded saline land.

Objective	Plant strategy
Lower saline water table	Deep rooted salt tolerant shrubs and other perennial plants to reduce saline water table recharge
	Ground cover to increase evapotranspiration
Reduce run-off of salt and sediment into local waterways	Deep rooted perennial shrubs and other plants to reduce water and sediment movement
	Deep rooted perennial shrubs and other plants to improve the growing environment for other plants
Improve soil properties	Revegetation to improve soil texture and microbial health
	Deep rooted perennial shrubs and ground cover to reduce soil erosion
Biodiversity and habitat	Volunteer native plants grow around shrubs.
	Potential habitat for native animals, birds and insects
Aesthetics	Improved visual amenity to address societal aspirations
Improve nutrition for grazing livestock	Salt tolerant perennials:
	Produce edible out of season biomass
	Respond to out-of-season rainfall
	Source of nitrogen, sulphur and vitamin E during the dry season
	Neutraceutical benefits such as anthelmintics, antibiotics, antimicrobials
	High salt intakes to decrease protein degradation in the rumen
	Improved growing environment to allow planting of short-season annual pasture plants
	Annual grasses and legumes:
	High nutritive value
	Increased edible biomass
	Fix nitrogen in the soil
Improve the grazing environment	Shrubs provide shade and shelter from wind and rain
Improve product quality	High salt increases efficiency of wool growth and decreases carcass fat
	Environmentally friendly products

they establish principles that are more broadly applicable. The plant assembly must have the potential to form a functional and stable community under grazing and the production potential must exceed the costs of establishment (or alternative land use).

Similar shrub or alternative forage systems have been advocated in Africa, the Middle East, Asia, North and South America (McKell, 1975; Qureshi et al., 1993; Assefa, 1998) with claims that they contribute to the alleviation of fodder shortage, soil degradation, low soil fertility and fuel wood scarcity.

These are examples of livestock-driven solutions to land degradation problems.

USING MIXED PLANT ASSEMBLIES WITH LIVESTOCK IN DEGRADED LANDSCAPES

Benefits from Plant Diversity in Grazing Systems

Developing and maintaining plant mixtures adds complexity to the feed base, and will require a change in skills for land managers. We contend that opportunities exist to capitalise on the capacity of livestock to cope with diversity of forage plants on offer. In fact, grazing herbivores seek diversity (Provenza, 1996) and, with appropriate management, can perpetuate it (Ngwa et al., 2000).

Experiments under a range of environmental conditions indicate ruminants have the ability to select foods with high digestibility and avoid those with poor digestibility and high fibre (Duncan et al., 1994; Provenza, 1995). Crude protein content also affects diet selection although its role is less clear. Growing lambs will select from a range of different crude protein levels to obtain a mixture of feeds that meet their crude protein requirement (Kyriazakis and Oldham, 1993) but avoid excessive intakes of degradable protein (Provenza, 1995). Ruminants will also select a diet with low or manageable concentrations of anti-nutritional compounds. The ability to metabolise such compounds is often related to the intake of both metabolisable energy and other non-nutritional compounds in the diet; loss of forage diversity will be accompanied by a decline in nutritive value, palatability and metabolic variety with a consequent reduced ability to deal with toxins (Provenza et al., 2003).

These grazing behaviours are consistent with the hypothesis that sheep have the ability to select across a range of feeds to balance nutritional needs or minimise metabolic cost (Forbes and Mayes, 2002). Metabolic cost is defined as the increase in energy or other nutrients that is required to maintain health and production under sub-optimal conditions. Increases in metabolic cost may be associated with diverse stresses such as imbalanced nutrient intake or parasite infection. The concept of minimisation of metabolic cost can be taken further. For example, if methane production is an indicator of the efficiency of conversion of digestible energy to metabolisable energy, then preference may extend past digestibility to include methane production. Preference testing on forage for or against

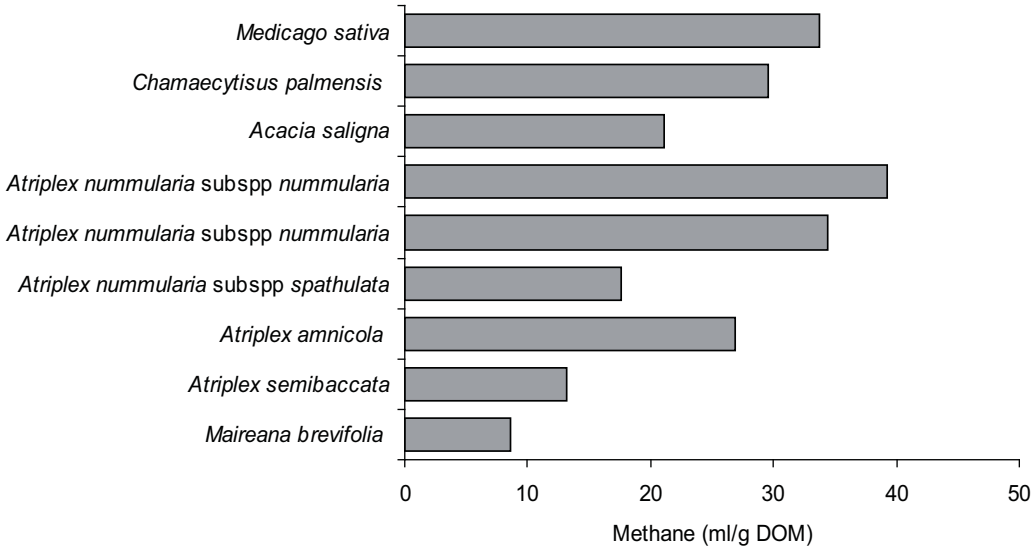

Figure 1. Methane production (ml/g DOM) measured *in vitro* (Busquet et al., 2006) for lucerne *(Medicago sativa)*, tagasaste *(Chamaecytisus palmensis)*, orange wattle (Acacia saligna), old man saltbush *(Atriplex nummularia)* river saltbush *(Atriplex amnicola)*, creeping saltbush *(Atriplex semibaccata)* and blue bush *(Maireana brevifolia)* (Norman and Durmic, unpublished).

plants causing high methane production in the rumen is worthy of investigation. *In vitro* studies have shown wide variation in methane production from different forage sources when expressed per unit of digestible organic matter (**Figure 1**). This is consistent with observed differences in the proportion of digested energy converted to metabolisable energy across a range of plant species (Minson, 1990).

Minimisation of metabolic cost is also significant because it suggests that ruminants have the ability to optimise their diet when provided with a choice but that the choices made are not always a simple reflection of the energy and protein content of the plant options. This has been demonstrated using $\delta^{13}C$ ratios in the faeces of sheep to estimate diet selection in a mixed halophytic shrub (with a C4 photosynthetic pathway) and annual legume (with a C3 photosynthetic pathway) system. Even during times of the year when the supply of feed from annual legumes growing between shrubs was unlimited and organic matter digestibility of the pasture plants was 10% higher than in the shrubs, sheep still chose shrubs as 13% of their total feed intake (Norman et al., 2010c). The reason for the selection in this case is not obvious. Increased salt intake is likely to trigger osmoreceptors and encourage the choice of low salt alternatives, this is consistent with the reduced rate of feed intake in goats fed diets with added salt (Morand-Fehr et al., 1997). Conversely, others have shown that over the longer term, sheep will select a combination of high and low salt feed, even when the feeds are otherwise the same (Thomas et al., 2007b). Importantly, salt intake at levels above published requirements increases the flow of undegraded protein from the rumen and therefore alters the amount and relative proportions of amino acids available for absorption and also the relative proportions of absorbed energy and protein (Hemsley et al., 1975; Thomas et al., 2007a). This balance of amino acids available for absorption could therefore potentially be manipulated by the animal through the chosen level of salt intake. While these are known nutritional consequences of elevated salt intakes, selection for salt is not the only possible explanation. These shrubs are higher in fibre than spring pastures and have an unusual nitrogen, mineral, vitamin and secondary compound composition (Masters et al., 2007; Mayberry et al., 2010; Norman et al., 2010b). These may also influence metabolic cost and subsequent selection.

A similar scenario may be expected when the forage mixture includes plants that contain tannins. Tannins at high levels will depress intake and the rate of ruminal digestion and may even cause death, but at lower levels they increase both microbial protein synthesis and amino acid flux into the small intestine (Makkar, 2003).

There are many other examples of ruminants showing preferences for dietary combinations that do not maximise the energy density of ingested feed but appear to have metabolic or health benefits that the grazing animal has the ability to recognise (Baumont et al., 2000). Rogosic et al. (2006) demonstrated that goats offered Mediterranean shrubs containing tannins and saponins consumed a variety of shrubs with different levels of the compounds in such a way as to increase shrub intake and avoid toxicosis. Goats grazing blackbrush (*Coleogyne ramosissima*), a poor quality feed that has high fibre, low crude protein and high tannins, selected older branches with lower tannins, lower energy and crude protein in preference to younger branches with higher tannin, energy and crude protein (Provenza and Malechek, 1984). Sheep grazing diverse populations of two species of saltbush were able to select and discriminate both within and between species, yet the selection could not be explained by any of the measured nutritive and anti-nutritive value characteristics of the plants (Norman et al., 2004).

This ability to utilise plants containing toxins may be facilitated through learning. Papachristou et al. (2007) demonstrated that when nutritious herbage was restricted, lambs were encouraged to learn to use different kinds of forages with a range of potentially negative plant secondary compounds. The lambs learned the benefits of mixing food with tannins, terpenes, and oxalates. Further, the lambs continued to include these compounds in their diet when they subsequently had *ad libitum* access to nutritious foods.

Meeting Nutritional Requirements

Meeting the nutritional requirements of livestock grazing in degraded environments will always be a challenge. This is primarily because

degraded landscapes produce low edible biomass. But, as discussed in the previous section, fragile or hostile environments are most likely to be converted to a functional system with a diverse plant mixture. Combining this approach with the grazing herbivore's capacity to seek nutrients and avoid toxins, there is an exciting prospect to develop sustainable grazing systems on degraded land by perpetuating diverse plant communities with managed grazing. Both overgrazing and undergrazing are likely to result in a loss of species diversity (Oba et al., 2001b) thereby reducing the ability of grazing ruminants to meet their nutritional requirements. This is perhaps even more important when considering differences between individuals in their nutrient requirements. Whilst we normally attempt to manage animals according to their average nutrient requirements, we know there are wide differences in requirements between individuals. Providing a 'cafeteria' of forages may help individuals meet their own requirements.

As livestock scientists, we have very limited knowledge of the range of nutritional and anti-nutritional compounds within many of the plants that flourish in degraded landscapes. While we may have the capacity to identify and perhaps even attempt to eradicate plants that are toxic or totally avoided by livestock, our best course of action may be to allow grazing livestock to use their sophisticated post-ingestive feedback mechanism to direct feed selection (Provenza, 1995; Weston, 2002). Within environments that are not conducive to manipulation we may need to learn from animal behaviour rather than attempt to control it.

Improving Livestock Production and Health

Complementary Feeding

Where loss of diversity is a consequence of previous overgrazing, complementary feeding can improve intake, feed conversion efficiency and therefore productive potential. Complementary feeding is defined here as a production response from two or more feeding sources that is greater than the sum of production expected when each feed source is used alone. It is a compromise between a prescriptive feeding approach, where we make all the decisions on what is best for the animal, and a system that relies on the innate ability of an animal to optimise diet selection from a range of dietary options.

The objective is to combine feed sources for improved production and can be based on cut and carry, concentrate feeds or even strategic grazing management. Examples include the provision of energy supplements to improve the utilisation of plants that contain high levels of non-protein nitrogen or to facilitate the breakdown of anti-nutritional compounds in the forage. The provision of small amounts of concentrate feeds may be all that is required. For example, growth of sheep is significantly improved when cactus cladodes, high in carbohydrate and water are fed with saltbush (*Atriplex spp*) that is high in salt, low in digestible organic matter and high in non-protein nitrogen (Ben Salem and Smith, 2008). Equally, barley grain has been shown to complement saltbush, increasing the digestibility more than would be expected from the weighted average of the two feeds (Van der Baan et al., 2004). Under grazing conditions, the provision of a barley supplement can improve live weight of sheep grazing saltbush (Franklin-McEvoy et al., 2007; Norman et al., 2008b).

Table 2. Mineral composition (on a DM basis) of 18 shrub species grown at one site Monarto, South Australia (D. Revell, J. Emms et al., unpublished data).

Species	Ca g/kg	P g/kg	Mg g/kg	Na g/kg	K g/kg	S g/kg	Cu mg/kg	Zn mg/kg
Approx. requirement[1]	1.5–4	1.3–3	1.2–1.9	0.7–1.2	5	1.5–2	5–10	20–30
Acacia saligna	6.5	0.9	3.5	4.1	15	6.3	3.3	14
Atriplex amnicola	6.8	1.9	10.5	—	28	6.1	5.0	20
Atriplex cinerea	12.4	1.7	7.0	64	33	4.7	11	19
Atriplex nummularia	14.3	1.6	7.8	73	25	7.5	8.4	42
Atriplex rhagodioides	7.1	1.9	8.2	63	26	5.9	4.8	22
Chameacytisis proliferus	6.8	1.2	4.55	1.50	8	1.2	4.0	22
Chenopodium nitrariaceum	6.1	2.4	5.6	11.9	53	4.7	3.3	24
Convolvulus remotus	5.3	1.6	3.2	1.3	16	1.6	5.3	12
Enchylaena tomentosa	3.4	1.1	1.8	—	17	2.1	8.9	15
Eremophila glabra	7.0	1.4	2.3	0.6	20	1.8	9.2	16
Eremophila longifolia	7.9	1.0	2.9	2.0	18	1.5	5.9	12
Eremophila maculata	4.3	1.4	2.4	18.8	13	1.6	4.0	14
Maireana tomentosa	3.1	2.3	2.5	—	26	3.4	8.1	15
Medicago strasseri	22.0	1.5	3.2	6.9	15	2.3	8.0	14
Rhagodia crassifolia	5.8	2.0	11.4	45	24	3.9	5.3	15
Rhagodia parabolica	5.3	1.4	6.0	30	27	3.0	4.4	16
Rhagodia preissii	7.1	1.4	8.6	31	24	3.9	13	23
Rhagodia spinescens	5.4	1.5	7.8	67	26	6.6	4.6	21

[1] Mineral requirements depend on physiological state, breed, sheep vs cattle and mineral balance. Values presented here are indicative only.

Although the focus of complementary feeding in degrading landscapes is usually on the macro-nutrients used as energy and protein sources, there are also consequences for mineral and vitamin nutrition. This can be viewed from two perspectives. One, the traditional view, is to provide limiting minerals or vitamins in order to increase forage utilisation and increase animal performance. The other is to use forages as the source of limiting minerals and vitamins. For example some shrubs are a good source of vitamin E (Pearce et al., 2005). The shrub species listed in **Table 2** show that adequate or high levels of some minerals can be provided by certain forages. The high magnesium and sulphur in particular in some plants means that even if these shrubs provide a small proportion of total intake they have the ability to reduce the risk of grass tetany and improve the digestibility of dry forage.

Alternatively, complementary feeding can be used to dilute out limiting characteristics or other anti-nutritional compounds in one or more of the feeding alternatives. A practical example of this strategy is the feeding of cereal residues to ruminants grazing high salt shrubs. With a shrub-only diet, high salt limits feed intake and production; similarly, with a diet high in crop residue, fibre limits intake. Feed intake and production are both improved by combining the two feed sources (Le Houérou, 1992; Warren and Casson, 1992; Alicata et al., 2002). Opportunities to improve the feeding value of low digestibility grasses through the strategic use of complementary native browse species have also been described (Yayneshet et al., 2009b). Similar observations have been made when livestock have access to forages containing different toxins. For some, but not all toxins, sheep will eat more when they are offered two feeds each containing a different toxin than when they were offered a single forage with only one toxin (Burritt and Provenza, 2000). Intake almost doubled when diets containing nitrates and oxalates were fed together rather than when sheep were offered each diet separately. There was no improvement in intake when the same comparisons were made using diets containing saponin and tannin (Burritt and Provenza, 2000; Makkar, 2003).

Supplementary Feeding

The term supplementary feeding is used here to define a strategy where the nutritive value of two or more components of the diet is additive when they are fed together. Supplementary feeding is intuitively unattractive in degraded landscapes and comes with connotations of artificially elevating stocking rates well past capacity. However, while this may be the case it does not need to be so. Supplements can be used for the spatial and temporal manipulation of foraging behaviour.

Supplementary feeding to defer grazing is one example. This involves the strategic use of supplements or even full replacement rations to keep livestock off degrading land and allow either rest or forage establishment during sensitive periods. For example, in environments with regular dry and wet seasons, there is benefit in restricting grazing after initial rains. Grazing at this time may compromise seedling establishment, reduce diversity and is usually characterised by increased livestock movement and trampling damage during the search for fresh growth. The concept of deferred grazing has been studied for more than 50 years (Frandsen, 1950), but the quantification of the potential benefits for later livestock or crop production within some fragile farming systems is still not well understood (Proffitt et al., 1993).

Supplements can also be used to manipulate grazing distribution patterns. Uneven grazing is a major problem in degrading landscapes. Management units tend to be large and there is a tendency for grazing activity to remain around an uneven distribution of watering points or within an area of preferred plant species. The development of new water sources and fencing can be used to change grazing distribution but are expensive options.

Uneven grazing patterns, while initially based around forage quantity and quality, water and landscape features, also may be exacerbated by spatial memory related to previous experience and training (Bailey et al., 1996). This complicates the prediction and management of grazing distribution, but also provides an opportunity for manipulation. Bailey (2004) reported that cattle spent more time and grazed more forage in areas where molasses blocks were provided than in areas with no supplement. Supplements changed grazing patterns away from sources of water. Grazing distribution has also been manipulated through the use of free-choice mineral supplements (Ganskopp, 2001). The learning and training processes that accompany the use of such supplements means that animals may then choose to visit these new foraging areas even when supplements are no longer provided there.

The benefits of improved grazing distribution for livestock production still need to be balanced against the possible decrease in ungrazed areas and the refuge they provide for grazing sensitive plants and the consequences for native plant and animal diversity (James et al., 1999).

Strategic Revegetation

Another option is to select plants for revegetation on the basis of their ability to complement the composition and availability of feed resources already available. Where revegetation or partial revegetation is an option, there are benefits in assessing plants for palatability, nutritive value, anti-nutritional properties, shelter and possible medicinal properties as well as ecosystem benefits. These do not need to be plant breeding projects but may be localised screening programmes based on indigenous plant species with known natural advantages. Small or large scale propagation programmes are both options to support distribution requirements. A programme in Australia designed to screen and select native chenopod shrubs has recently demonstrated that there is significant variation in biomass production, palatability and nutritive value within a single species of these plants with organic matter digestibility ranging from 51% to 67% and crude protein from 12% to 19% across provenances (Norman et al., 2010a). Seeds were collected from 600 female *Atriplex nummularia* plants across southern Australia and grown together on four diverse assessment sites. This allowed prediction of heritability and indicated much of the variability in nutritive and feeding value was due to genetic rather than environmental factors. There is clearly potential to improve profitability through the selection and distribution of superior plants.

In another study, Bennell et al. (2009) screened more than 100 woody Australian plant species for nutritive value. Many were high in predicted digestibility and crude protein and have the potential to play a role in livestock-based revegetation programmes. There appears to be very few examples of plant improvement programmes of this scale that are based on the screening of native plants for livestock production. Many others have analysed and/or reviewed the nutritive value of native species, particularly shrubs (Long et al., 1999; Foster et al., 2007; Gasmi-Boubaker et al., 2008) and in some cases presented a strong case for selection or propagation of species or lines suitable for livestock production (Dynes and Schlink, 2002; Yayneshet et al., 2009a). Invariably, in these studies there is a two-fold variation in digestibility, and crude protein can vary by an order of magnitude. At the same time, the seasonal patterns of change in nutritive value can be quite different across plant species. If genetics plays a significant role in determining nutritive value in native forages

then *in situ* screening programmes, designed to identify superior plants within the relevant environment, are justified.

Complementary revegetation is not simply about the amount and nutritive value of forage produced as it will have a temporal component. Seasonal distribution has significant consequences for both the environment and animal. Seasonal variation in feed supply is a feature of many degrading environments, with periods of rapid forage growth and high feeding value followed by periods of dry, low quality feed, reduced ground cover and exposed soil. A diverse assembly of plant types that include perennial and annual species of both native and introduced plants provides a greater opportunity to develop a stable livestock system and reduce the grazing stress that often occurs during the dry season or during extended periods of low rainfall (Revell et al., 2008). Some native plants in particular have the ability to survive long periods of low rainfall and to respond quickly to rain. Providing an improved seasonal distribution of quality feed by including plants with seasonal complementarity will have both livestock and environmental benefits. While total dry matter production per year may decrease with a mixture designed to provide feed throughout the year, feed utilisation rates may well improve with animals selecting forage that would normally not be eaten, thereby increasing grazing production over the full year. From an economic point of view, the value of feed during periods of shortage far exceeds that of feed grown at other times. In the Mediterranean environment for example there is a ten-14 fold difference in marginal value of additional forage in autumn (a time of low feed quality and availability) compared with spring (high quality and availability) (Morrison and Bathgate, 1990).

Previously the combination of low edible biomass and low-moderate nutritive value of native plants, when considered as the sole source of forage, discouraged interest in their use or potential for improvement (Lefroy, 2002). Clearly a broader view that considers these plants as part of a diverse nutritional mixture with ecosystem benefits means reconsideration is necessary and may have significant benefits for agricultural systems of the future.

This is particularly the case given the challenge of using new feeding or revegetation strategies to improve livestock production and health without exacerbating landscape degradation. Landscape degradation is a possibility but not an inevitability. Many of the forage systems that are appropriate for degraded landscapes are inherently low in nutritive value (Minson and McLeod, 1970; Skarpe and Bergström, 1986; Rubanza et al., 2003). At best, the forage for much of the year is so low in digestibility that it will only support liveweight maintenance. Under such conditions there is little incentive to reduce stocking rate because nutritive value, rather than the amount of edible biomass defines the production per animal. Increasing the base nutritive value could simply be used to increase stocking rate with consequential trampling and camping damage. Alternatively, if stocking rate was maintained or even lowered, options for growth, reproduction or even lactation become realistic and greenhouse gas production per unit of production can be reduced. Greenhouse gas emissions will be lower per unit product in livestock systems with improved growth and reproduction rates. In some cases there may even be an opportunity for system change with alteration to the timing of reproduction and turnoff. Even small changes in digestibility of the edible biomass may be sufficient to encourage and support system change. Overstocking becomes a consequence of social, cultural and educational experience and not necessarily production economics.

Beyond Nutrition

The discussion to this point has focussed on improving the intake of metabolisable nutrients from forages that grow on marginal land and in minimising toxin intake or maximising the ability of the animal to detoxify anti-nutritional compounds. While this provides a broad perspective on the design and management of livestock systems in degraded landscapes, the perspective is still too narrow. There are other implications that are too frequently ignored or dismissed as too difficult. In particular, the potential neutraceutical properties of plant secondary compounds. By far the best known of these is the effect of tannins on internal parasites. Worm burden and faecal egg counts in naturally parasitised sheep are significantly reduced and growth increased by the consumption of high tannin plants (Niezen et al., 1995). In recent studies, extracts from 85 native Australian plants were screened for their ability to inhibit the development of *Haemonchus contortus* larvae *in vitro*. More than 40% of the plants showed anthelmintic properties, and tannins were the bioactive agent in only some of these plants (Kotze et al., 2009).

What is not well understood is whether parasitised sheep and cattle will actually seek out and preferentially consume plants that contain tannins or other anthelmintic compounds - are they capable of self-medication? There are many well documented examples of self-medication in non-domesticated mammals. These can be for either prevention or therapy and may play a role as stimulants, anthelmintics, laxatives, antibiotics, or as antidotes for previously consumed toxins (Lozano, 1998). Although there does not appear to be published examples of parasitised sheep or cattle selectively consuming high anthelmintic plants, there are reports that they will seek out and consume nutrients that have been depleted through parasitism such as protein (Kyriazakis et al., 1994) and sodium (Suttle et al., 1996).

Villalba and Provenza (2007) suggested that plant-derived alkaloids, terpenes, sesquiterpene lactones and phenolics may also benefit herbivores by combating internal parasites, controlling populations of fungi and bacteria, and enhancing nutrition. Vercoe et al. (2009) described the effects of 'natural bioactives' in Australian plants on acidosis and biohydrogenation *in vitro.* There are likely to be many compounds that influence immune function and the incidence of metabolic disease (Provenza and Villalba, 2010) and, while the identification and characterisation of such compounds through traditional research methods may be prohibitively expensive, the potential ability of grazing livestock to select on the basis of minimisation of metabolic cost means that over time, ingestion of compounds that reduce the need to mount a metabolically expensive immune response would be expected. This presents a logical explanation for 'self-medication'.

LIVESTOCK SELECTION AND PREPARATION

Genotype and Phenotype Selection

Just as plants species that have been bred and selected for high production systems are usually inappropriate for degraded landscapes so too are livestock. Livestock developed for high productivity systems are often not trained to cope with diversity of feed sources and not bred to thrive with between- and within-season variability.

Traditional breeds or native animals may be less selective and therefore less likely to reduce forage heterogeneity (Dumont et al., 2007) are better able to utilise rugged terrain (Bailey, 2004), have more efficient use of feed energy during periods of low feed availability or have an improved ability to metabolise and detoxify plant secondary compounds (Mead et al., 1985; Jones and Megarrity, 1986). For example, after decades of beef breeding research in Africa that

commenced in 1930, an optimum cross-breed was identified (62.5% Afrikander and 37.5% exotic Shorthorn or Hereford), which was later developed into a breed known as the Bonsmara (Cronjé, 2000).

In taking an approach to select 'appropriate animals' for the environment, it may be necessary to avoid selecting animals that partition a greater proportion of available nutrients to production only when feed supply is abundant, because these same animals are likely to be compromised when nutrient availability is limiting or variable, as is often the case in degraded landscapes (Cronjé, 2000). Consequences can include reduced reproductive and survival rates when nutrient supply is low or when other stressors occur (such as thermal stress or challenges to the immune system). This case has recently been made with high wool producing sheep in Australia. High genetic values for wool growth were accompanied by lower fat stores, lower plasma glucose and insulin and reduced reproductive turnoff — reduced fitness characteristics for survival in a harsh environment (Adams et al., 2006).

It is important to identify genotypes that are adapted to, or can cope with, the range of nutritional conditions they will be exposed to (Sinclair et al., 1998), including seasonal variation and drought events. A key point here is that where limited nutrient availability and drought events are probable, the strategy of running high stocking rates to maximise returns is risky and so less attractive. This, then, places a greater emphasis on the performance of individual animals.

While it is not always easy to identify better adapted animals within the applicable grazing environment, it may be possible to characterise local breeds for energy use and changes in body composition using ^{13}C-bicarbonate and ^{3}H-water (Makkar, 2008).

Foetal Programming

The ability of livestock to cope within a sometimes hostile environment may also be subject to gestational influence. Nutritional status at critical stages in pregnancy has the ability to have profound effects on the foetus that sometimes persist through later life. Folic acid, zinc, iron copper, vitamin A and vitamin E are of particular importance (Ashworth and Antipatis, 2001). Low birth weight will also influence glucose tolerance and response (Oliver et al., 2002). More recently there has been interest in whether exposure to nutritional or environmental stressors during gestation will result in metabolic or epigenetic changes in the offspring that will improve their ability to deal with the same stressors during later life. For example, feeding pregnant ewes salt or saltbush (*Atriplex spp*.) during part of pregnancy may lead to potentially permanent changes to the regulation of salt and water balance in the offspring (Digby et al., 2008; Chadwick et al., 2009b; Chadwick et al., 2009c). Offspring born to ewes fed saltbush during pregnancy and early lactation managed to gain body weight as weaned ten-month-old animals when they grazed saltbush for eight weeks, whereas offspring from control ewes (not fed saltbush) lost body weight (Chadwick et al., 2009a). Early life experience can also affect a ruminant's ability to deal with challenging diets. For example, lambs fed a low quality grass from one to five months of age, had a higher voluntary intake, increased nitrogen retention and higher liveweight gain when fed sorghum hay compared to conspecifics fed a higher quality diet in early life (Distel et al., 1994).

Training

While there is evidence that sheep and cattle will select diets to optimise energy, protein and toxin intake (Forbes and Mayes, 2002), such behaviour may still be affected by previous experience, at least in the short term. Small differences in grazing experience can result in large and persistent differences in grazing preference. This difference in experience can be reflected in different feed intake and liveweight patterns in animals grazing the same area. Differences tend to be more evident in animals moving from harsh grazing environments to ones that have been improved (Arnold and Maller, 1977). Training and manipulation of experience may therefore be required under some circumstances. This may be gained from other animals (e.g. maternal influence) or may be applied externally for example, short-term elevations in stocking rate to encourage stock to try unfamiliar foods (Provenza et al., 2003) or simply through herding livestock towards or away from particular areas. Although the effectiveness of herding has been questioned, it has sometimes proved effective, particularly if combined with strategic supplement locations (Bailey, 2004).

The ability to use selection and training for the exploitation of environmental variability, rather than using technology to neutralise or avoid variability is inconsistent with modern agricultural practice, but is successfully practised in some traditional livestock systems (Krätli, 2008).

REMOTE MONITORING AND MANAGEMENT OF LIVESTOCK AND LANDSCAPE

Livestock Monitoring and Control

Strategies to improve the intake and utilisation of forages in degraded environments have the potential to induce further degradation if not accompanied by improved grazing management. Technologies are now available that allow remote monitoring of livestock condition and behaviour. This type of information provides incentive and opportunity for livestock managers to tactically manipulate feeding and production. For example, monitoring change in both livestock and forage are grazing management options. While monitoring feed supply is the more traditional method advocated in high input grazing and may also be an option for degraded environments through the use of remote sensing (Hill et al., 2004), more information and control may be available from direct livestock monitoring. Livestock will begin to lose weight, or gain weight at a reduced rate long before feed supply is exhausted, and live weight change may provide information on both available biomass and diversity of feed sources. Remote and automatic monitoring of live weight has been successfully used experimentally (Rowe et al., 2005) and can provide information on nutritional status well before it becomes clear through visual observation. Similarly, animal behaviours, such as time spent walking, grazing and preferred feeding sites are all related to feed supply and diversity. Within rangeland and Mediterranean grazing systems, walking velocity and grazing time increases and intake rate decreases when forage supply decreases (Allden and Whittaker, 1970; Bailey et al., 1996). It is now possible to monitor these behaviours remotely on at least a small number within a flock or herd (Ganskopp, 2001; Ungar et al., 2005; Thomas et al., 2008; Handcock et al., 2009). Opportunities exist to directly relate measured characteristics of animal behaviour to more specific features of ecosystem change such as the consumption of less preferred but more for vulnerable plant species or soil damage. This information on livestock condition and behaviour can then provide the basis for well informed decisions on stocking densities or relocation.

The development of virtual fencing has the potential to control the movements of sheep and cattle in extensive grazing environments. This option for remote control will complement remote animal and livestock monitoring. Although this technology is still in the very early stages of development, a prototype system has been used to successfully modify the behaviour of cattle (Bishop-Hurley et al., 2007).

Application of these techniques has the potential to optimise the combination of livestock production and ecological stability in a way that will allow the long term productive use and revegetation of degraded landscapes.

CONCLUSIONS

Degrading environments provide an opportunity for the profitable production of food from livestock. In many circumstances, a livestock system can also retrieve or sustain functionality. Whether the outcomes of profitable livestock and sustainable landscape are both achieved depends on the design and management of the system. System management within these environments requires an innovative approach integrating the skills of animal physiology and behaviour, agronomy, plant ecophysiology, soil science and ecosystem ecology and management. This integration must operate outside the narrow perspectives that often characterise these disciplines.

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Breed Diversification in South Western Uganda: Characterisation of a New Cattle Farming System

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ABSTRACT

In response to increasing land pressure due to rapidly growing population, growing demand for livestock products in urban centres and new land policies which encourage individual land ownership in Uganda, pastoralists rearing the long horned Ankole cattle in south western Uganda have now become sedentary and less dependent on communal grazing systems. Crossbreeding of Ankole cattle with the Holstein Friesian for increased milk production is taking place at a very fast rate. A new production system in which pure bred Ankole and crosses of Ankole with Holstein Friesian are reared in separate herds on one farm has now emerged in the area. As part of a programme evaluating the ecological and economic sustainability of breeding in pastoral systems, a survey of sixteen farmers selected from three sub-counties in Kiruhura District in south west Uganda was undertaken. Two sets of detailed structured questionnaires were used to collect information from the farmers. Set one was administered at the beginning of the study in April 2007, while set two was administered on a monthly basis for a period of 12 months. In addition, production data from the animals was collected monthly. Results show that crossbreeding is taking place with no defined programme, farmers still have an attachment to the Ankole cattle and that the most important challenges to the production system are insufficient pasture during the dry season and livestock diseases. The crossbreeds produce significantly more milk than the Ankole and have higher live weights. There is need to formulate appropriate breeding programmes for the farmers and to develop guidelines for suitable stocking densities.

Key words: *Ankole cattle, crossbreeds, farming system, breeding programme.*

INTRODUCTION

For many years the Bahima, a pastoralist community found in south western Uganda have kept the Ankole cattle. This cattle breed is characterised by a relatively large body frame with long white horns. Their coat colour is usually solid cherry red but other colours like light brown with black stripes, red with white spots and black also exist. Ankole is a stabilised interbreed of *Bos indicus* (Zebu) and *Bos taurus* cattle (Mbuza, 1995). Traditionally the Ankole cattle play a central role in the lives of Bahima who have kept and continue to keep these animals as a source of milk for the owners, a store of wealth and pride. Typically for every 100 animals that one has, an iron bell also known as *omurebe* is tied around the favourite animal in the herd. The Ankole is adapted to the seasonal harsh climatic conditions prevalent in the south western range lands of Uganda which include low rainfall regimes leading to frequent droughts (Kisamba, 2006). In addition these animals have the ability to produce in situations where diseases and parasites are prevalent (Petersen et al., 2004; Kabi et al., 2008). It is because of these qualities that pastoralists have been able to keep this cattle breed on an extensive grazing system with minimal or no supplementation and with irregular supply of water.

According to official estimates (MAAIF, 2002) the cattle population in Uganda is around 6.1 million. Of this 50% is Long Horn Ankole, 30% the East African Short Horn Zebu, 16% Nganda, an intermediate breed of the Ankole and East African Shorthorn Zebu and the rest exotics and crosses of indigenous cattle with exotics. At a national level it is estimated that the local breeds contribute 75% of the domestic milk supply and more than 95% of all the total beef production in the country. Increasing pressure on land due to the rapidly growing population, growing demand for livestock products in urban centres and new land policies in Uganda are changing the life styles of the hitherto extensive Ankole cattle grazers. Large tracts of what used to be communal grazing land have now been fenced off and there is a shift from extensive to intensive production systems. To obtain animals with higher output, crossbreeding of Ankole with exotic breeds mainly the Holstein Friesian has begun and is taking place at a very fast rate. This has led to the emergence of a new production system where two separate herds i.e. a pure Ankole herd and a herd of Holstein Friesian–Ankole crosses are kept on one farm. In this system the Holstein Friesian-Ankole crosses are kept for commercial milk production, while the Ankole are kept for multiple reasons namely: cultural, a buffer against shock in case of prolonged drought and disease outbreak and for income through sale of live animals.

With the continuous improvement of rural infrastructure, for example through increased availability and accessibility to milk coolers and specialised milk transport vehicles to the urban centres, it is likely that cross breeding will continue at a fast rate. According to a recent report (Balikowa, 2004), south western Uganda had 61 milk collection centres with an installed capacity of 332 700 L. Since that time there has been additional investments in milk collection facilities (Wamboka, 2006) creating an even bigger demand for milk in the area. The fast rate of crossbreeding is of major concern to many and it is now believed that the Ankole breed famous for its gracefulness and long horns is now threatened with extinction (Rice, 2008).

Whereas the crossbreds produce more marketable product, they can only do so under good climatic conditions, low disease pressure

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N.E. Odongo, M. Garcia & G.J. Viljoen (eds), Sustainable Improvement of Animal Production and Health. Food and Agriculture Organization of the United Nations, Rome, 2010: **269–273**

and short drought periods. This necessitates considerable investments in the control of external and internal parasites, overall disease control and in the excavation of water holding facilities. The animals need to be well managed so that there is a right balance between available pasture and the needs of the animals. The emerging production system therefore raises a number of questions. Firstly, is it economically viable? Secondly, is it sustainable in the existing environment? This study was conducted in Kiruhura District in south western Uganda with the objectives of understanding the new production system, husbandry practices involved and the challenges faced by farmers.

MATERIALS AND METHODS

The Study Area

Kiruhura District falls within an area referred to as the cattle corridor that stretches from the south western part of Uganda to the North East part of the county. Kiruhura is home to the ethnic pastoralist group called Bahima. The area has two rainfall seasons with peaks from March to May and from September to November and two dry spells between June to September and from December to January (Okello et al., 2005).

Data Collection

The survey was conducted through two sets of questionnaires administered to farmers keeping two genotypes i.e. the Ankole and crosses of Ankole with Holstein Friesian in separate herds on one farm. The farmers were selected randomly from three sub-counties in Kiruhura District in south west Uganda. The first set was administered at the beginning of the study in April 2007 to 16 farmers, while the second set was administered to 17 farmers on a monthly basis for a period of the 12 months. The group included the initially selected 16 farmers and an additional one. The questionnaires were designed to obtain a wide range of information which included: attitude towards crossbreeding, land size, herd size and structure, herd management, disease prevalence and production challenges. On each of the participating farms production traits such as milk yield (MY) taken once a month were recorded. A correction factor of (M x 1.65) where M is the single morning milk record was used to adjust milk yields for animals milked twice daily as observed by Erdman et al. (1995). Other production traits collected included weight of the animal estimated by heart girth measurement (HG) and body condition score (BCS) In addition calving dates and dry off dates were recorded. Some of the information collected was supported by observations during the visits.

Data Analysis

Frequency counts and means for some of the observations were calculated using SAS (2002). For analysis of BCS and HG the General linear model SAS (2002) was used. The model included fixed effects of genotype, season, farm, interaction of breed and farm, interaction of breed and season. Covariate (age) was used to adjust for differences in ages of the cows. For analysis of milk yields the General linear model SAS (2002) was used. The model after appropriate changes included the independent variables genotype, lactation number, and farm, breeds nested in farms, interaction of breed and lactation number, season and breed, farm and season. Covariate (days in milk) was used to adjust for stages of lactation.

RESULTS AND DISCUSSION

The Nutrition and Management of Animals

In the new farming system, grazing of the animals is usually on enclosed farms. These are enclosed either by a natural fence or barbed wire. Apart from one farmer who provided hay to his animals during the dry period, the rest of the farmers grazed their animals on natural pasture and only mineral lick was provided as a supplement.

Water was supplied to the animals once daily from valley tanks that existed on the farm. Typically the water is scooped into a drinking trough by a stockman. Care is taken to ensure that the two genotypes are taken to the watering points at different times.

About 50% of the farmers interviewed stated that they kept the two cattle types because the crossbreds gave them more marketable milk, while the Ankole provided security in case diseases or prolonged drought affected the crossbreds. Another group (19.9%) stated that they still preferred to keep Ankole cattle besides the crosses because they were hardy, while others (13.3%) stated that they kept Ankole for beef production because they were easier to sell for this purpose and the crosses for milk production. Another 13.3% stated that the crossbreds were kept for income through milk sales and Ankole were kept for cultural reasons. One farmer informed the interviewer that he started crossbreeding in 1974 and that during the civil war in Uganda in 1978/9, essential inputs like acaricides were not available; he lost his entire herd of crossbred animals to tick-borne diseases. Some of his Ankole survived and he was able to re-establish his crossbreeds from this surviving group.

The majority of the interviewed farmers started crossbreeding between 1990 and 2000 (**Table 1**).This period coincided with the time when new land policies were introduced and with improvements in rural infrastructure like improved road networks and rural electrification (Sserunkuma, 1998).

The farmers interviewed all kept the two genotypes in separate herds. On some farms, grazing areas for the two genotypes were demarcated while on others the same grazing land was used but at different times. Different reasons were given for rearing the animals in separate herds: 31.25% stated that the two genotypes required different levels of management, 25% stated that this was to control breeding, 12.5% stated that the animals had different grazing behaviour. One of the farmers informed the interviewer that the Ankole tended to graze together in one pack while the crossbreds spread out in the fields while grazing. Utilisation of the pastures was therefore different for the two genotypes. This observation is also supported by a study (Huber et al., 2008) who found that Ankole heifers tended to graze in higher densities than did the crossbred heifers as they had more animals within 5 meters around focal animals than was the case with the crossbreeds. Other reasons advanced for keeping the animals in separate herds were (i) Ankole animals scare the crossbreeds, (ii) different production costs are involved for the two genotypes, and (iii) crossbreds are kept purely for milk production while the Ankole are kept for meat production in which case the Ankole could be reared at places distant from the homestead.

Table 1. Frequencies of when farmers started crossbreeding.

Period (y)	Percent
1970–1979	7.69
1980–1989	15.38
1990–2000	76.92

Only 50% of farmers indicated that they carried out pasture improvement activities such as bush clearing, removal of unwanted plants, burning, planting pasture, and a combination of two or more of the above activities.

Land Size and Animal Numbers

Most farmers interviewed owned the land on which the animals were grazed. Two farmers stated they rented additional land to ensure that they had enough pastures. Land sizes ranged between 57 ha and 750 ha. Farmers were asked to indicate how much of their land had been fenced off (all of if, part of it and/ none of it). Most (82%) indicated that only parts of the land were fenced off. All farmers were settled in one part of the farm with permanent homes. However, it was clear from the discussions that there was still some degree of movement of animals taking place by some farmers. For example, animals were shifted to distant areas during periods of prolonged drought. The selected farms had a combined total of 4 886 animals of which 56% were Holstein Friesian Ankole crosses and the rest Ankole. The combined herd sizes were between 91 and 725 animals **Table 2**. On average, herd sizes of the Ankole were smaller those of the crossbreds.

Other Farm Enterprises and Sources of Labour

Farmers indicated that in addition to keeping cattle, they kept sheep and goats and had banana plantations.

On all the farms both family and hired labour was used. The number of family members engaged in the day-to-day running of the farms was between one and four, although this number increased during school holidays when additional family members were available. The number of hired labour ranged from 1–20 depending on the number of animals owned.

Mating Systems

All farmers on the study used natural mating and each farmer owned at least one Ankole and one Holstein-Friesian bull. The maximum number of Ankole and Holstein Friesian bulls was three and four respectively. Some farmers informed the interviewer that the Holstein-Friesian bulls and some of the crossbred cows had been bought from other areas where intensive farming is well established. This included areas near to Kampala (the capital city) and Kashari (near Mbarara town). On all farms there was continuous upgrading of the crossbred animals to a higher exotic grade without a defined crossbreeding programme. This is likely to result in undesirable effects in future if management is not improved in line with changes in the genotype.

Table 2. Number of animals of the different genotypes.

Ankole (n = 16)					Crossbreds (n = 16)				
Category	Mean	Min.	Max.	S.D.	Category	Mean	Min.	Max.	S.D.
Herd	134.5	59	453	93.02	Herd	171	32	325	110.5
Bulls	1.5	1	3	0.63	Bulls	1.7	1	4	0.85
Cows	59.2	20	200	41.9	Cows	73.4	15	150	48.4
Heifers	32.8	2	80	21.93	Heifers	37.3	3	100	25.49
Steers	17.90	2	42	17.50	Steers	17	2	60	23.04
Calves	32.06	6	130	11.56	Calves	44	2	148	40.86

Table 3. Disease occurrence in the two genotypes over a 12-month period.

Ankole (n=16)		Crossbreds (n=16)	
Disease	Percent	Disease	Percent
Ephemeral fever	45.45	East Coast fever	21.8
Lumpy skin disease	27.3	Brucellosis/abortions	15.6
Internal parasites	9.09	Lumpy Skin disease	15.6
East Coast fever	9.09	Mastitis	9.4
Salmonellosis	4	Eye infections	9.4
		Ephemeral fever	6.3
		Anthrax	6.3
		Anaplasmosis	3.1
		Mange in calves	3.1
		Salmonellosis	3.1
		Internal parasites	3.1
		Dystocia	3.1

In the Ankole herd only pure breeding was practised. It was not clear from where bulls or replacement animals in this herd were obtained, although according to a similar study in the same area (Wurzinger et al., 2008) farmers are likely to replace their Ankole breeding stock from their own herd or from neighbours, friends, or relatives.

Prevalence and Control of Disease

During the monthly farm visits farmers were asked to indicate diseases that had occurred in the herd and the number of cases observed (**Table 3**). The results show that in the Ankole, ephemeral fever was most common followed by lumpy skin disease. In the crossbreds there were more disease conditions observed than in the pure bred Ankole. In this group of animals East Coast fever was the most important disease followed by brucellosis and lumpy skin disease.

All farmers had special arrangements for the control of ectoparasites. Eighty eight percent sprayed the animals against ticks using a bucket spray while 12% of the farmers indicated that they used a plunge dip. The frequency of acaricide application varied between the farms. On 81.25% of farms all the genotypes were sprayed once a week, 12.5% indicated that the animals were sprayed twice a week while the rest indicated that they sprayed Ankole weekly and the crossbreds twice weekly. On some of the farms expensive acaricides were used only on the crossbreds, while ordinary types were used on the Ankole. There was a wide variation in the type of acaricide used on different farms. However judging from the frequent use of acaricides it is likely that the success of the production system depended heavily on the availability of these chemicals. Frequency of use of drenches to control internal parasites varied widely from one to four times annually.

Production

Production and body linear traits of the adult cows are given in **Table 4**. The crossbreds had significantly ($P < 0.001$) higher daily milk yields and had higher bodyweights than the Ankole. The higher milk production observed in crossbreds is supported by other research findings (Rege, 1998) which showed that where management is good, there is improvement in performance with increasing *Bos taurus* genes with 50% and 75% performing better.

According to statements by the farmers total milk production/d on the farms from crossbreds ranged between 260 L and 900 L while that of the Ankole ranged between 34 L and 80 L. The average price of milk/L at the time the questionnaire was administered was approximately eight US cents. It is therefore clear that the crossbreds contributed greatly to daily house hold incomes and as such are very attractive to the farmers.

The higher body weights observed in the crossbreds indicate that this group of animals will require different stocking densities than the Ankole to obtain maximum production.

Challenges

On each of the monthly visits farmers were asked to indicate the challenges they faced on their farms since the last visit. Responses were grouped into six categories (**Table 5**).

Animal health was the single most important challenge on the farms, followed by inadequate pasture during certain periods of the year. This could be an indication that farmers were overstocking or did not have adequate arrangements to feed the animals throughout the year. This was confirmed by a study (Mulindwa et al., 2009) that simulated the long term pasture production dynamics and carrying capacity of the study area. Unstable labour was also of great concern to the farmers with frequent change of stockmen on most

Table 4. Least square means of daily milk yield, body condition score and body weight.

Daily milk yield			BCS			Body weight (cows)		
Genotype	Litres	S.E.	Genotype	Score	SE	Genotype	kg*	SE
Ankole (n = 91)	2.2[a]	0.5471	Ankole (n = 1576)	3.36	0.0283	Ankole (n = 1 622)	334.[a]	2.0167
HF50% (n = 158)	10.6[b]	0.5132	HF 50% (n = 345)	3.28	0.0915	HF 50% (n = 361)	398.2[b]	6.5442
HF > 50% (n = 625)	10.1[b]	0.1758	HF > 50% (n =1602)	3.24	0.0297	HF > 50% (n = 1 641)	395.6[b]	2.1036

HF 50% — F1 Ankole Holstein; Friesian HF > 50% — crossbreds of greater than 50% Holstein. Friesian kg* converted from heart girth measurement; SE — standard error; [a b] means in a column with different superscripts differ significantly ($P < 0.0001$), n — number of observations

Table 5. Challenges faced by farmers over a 12-month period.

Challenge	Frequency (%)
Animal health (diseases in the herd)	54.2
Animal nutrition (inadequate feed for animals)	24.1
Unstable labour	18
Unstable price of milk	1.8
Security (theft of stock)	1.2
Poor milk production	0.6

farms being noticeable. This had a negative effect on routine farm operations and much time was spent on orientation of new staff. The high turnover of staff on the farms could be due to poor remuneration and working conditions. Although unstable milk prices were not given as one of the top challenges to production, farmers complained about changes in milk prices during the different seasons. Prices were highest during the dry period when there was less milk available from the farms and then dropped during the wet seasons when supply to the collection centres exceeded capacity.

CONCLUSIONS

Cattle continue to play an important role in the lives of the Bahima by providing income from the sale of milk and a store of wealth. The changing production system is in response to the changing land policies in south western Uganda and due to demand for milk products in the urban centres. With the rapidly growing population it is likely that demand will continue to grow and crossbreeding of Ankole will continue at a very fast rate. There is therefore no guarantee that the new production system will continue unhindered. Efforts by The National Animal Genetic Resources Centre and Data Bank (NAGRC & DB) to conserve the Ankole cattle through the Ankole open nucleus breeding programme in collaboration with pastoralists should be well funded by government.

The crossbreds are very attractive to farmers in south western Uganda because of their high milk yields but there is no proper breeding programme in place to support farmers in selecting their replacement stock or guiding them on the appropriate level of crossing. Simple recording systems need to be developed and introduced to the farmers so that they have a reference point from which to make choices.

The high prevalence of diseases in cattle herds remains a very important challenge to the farmers by affecting productivity of the animals. Proper disease control strategies need to be instituted by the local authorities in the area in collaboration with the central government.

The fact that farmers are facing periods of inadequate pasture availability for their stock could be an indication that at certain times of the year these farms are over stocked. Guidelines need to be developed to enable decision-making by farmers on appropriate stocking densities and appropriate actions to reduce seasonal fluctuations in production.

The high milk yields in crossbred animals do not necessarily mean that they are profitable. An in-depth cost-benefit analysis needs to be undertaken to determine the actual costs involved in producing milk under the production system.

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SESSION 3

TRANSBOUNDARY, EMERGING AND ZOONOTIC DISEASES

The Importance of Emerging and Re-emerging Zoonotic Diseases: Recognition, Monitoring and Control

S. Mas-Coma[1]

ABSTRACT

Whilst communicable diseases mainly affect the developing world, new and emerging diseases have re-awakend the developed countries to the importance of these infections. Concern has been raised that climate change and other man-made changes to the environment could provide conditions for diseases to increase their range and affect countries where they have not normally been a problem. The zoonoses, infections naturally transmitted between vertebrate animals and humans, present considerable difficulties because eradication is almost impossible and control is problematic. However, control of many zoonoses can be affordable and reduction in human morbidity needs to become a priority task in many countries and regions. Many global, regional or locally occurring emerging or re-emerging infectious diseases are caused by zoonoses, including both vector-borne and non-vector borne diseases. Molecular biology has been instrumental in providing an understanding of zoonotic infections in relation to their transmission, epidemiology, clinical and pathological effects, treatment, development of vaccines and diagnosis and control. Examples of zoonoses in which molecular tools have helped decisively are mentioned in this review and examples are given on how molecular markers may help in the assessment and control of zoonotic diseases as illustrated in the insect vector-borne protozoan zoonosis, Chagas' disease and a snail-borne helminthic zoonosis, fascioliasis. Finally, emphasis is placed on the need to increase studies on animal reservoirs, to improve teaching and understanding of 'old-fashioned' disciplines such as medical malacology and entomology, as well as training and technology transfer and to actively pursue field work. Only by investing in these disciplines will we be in a position to go to the field, perform surveys, and acquire the data that will enable us to determine the presence of emerging or re-emerging infectious diseases.

Key words: *Emerging and re-emerging zoonoses, molecular biology tools, Chagas' disease, fascioliasis, animal reservoirs.*

INTRODUCTION

A communicable disease is one that is transmitted from one individual or inanimate source to another either directly, through a vector, or by some other means. Communicable diseases cover a wider range than the person-to-person transmission of infectious diseases: they include vector-borne parasitic diseases, zoonoses and all other transmissible diseases. Recently, communicable diseases have caught the attention of the world with the appearance of: avian flu caused by the H5N1 virus, severe acute respiratory syndrome (SARS), bovine spongiform encephalopathy (BSE or mad cow disease) and new variant Creutzfeld-Jacob disease (CJD), the relentless increase in human immunodeficency virus (HIV) infection, the use of anthrax and other microorganisms as biological weapons and more recently the new 'swine' flu A/H1N1. Communicable diseases have always been with us and although they are not considered a serious problem in developed countries, they are the main cause of death and infirmity in the developing world.

The impact of a communicable disease depends on the agent, its mode of transmission, the host and the environment as taken together they will determine the outcome of infection. The range of communicable diseases occurring throughout the world is considerable. Numerous types of agents are involved: Prokaryotes (microorganisms) including arboviruses and other viruses, bacteria, rickettsiae and spirochaetes; Eukaryotes (parasites) comprising Protozoa (with Sarcomastigophora, Apicomplexa, Ciliophora and Microsporidia), Helminths (with Trematoda, Cestoda, Nematoda and Acanthocephala), and Ectoparasites (with Arachnida and Insecta) and also Fungi. Others to be added are prions and toxins.

EMERGING AND RE-EMERGING ZOONOSES

Whilst communicable diseases mainly affect the developing world, new and emerging diseases have re-awakend developed countries to the importance of these infections (Morens et al., 2004). Although the major impact of these diseases arise within a developing country, the problem assumes international importance as more people travel to affected countries and incidents occur where exotic diseases are imported to the developed world. Concern has been raised that climate change due to global warming could provide conditions for diseases to increase their range and affect countries where they have not normally been a problem (Harvell et al., 2002). Similarly, global change factors, including increasing man-made modifications of the environment (Patz et al., 2000) and import/export of mainly domestic animals (farm animals, pets) and also exotic, sylvatic animal species, is also playing a role in the spread of infectious diseases (Chomel et al., 2007).

The key to the control of communicable diseases is the method of transmission. Communicable diseases fall into a number of transmission patterns: (i) direct transmission: without intermediate hosts (e.g. human to human, animal to animal, animal to human); (ii) human

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reservoir with intermediate invertebrate host: the causal agent must undergo developmental stages in an intermediate host (e.g. snails in schistosomiasis); (iii) animal as intermediate host or reservoir: vertebrates play the role of intermediate host (e.g. taeniasis) or that can be reservoirs (e.g. Chagas' disease), and (iv) vector-borne transmission: an arthropod carries the infection from one host to another (e.g. *Anopheles* mosquitoes in malaria. Although often called vectors, snails are only intermediate hosts and not true vectors because they do not carry the infection from one host to another.

Communicable diseases fall into two main groups based on their transmission cycle, i.e. diseases in which only humans are involved and diseases in which there is an animal reservoir or intermediate host. The latter are the zoonoses, infections that are naturally transmitted between vertebrate animals and humans. According to the focus of the disease (intimacy of the animal to the human being), zoonoses can be grouped into the following types: (i) domestic: animals that live in close proximity to man (e.g. pets and farm animals); (ii) synanthropic: animals that live in close association with man, but are not invited (e.g. rats); and (iii) exoanthropic: animals that are not in close association with man and are not invited (e.g. monkeys). In a zoonotic disease, the animal reservoir is of prime importance to the success of any control measures. The most important difference between human diseases of zoonotic origin and those in which animals do not play a role as reservoirs is that in zoonoses, opposite to the latter, eradication becomes almost impossible and elimination becomes a task always believed to be far from affordable. Therefore, the greatest efforts by international agencies and national/international funding institutions are nowadays concentrated on human diseases of non-zoonotic nature/source, that is, in which there is no animal reservoir. The so-called big three, namely malaria, HIV/AIDS and tuberculosis, and the so-called neglected diseases including schistosomiases, filariases, onchocercosis, ascariasis, trichuriasis and ancylostomiasis-necatoriasis are at present priorities for the World Health Organization (WHO).

Additionally, there are other neglected parasitic diseases which need to be added to this priority list. These are diseases for which it is very difficult to get funds for research, despite being of high human impact globally, regionally or locally. Most of them are zoonoses which are emerging or re-emerging at present, including both vector-borne and non-vector borne diseases. They include (i) intestinal protozoal diseases such as giardiasis, cryptosporidiasis and amoebiasis, (ii) vector-borne protozoal diseases including leishmaniases, sleeping sickness and Chagas' disease, (iii) food-borne trematodiases such as fasciolosis, fasciolopsiasis, clonorchiasis, opisthorchiasis, paragonimiasis and gastrodiscoidiasis, (iv) cestodiases like taeniasis/cysticercosis, hidatidosis and alveococcosis, and (v) nematodiases as trichinelliases (or trichinosis) and strongyloidiasis.

For most of these zoonoses, the crucial facts are already known: (i) the characteristics of the disease including the transmission cycle of the causal agent are known; (ii) there are tools for the diagnosis of the disease in both humans and animal reservoirs, as well as in the intermediate host or vector in vector-borne diseases, and (iii) effective drugs for both animal and human use are available. The control of many of these zoonoses appears, therefore, affordable and a reduction of human morbidity may become a realistic goal for several of them in many countries and regions.

USE OF MOLECULAR BIOLOGICAL TECHNIQUES FOR UNDERSTANDING ZOONOTIC DISEASES

Molecular biology is a very broad field which has quickly evolved in recent years. It provides molecular tools for the genetic characterisation of living organisms and, through gene expression, the baseline for phenotypical analyses. There are many kinds of molecular approaches with different degrees of resolution, including methods and techniques for the genetic characterisation of: individual specimens, strains, populations, species and supraspecific taxa. Bioinformatics is a modern computer science which has evolved in parallel to molecular biology with the main objective of furnishing high capacities for the mathematical analysis of genetic data (mainly nucleotide and amino acid sequences of DNA) for both molecular phylogenetics and population studies.

Molecular marker combinations, including from high resolution DNA sequencing such as single nucleotide polymorphisms or SNPs (Mas-Coma and Bargues, 2008) up to less detailed techniques, such as banding analytical methods like random amplified polymorphic DNA or RAPD (Pacheco et al., 2003), restriction fragment length polymorphism or RFLP (Marcilla et al., 2002) or microsatellite markers (Hurtrez-Bousses et al., 2004) are very useful tools for studying zoonoses. For instance, in epidemiology they enable distinguishing different strains of the causal agent and their relationships with higher/lower prevalences and intensities in humans and animals, animal species which constitute the reservoirs and infection sources for humans, intermediate hosts or vector species which constitute the transmission sources for humans, climatic factors and environmental characteristics, geographical distribution and spreading capacities. In clinical studies and pathology, they enable distinguishing between different strains of the causal agent and their pathogenicity and immunogenicity. In diagnosis, they are useful for the highly sensitive and specific diagnosis of the causal agent in humans, reservoir animals and intermediate hosts and vectors. In treatment, they are used for the characterisation of resistant and susceptible strains. In control and surveillance, they furnish tools for the development of vaccines and the follow up of postreatment re-infections. The application of molecular tools in studies of avian influenza caused by the H5N1 virus is an excellent example of the application of this technology (WHO Global Influenza Program Surveillance Network, 2005).

Examples of zoonoses in which molecular tools have decisively helped in clarifying disease epidemiology and transmission are numerous. In cryptosporidiasis, molecular tools have proved that there is a higher number of different human-infecting species and specific reservoir hosts than initially believed (Xiao et al., 2000). In hidatidosis, different *Echinococcus granulosus* strains (genotypes) with different host ranges and geographical distributions are at present differentiated: sheep-dog, horse-dog, cattle-dog, camel dog, pig-dog, cervid strains (Le et al., 2002). Trichinosis was a disease in which only one species, *Trichinella spiralis*, was believed to be the causal agent but now it is known that there are in fact different *Trichinella* species with different sylvatic cycles and geographical distributions (Murrell et al., 2000).

Molecular tools are furnishing very useful information on the insect-borne protozoan zoonosis Chagas' disease and the snail-borne helminthic zoonosis, fasciolosis. These two diseases are reviewed in more detail to illustrate how molecular markers may help in the assessment and control of zoonotic diseases.

CHAGAS' DISEASE, AN EXAMPLE OF AN INSECT-BORNE PROTOZOAL ZOONOSIS

American trypanosomiasis or Chagas' disease caused by the haemoflagellate protozoan species *Trypanosoma cruzi* is widespread in Latin America from Mexico to Chile and southern Argentina. Although present estimates of 10 to 12 million people infected represent between six and eight million cases fewer than those reported in the 1980s, it is still the most serious parasitic disease of the Americas for its social and economic impact. Although it can also be transmitted by blood

transfusion and transplacentarily, human contamination usually occurs by vectorial transmission in poor rural or periurban areas of Central and South America (Schmunis, 2004). Moreover, the disease is recently emerging in western European countries such as Spain and France, but also Portugal and Italy, as a consequence of the present high immigration from Latin American countries- Although vector-borne disease transmission cannot take place in Europe because of the absence of triatomine vectors (Bargues et al., 2000), blood and vertical transmission are issues of concern (Schmunis, 2007).

Chagas' disease vectors are haematophagous reduviid (Hemiptera: Heteroptera) insects belonging to the subfamily Triatominae. The 138 species currently recognised within Triatominae are grouped into 17 genera forming five tribes, with all species appearing capable of transmitting *T. cruzi*. Among the triatomines, most of the species (76) are included in the genus *Triatoma*, within different complexes and subcomplexes in a classification which is progressively updated according to new genetic and morphometric data (Dujardin and Schofield, 2004).

In Chagas' disease, recent molecular results have shown that the causal agent *Trypanosoma cruzi* is very heterogeneous throughout the endemic countries of Latin America, including two main phylogenetic groups, I and II which diverged very long ago and in turn also appear to comprise different lineages (Briones et al., 1999). This, together with molecular studies showing that triatomine vectors are also more complex than previously believed, is giving rise to a completely new frame which indicates that known transmission patterns, clinico-pathological pictures, diagnostic kits and traditional control strategies will be in need to be reassessed.

The *Trypanosoma cruzi* Complex and the Heterogeneity of the Disease

Heterogeneity of *T. cruzi* does not only concern a different geographic distribution of the clinical presentation of the disease in the chronic stage and of the transplacentary transmission from mother to foetus, but also the different response to treatment and the different behaviour of the trypanosome in mice and triatomine bugs (Miles et al., 2004).

DNA-based techniques including RFLP, analysis of DNA of the kinetoplast (kDNA), RAPD, zymodeme analysis, comparison of polymorphisms between rDNA and miniexon gene, and analysis of the two internal transcribed spacers of the rDNA (ITS- and ITS–2), have shown that there are two main phylogenetic groups within *T. cruzi*, which by consensus have been designed *T. cruzi* I y *T. cruzi* II (WHO, 2002). Additionally, 5 subgroups have been distinguished among the second group and named *T. cruzi* II a-e (Tibayrenc *et al*., 1993; Fernandes *et al*., 1998; Oliveira *et al*., 1998). *T. cruzi* I has been linked to the sylvatic life cycle of the parasite and to strains showing low morbidity in humans, whereas *T. cruzi* II has been related to the domestic life cycle of the parasite and the chronic form of the disease in countries of the Southern Cone (Fernandes *et al*., 1998 and 1999). The subdivision of *T. cruzi* II into 5 subgroups was recognised from analysis of fragments of the 18S and 24S α genes of the rDNA (**Figure 1**) (Brisse et al., 2001).

T. cruzi I, initially considered uniform, is also progressively proving to be complex. When analysing *T. cruzi* I specimens from humans, domestic and sylvatic animals and also triatomine vectors, different lineages were found by intergenic miniexon gene sequencing in northern South America (Herrera et al., 2007) and also Mexico

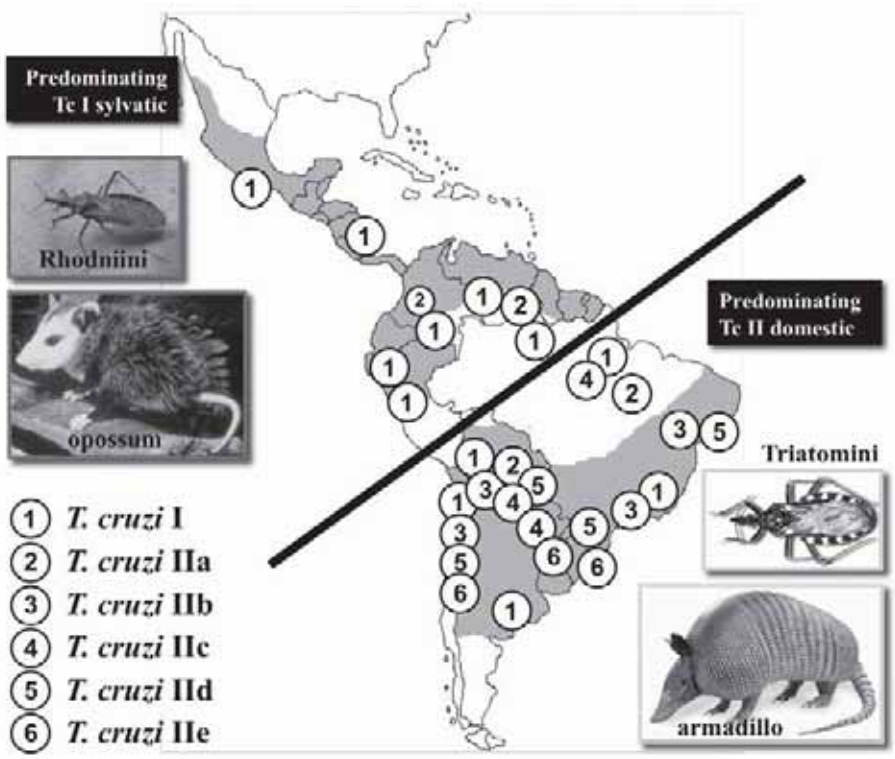

Figure 1. Geographical distribution and relationships with pressumably original definitive mammal hosts and triatomine vector groups of the causal agents of Chagas' disease, *Trypanosoma cruzi* I and *T. cruzi* II. Grey shading includes areas with Chagas' disease endemics or human reports.

(O'Connor et al., 2007). These lineages are also showing apparent links to the domestic, peridomestic and sylvatic life cycles of the parasite. These results indicate that *T. cruzi* I may also be as heterogeneous as *T. cruzi* II. All suggest that subgroups will need to be established within *T. cruzi* I, once more information from other molecular markers obtained in different geographic areas becomes available. So, there is still a long way to go until a sufficiently detailed mapping of the distribution of both *T. cruzi* I y *T. cruzi* II, as well as relevant information about relationships of the groups and subgroups with the differences in biology, transmission, epidemiology, clinical picture and pathology known to exist within Chagas' disease are obtained throughout their wide distribution in the Americas and which until now could never be satisfactorily explained.

Problems of Systematics in Triatomine Vectors, Disease Transmission, Epidemiology and Control

The geographical distribution of Chagas' disease, its local transmission patterns and main epidemiological characteristics depend on the vectors. The absence of effective drugs and an available vaccine mean that vectors become the main target for control measures. This is why great multidisciplinary efforts have always gone into understanding and cataloguing the triatomines, both vector species and those never found to be infected or involved in transmission. Vector systematics always plays a key role in vector-borne diseases. The cataloguing of vector characteristics related to disease transmission, human contamination, and epidemiology in general within different systematic units greatly facilitates the tasks.

The capacity of a triatomine species to become close to humans in an intradomiciliation process and feed on human blood is the key to becoming a good vector of the disease (Schofield et al., 1999; Dujardin and Schofield, 2004). A distinction is made between (i) intradomicilary vectors having colonised human dwellings (domestic vectors), (ii) sylvatic species in the process of adaptation to human dwellings (candidate vectors), and (iii) sylvatic species that remain closely associated with wild mammals (potential vectors) (Dujardin et al., 2002; Dujardin and Schofield, 2004). Thus, plasticity, adaptability and movement capacity of triatomines within the triangle constituted by the intradomicile, peridomicile and sylvatic habitats is crucial in the transmission, epidemiology and control of the disease (Schofield et al., 1999; Dujardin and Schofield, 2004).

Sequencing of nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) is the best method for providing information concerning triatomine systematics and vector characteristics up to the present. Both rDNA and mtDNA include neutral markers whose usefulness has already been emphasised in understanding Chagas' disease vectors (Monteiro et al., 2001; Bargues et al., 2002). Examples of aspects of Triatominae bionomy and of their relationships to Chagas' disease transmission, epidemiology and control that can be addressed from DNA sequence data are: (i) systematics and taxonomy; (ii) origin; (iii) evolutionary traits; (iv) evolutionary rates; (v) biogeography; (vi) specimen classification; (vii) population delimitation; (viii) hybrid characterisation; (ix) population changes; and (x) disease transmission (Bargues et al., 2002). Studies on the major vector species such as *Triatoma infestans* (Bargues et al., 2006), *T. dimidiata* (Bargues et al., 2008) and *Rhodnius prolixus* (Monteiro et al., 2000), have shown the valuable information that molecular techniques can furnish.

The broad usefulness of nuclear rDNA and mtDNA sequences explains why the number of studies using these markers published has increased so markedly in recent years. A review on selected, updated knowledge about nuclear rDNA and mtDNA in insects, concentrating on aspects useful for research on triatomines has recently been published (Mas-Coma and Bargues, 2009). This study analyses the efficiency, importance of their different characteristics, and the limitations and problems of each type of marker in the light of the results obtained in studies on populations, hybrids, subspecies and species of Triatominae, including several crucial genetic aspects newly or very recently detected. Emphasis is given to taxonomic units and biological entities presenting well-known problematics, as well as to molecular situations which can give rise to erroneus conclusions. The purpose of this review is to offer a baseline for future research on triatomines and their relationships to transmission, epidemiology and control measures of Chagas' disease, thereby facilitating future work on triatomines. This review highlights (i) present gaps, (ii) choice of the most appropriate markers, and (iii) marker aspects which must

Figure 2. Global geographical distributions of fascioliasis.

be taken into account in sequence studies and phylogenetic analyses to obtain appropriate results and correct interpretations (Mas-Coma and Bargues, 2009).

HUMAN AND ANIMAL FASCIOLIASIS, AN EXAMPLE OF A SNAIL-BORNE HELMINTHIASIS

Molecular results are also giving rise to a revolution in the traditional knowledge on fascioliasis. Fascioliasis is an important disease caused by two digenetic trematode species of the genus *Fasciola*: *F. hepatica* distributed in the five continents and *F. gigantica* mainly in Africa and Asia, and transmitted by different freshwater snail species of the family Lymnaeidae (**Figure 2**) (Mas-Coma, 2004; Mas-Coma et al., 2005). The impact of this disease is markedly higher than that of Chagas' disease due to (i) its worldwide distribution, (ii) the present emerging situation almost everywhere, (iii) its great pathogenicity and adverse impacts on development, and (iv) the large number of around 17 million people affected, mainly children and females. All of this indicates that the large intraspecific variability of liver flukes and their lymnaeid snail vectors is important in determining the capacity of this disease to spread and emerge in very different areas and environments despite the different human behaviours. All in all, molecular tools appear to be in the forefront in ascertaining transmission patterns, human and animal infection sources, and epidemiological situations, as well as for establishing the appropriate global strategies and local measures of control.

Fasciola hepatica is present in the five continents, transmitted by lymnaeid vectors of the Galba/Fossaria group excepting in Oceania where it is transmitted by authochthonous as well as introduced lymnaeid vector species. *Fasciola gigantica* is transmitted by lymnaeid species of the Radix group and is distributed in sub-Saharan Africa, the Nile bassin, the Near East and overall southwards from the Himalayas and also partly northward from this mountainous chain, up to the Far East and Pacific islands as the Philippines and Hawaii. There is overlap of both species and respective lymnaeid vectors in eastern Africa and large parts of Asia. The molecular difference between both fasciolid species is only 10 mutations throughout the intergenic ITS–1–5.8S - ITS–2 region of the nuclear rDNA operon. Countries in white = presence of only *F. hepatica*; countries in grey = presence of only *F. gigantica*; countries in blackish = overlap of the two fasciolid species.

The Heterogeneity of Human Fascioliasis

In the last two decades, field studies have shown that human fascioliasis evolves very differently depending on the geographical area. Thus, epidemiological scenarios range from hyperendemic, mesoendemic and hypoendemic situations, up to epidemics in human endemic areas and in animal endemic areas, as well as areas with authochthonous, isolated, non-constant cases and others with only imported cases (Mas-Coma et al., 1999).

Moreover, different transmission patterns may be distinguished within the different human endemic areas: a very high altitude pattern in Andean countries, a Caribbean insular pattern, an Afro-Mediterranean lowland pattern in Egypt, and a pattern related to areas surrounding the Caspian Sea (Mas-Coma, 2005), to which another recently detected in Southeast Asia may be added. The relationships between the lymnaeid vector species and a specific transmission pattern should be emphasised. Lymnaeids show markedly different ecological and ethological characteristics depending on the species, with different factors being crucial in determining the characteristics of the disease, such as type of water collection, habitats, population dynamics, temperature thresholds, seasonality, or susceptibility regarding liver fluke infection. This indicates that, as in other vector-borne parasitic diseases, the lymnaeids may constitute excellent markers of disease characteristics useful for differentiating between different human fascioliasis situations and patterns. Consequently, an accurate classification and adequate genetic characterisation of the lymnaeid vectors is of the highest importance (Bargues and Mas-Coma, 2005).

The aforementioned heterogeneity of the disease in transmission and epidemiological characteristics is of such a level, that establishing convenient, simplified and uniform control programmes for the different endemic areas does not appear feasible. For many areas or countries, specific control measures should be recommended which may include several measures peculiar to that given place. Pragmatism demands the convenience of looking for markers which could easily and quickly distinguish each type of transmission pattern and epidemiological situation (Mas-Coma et al., 2009a). Genetic markers appear to be the frontline targets, since all the above-mentioned characteristics may be related to different combinations of species and strains of both liver flukes and lymnaeid vectors. Climatic-physiographic markers appear in the second line, because they need a previous characterisation of flukes and snails, both in the field and experimentally in the laboratory, to enable the application of accurate methods like mathematical modelling and remote sensing and GIS (Mas-Coma et al., 2008, 2009b). Summing up, molecular markers are key for an adequate characterisation of both parasites and vectors.

Molecular Markers to Assess Species and Strains of the Causal Agents of Fascioliasis

Among the different techniques used for the genetic characterisation of fasciolids, sequencing of nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) appears to be the best technique (Mas-Coma et al., 2009a). Among the nuclear rDNA operon, the 5.8S gene and the 18S gene are not useful markers due to their slow evolutionary rates (Mas-Coma and Bargues, 2009). The 28S rDNA is also highly conserved in *F. hepatica* and *F. gigantica*, with only few interspecific nucleotide differences. In spite of this, a 618-bp fragment was successfully selected for a PCR-RFLP assay using the restriction enzymes *Ava*II and *Dra*II to differentiate both fasciolid species (Marcilla et al., 2002). More recently, a nucleotide position among a 510-bp-long fragment of the 28S gene allowed differentiation between anthelmintic-resistant and susceptible fluke specimens in Spain (Vara-del-Rio et al., 2007).

The intergenic transcribed spacers ITS–1 and ITS–2 are the best markers for the differentiation of species (Mas-Coma and Bargues, 2009) and have also shown their usefulness for liver flukes. Both ITSs appear to be intraspecifically very conserved and with only five nucleotide mutations differentiating the two fasciolid species (Mas-Coma et al., 2009a).

With regard to mitochondrial gene markers, only partial sequences of two genes were initially used, cytochrome c oxidase subunit I (*cox*1) and the NADH dehydrogenase subunit I (*nad*1) (Hashimoto et al, 1997; Itagaki et al., 1998). Surprisingly, despite the availability of the whole mitochondrial genome sequence of *F. hepatica* for several years (Le et al., 2001), only short sequences of *nad*1 and *cox*1 have been used until recently, with the additional problem that the different fragments and sometimes their different lengths make comparative analyses difficult. However, recent work including complete *cox*1 and *nad*1 sequences and analysis of specimens from numerous countries of all continents offers opportunities to set new baselines for future studies involving (i) a global framework on the evolution of the disease, (ii) molecular characterisation of 'pure' *F. hepatica* and 'pure' *F. gigantica* and analysis of their respective intraspecific variabilities and also a detailed assessment of their interspecific differ-

ences; (iii) an exhaustive review of all sequences available so far, (iv) standardisation of methods and techniques, and (v) standardisation of DNA sequence nomenclature (Mas-Coma et al., 2009a).

The combined use of markers from both nuclear rDNA and mtDNA has proven to be very useful in fascioliasis, because it allows the detection of hybrid forms. The two fasciolid species are characterised by presenting introgression at the level of the mitochondrial genome. This enables an understandng to be gained of intermediate forms between the two fasciolid species known to affect animals and recently also detected in humans (Periago et al., 2007; Le et al., 2008; Mas-Coma et al., 2009a), as well as rare phenomena described in Asia, such as abnormal ploidy and aspermic partenogenesis (Terasaki et al., 2000).

Molecular Tools to Characterise Lymnaeid Snail Vectors

The role of lymnaeid vectors in fascioliasis transmission, epidemiology and control demonstrates the importance of getting new tools which could help in specimen classification, genetic characterisation of natural populations and laboratory strains, and in arranging the systematics and taxonomy of Lymnaeidae. The failure of all malacological and non-malacological tools used for these purposes up to the present suggests the convenience and usefulness of such tools to analyse whether DNA sequences and phylogenetic methods could be useful. The first attempt to develop a research collaboration of parasitologists, molecular biologists and malacologists was successful (Bargues et al., 2001).

A worldwide lymnaeid molecular characterisation initiative began progressively to make steps forward, including large, transboundary studies (Bargues and Mas-Coma, 2005; Mas-Coma et al., 2009b). The great spreading capacity of lymnaeids is evidence that sometimes not even a continental scale is sufficient, and intercontinental sequence comparisons are needed to correctly classify specimens. The intercontinental spreading of lymnaeids and its role in fascioliasis dissemination is well known (Mas-Coma et al., 2003, 2005; Pointier et al., 2007).

Among the different DNA markers used so far in lymnaeids, the 18S rRNA gene appears to be too conserved and its few variable positions may only be useful at generic and suprageneric taxon levels (Bargues and Mas-Coma, 1997, 2005). The ITS–2 and secondarily ITS–1 are the most appropriate for: (i) classification of lymnaeid specimens, (ii) characterisation of lymnaeid intraspecific genetic interpopulational variablity to furnish the genetic base on which to understand fasciolid-lymnaeid specificity, different susceptibilities or compatibilities of geographical strains or even resistances, (iii) establishment of valid species and their geographical distributions, and (iv) assessment of species interrelationships to arrange a natural systematic-taxonomic classification which would allow an analysis of coevolution with fasciolids (Bargues and Mas-Coma, 2005). One mutation at the level of the ITS–1 and another at ITS–2 have proven useful to distinguish between resistant and susceptible populations of *Pseudosuccinea columella* in Cuba (Gutierrez et al., 2003), although nothing evidently suggests that these mutations are linked to the resistance/susceptibility duality.

Within mtDNA, only fragments of the 16S and *cox*1 have been sequenced in lymnaeids (Remigio and Blair, 1997a; Remigio, 2002; Bargues et al., 2007). Recent knowledge indicates that mtDNA markers, including both mitochondrial genes and the ribosomal 12S and 16S genes, should be used with great caution when dealing with species belonging to different genera and even those well separated within the same genus (Mas-Coma and Bargues, 2009). Consequently, the use of mtDNA markers for this initiative are restricted to (i) sequence comparisons and phylogenetic analyses of only close species within the same genus, (ii) studies of intraspecific variability of species by sequence comparisons of individuals and populations, (iii) genetic characterisation of laboratory strains, (iv) studies on the spread of populations of a species, and (v) studies on genetic exchange between different neighbouring populations.

Very recently, the standardisation proposed for lymnaeids is expected to furnish the baseline needed to clarify the present chaotic systematics of this molluscan family and thus raise the possibility of moving forward in fascioliasis assessment. It should be borne in mind that fasciolid flukes show a strict specificity regarding lymnaeid species and sometimes apparently also geographic strains within a given lymnaeid species. Hence, molecular markers become crucial for assessing fascioliasis transmission both in the field and also experimentally in the laboratory, as well as in assessing fasciolosis transmission and establishing adequate control measures (Bargues and Mas-Coma, 2005; Mas-Coma et al., 2009a).

FUTURE EFFORTS IN VECTOR-BORNE ZOONOSES

The complexity of zoonotic infectious diseases poses a number of problems which must be solved. Although overall knowledge of the epidemiology and transmission of these diseases is available, specific knowledge relating to the local epidemiology and transmission characteristics is still lacking in many cases. Multisdisciplinary approaches and cross professional team networks are needed for both research and training. Efforts will be needed to convince different responsible ministries and health responsibles to work together and any political-strategic difficulties must be solved. Funding agencies need to be convinced about the need for increasing efforts at the animal level since studies on geographical distribution and epidemiology of zoonoses using modern tools are crucial to establish the appropriate local control measures.

The need for 'old-fashioned' disciplines such as medical malacology and entomology needs to be emphasised. Field work should again be encouraged. Today, one of the greatest problems is that in many developing countries little is known about disease epidemiology and therefore effective control methods cannot be applied.

Moreover, control of all kind of infectious diseases needs sustainability. Sustainability requires specifically trained scientists in endemic countries and areas. Consequently, training and technology transfer should be high on the agendas of research projects on zoonotic diseases.

Interestingly, results from field surveys usually suggest that many diseases are emerging or re-emerging. This could be related to the higher performance of today's diagnostic methods compared with older ones; however, one conclusion is evident: all these diseases are still there and continue to be as prevalent as ever!

CONCLUSIONS

Communicable zoonotic infectious diseases are very problematic due to (i) the complexity of the different organisms involved, (i) the difficulties posed by the numerous and changing biotic and abiotic factors influencing their epidemiology and transmission, and (iii) the huge challenges they pose for control. However, our capacities to tackle them in a multidisciplinarily manner have never been so strong. The moment to take decisive steps against these diseases by taking advantage of all these techniques simultaneously has arrived.

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An Efficient Stakeholder Driven Approach to Disease Control

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ABSTRACT

The objective of this paper is to describe the work of the European Technology Platform for Global Animal Health (ETPGAH). The ETPGAH is a stakeholder driven initiative aimed at agreeing on the most important diseases, the most important gaps in our ability to control those diseases with the intention of focusing animal health research on filling those gaps. The stakeholders identified the need to prioritise animal diseases, carry out gap analysis, explore our fundamental research capacity, address enabling factors, review regulatory issues and take a global view as diseases do not respect borders. An Action Plan addressing all of these issues was published in 2007. The Action Plan identified 30 activities that need to be pursued. For each activity, the objectives, deliverables and tasks are stated. The ETPGAH must now oversee the delivery of the Action Plan and much progress has already been made. Mirror Groups have been established in a number of countries to communicate the Action Plan and to link its priorities into national research programmes. An ERA-Net has been created as recommended in the Action Plan and is launching research projects that will deliver additional actions contained in the Action Plan. The DISCONTOOLS project is aimed at developing a more sophisticated methodology for gap analysis and disease prioritisation. In conclusion, the ETPGAH has been successful in getting stakeholders to agree on priorities and is now actively delivering the Action Plan.

INTRODUCTION

The European Technology Platform for Global Animal Health (ETPGAH, http://www.ifaheurope.org/CommonTP.aspx?SubMenuId=47&MenuId=17) was established in December 2004 with the objective of identifying the most critical issues that need to be addressed in order to control diseases in animals. The ETPGAH was funded by the European Commission and as required by the Commission, was led by industry. European stakeholders and International organizations participated in the work of the Platform and developed a Vision, Strategic Research Agenda (SRA) and an Action Plan (http://www.ifaheurope.org/upl/4/default/doc/ETPGAH_ActionPlanAug07.pdf).

RESULTS AND DISCUSSION

The Vision developed is as follows 'To facilitate and accelerate the development and distribution of the most effective tools for controlling animal diseases of major importance to Europe and the rest of the world, thereby improving human and animal health, food safety and quality, animal welfare, and market access, contributing to achieving the Millennium Development Goals'.

The Vision foresees the speedier development of tools for disease control by focusing our research effort on the most important gaps in the most important diseases. These may be emerging, established or zoonotic diseases. By delivering better disease control, animal health and welfare is protected, human health benefits in terms of zoonotic disease control but human health also benefits from food security, safety and quality. Indeed, poverty and famine may be averted. The stakeholders to the ETPGAH recognise that diseases do not respect borders and take a global perspective in the knowledge that a global reduction in disease is to the benefit of everybody.

In developing the SRA, the stakeholders identified six major themes:

- prioritisation of animal diseases
- gap analysis
- fundamental research
- enabling factors
- regulatory issues
- global perspective

It was recognised that we need to prioritise our effort and focus on finding new disease control tools by collaborative research on a limited number of critical targets. By this mechanism, we can attempt to make greatest progress in the least amount of time. Critical to this concept is the need to identify and prioritise the most important gaps in our ability to control the critical diseases.

Having identified our targets, it is then vital that we have the fundamental research capacity — infrastructure and people — to carry out the necessary research. Establishing our research capability necessitates the creation of a database with the relevant information with gaps then being addressed. Along with filling gaps, efficiency can also be improved by avoiding unnecessary capacity development.

Enabling factors such as quality assurance, intellectual property rights and facilitation of technology transfer are critical components in moving from basic research to the development of a tool that can be used to fight a disease. Financial support at critical points in the development chain is also vital. Too often, projects are dropped because intellectual property rights have not been secured and nobody is willing to invest perhaps €100 million in taking the project from the laboratory bench through development and into the market.

The correct regulatory environment is vital to stimulate innovation. A balance needs to be reached between protecting human and animal health from the risks associated with a product versus the wish to eliminate all hazards. In addition, regulation needs to be focused on the needs of the veterinary sector which may be quite different to those of the human sector.

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From a global perspective, it is in the interests of everybody to reduce the global burden of disease. It may be much more beneficial to tackle disease at its source. This approach facilitates cooperation across the globe including capacity-building.

Having explored the broad areas that need attention in the SRA, the Action Plan was then developed and published in July, 2007 identifying the actions — research or information gathering — that needs to be carried out in order to deliver the SRA. The Action Plan follows the themes identified in the SRA with 30 activities outlined. For each activity, the objectives, deliverables and tasks are stated.

The purpose of the ETPGAH is to now oversee the delivery of the Action Plan. Each activity needs to be progressed and funding is an important factor. Funding from the European Commission is important as it stimulates collaborative research. However, nation states are the main source of research funding with more than 90% of funding coming from this source. As such, the ETPGAH has stimulated the creation of 'Mirror Groups' and seven have been formed to date (http://www.ifaheurope.org/CommonTP.aspx?SubMenuId=50&MenuId=17). The purpose of a Mirror Group is to communicate the content of the Action Plan to a national level and also to encourage the use of national funding to deliver some of the activities from the Action Plan best suited to that country. The Mirror Groups meet occasionally and now wish to develop common calls for research that may be funded by DG Research, individual Member States or a number of Member States.

In the overall context of the ETPGAH initiative, progress has been encouraging to date with many activities being funded by the European Commission.

A very important development has been the creation of the Emerging and Major Infectious Diseases of Livestock European Research Area Network (EMIDA ERA-Net). The EMIDA ERA-Net involves 19 countries with annual animal health research budgets of € 270 million. The EMIDA ERA-Net is progressing many of the information gathering exercises such as those related to infrastructure and human capacities. In addition, it is building a database of research carried out since 2005 along with information on ongoing research facilitating those who wish to make contact with and collaborate with scientists active in a given research area. The EMIDA ERA-Net has recently issued calls for research that will see projects being run across a number of countries thus contributing greatly to collaborative research across the countries involved in the initiative.

The DISCONTOOLS project commenced in March, 2008 and will run for four years. It is focused on developing a sophisticated prioritisation model based on a database of information in relation to approximately 45 diseases highlighted in the ETPGAH Action Plan. The database will be created by expert groups who will also be asked to propose prioritised gaps in relation to each disease. This information will then be scored via the prioritisation model and the output should be agreed priorities that should be the subject of focused research in order to lead to technological breakthroughs giving us the tools to control these disease more effectively. The technology will be web based and will be publicly available for comment and input thus leading to a database and prioritisation mechanism that will be continuously updated over time.

CONCLUSIONS

The ETPGAH has stimulated stakeholders to agree on a common Vision, Strategic Research Agenda and Action Plan. With the stimulus of DG Research in funding relevant research projects, the creation of the EMIDA ETRA-Net and the work of DISCONTOOLS, it can be concluded that much progress is being made in focusing our research effort on the priority gaps. In turn, this focus will lead to significant breakthroughs in technology at an earlier date than otherwise would have been possible leading to our earlier ability to control the diseases with which we currently struggle.

From the viewpoint of society, the ETPGAH initiative will help to protect animal health and welfare, as well as society from zoonoses. Most importantly, the aim of reducing the global disease burden should help to alleviate poverty and malnutrition in many parts of the world where endemic and epizootic diseases currently wreak havoc.

Development, Validation and Implementation of Animal Health Information Systems in an Environment without Uniquely Identified Animals in Transitional Countries

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ABSTRACT

Two animal health information systems were developed in Macedonia, the National Epidemiological Information System (NEIS) and the Laboratory Information System (LABIS). Both systems were aimed at collecting/interpreting animal disease data in a country where animals are not uniquely identified. The development of NEIS was based on the existing legislation of compulsory notification of infectious diseases. Field records are collected via the designated veterinary practices and entered into the NEIS via the veterinary inspectors who are employees of the Ministry of Agriculture, Forestry and Water Economy. Sources of data for NEIS are obligatory disease control programmes (Annual Order), endemic diseases, outbreaks, slaughterhouses and laboratory results of annual surveys. LABIS is a separate database for managing laboratory results. It collects data from samples submitted by Designated Veterinary Practices (DVPs). The samples can then be analysed in different laboratories using different methods and given a 'final status' by an authorised person. The final status is linked to the previously performed tests and entered into the NEIS. Using this concept, the Veterinary Department can trace back the background for each individual sample by reviewing the analyses performed on it. Both systems are designed as a referential integrity databases, where the field result is linked to the animal, owner, village (n = 1 803), epidemiological unit (n = 123) and epidemiological area (n = 30) in the country. NEIS can also present the same data in geographical maps, showing the infected village as the smallest unit of observation. Both systems have also different levels of authorisation access, allowing precise tracing of entered data.

Key words: *information systems, legislation, annual order, epidemiology, data entry, ISO 17205.*

INTRODUCTION

Veterinary activities for the control of animal diseases in Macedonia are based on the Law for Veterinary Health (Official Bulletin of R.M., 2007a), the Programme for control and eradication of especially dangerous diseases in animals (Official Bulletin of R.M., 2007b) and special programmes designed for specific diseases which are consistent with EU legislation. Under this legislation the country is divided into 30 Epidemiological Areas (EAs) and 123 Epidemiological Units (EUs). Each village belongs to a defined EU, which further belongs to a defined EA.

The Head Veterinary Office (HVO) comes under the Ministry of Agriculture, Forestry and Water Economy (MAFWE) and is divided into 5 sectors: Animal Health (AHS), Public Health, Border Inspection and Veterinary Legislation. It is lead by the Chief Veterinary Officer and the sectors are lead by the Heads of the sectors. The AHS is further divided into four departments: the Animal Health, Animal Welfare, Identification and Registration of Animals (I & R) and Veterinary Inspection in Animal Health (VIAHD). The veterinary inspectors (VIs) are employees of the VIAHD and are responsible for the animal health issues at the level of one or more EU.

The Designated Veterinary Practices (DVPs) are private enterprises, registered by HVO and licensed by the Veterinary Chamber. They perform field veterinary activities of Government interest. The designation is based on a public tender and is valid for five years. The DVPs are also responsible for one or more EU and can perform veterinary activities of government interest, only upon authorisation of the appropriate VI.

The Veterinary Institute is part of the Faculty of Veterinary Medicine in Skopje (FVMS) and is designated for official testing related to the animal health/public health issues. This testing is performed in the laboratories of the FVMS and are accredited under ISO 17025. Some of the testing methods (mainly upon request of the HVO) are accredited under ISO 17025.

In practice, the HVO publishes an 'Annual Order' (AO) based on the Programme for control and eradication of especially dangerous diseases in animals (Official Bulletin of R.M, 2007b). The AO defines all activities (examinations, vaccinations, checkups in the slaughterhouses, samplings, testings etc) which should be performed in a defined period of the year. Upon this order, the local VIs issue written orders to the appropriate DVPs. Activities are reported back to the local VIs, which forward the information to the HVO.

In case of laboratory testing, the samples are taken by DVPs, brought to the laboratories of FVMS and tested in the appropriate laboratory using predefined method/methods. The results obtained are sent back to the appropriate DVP, VI and at the HVO.

Until 2002, the results of all these activities were recorded on paper forms, cumulating progressively when collected at the level of

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N.E. Odongo, M. Garcia & G.J. Viljoen (eds), Sustainable Improvement of Animal Production and Health. Food and Agriculture Organization of the United Nations, Rome, 2010: **287–293**

HVO and causing continuous problems when certain data analysis were needed. Additional problems have arisen because of the lack of permanent identification, control of movement and definition of the target group for examination (flock, backyard animals, mixing between flocks and houses etc.). During 2004, a Law for Identification and registration of Animals (Official Bulletin of R.M, 2004) was issued which is supposed to cover all domestic animals with permanent identification numbers, with strengthened, computerised management of farming, animal health and public health control. Up to now, only cattle have been permanently tagged; sheep and goats, as well as pigs are in the process of being tagged.

To solve these problems, we have developed the NEIS, a computerised system for animal disease data collection, which imitates the original flow of information. The system is based on collection and recording of data by the local VIs, which is later transferred to the HVO, enabling immediate follow up of the planned activities.

As the majority of the results originate from the laboratory, there have been problems with entering the huge amount of results into the NEIS. A solution was the development of LABIS (MAFWE, 2004), a system which can store different laboratory analyses performed on a single sample, deliver the final status (result) and transfer it into the NEIS.

Using this concept, we have succeeded in improving the collection of epidemiological data, enabling their urgent analyses and undertaking appropriate measures in the field. As both systems are created for environments lacking strict animal breeding and disease control, they are suitable for application in developing and transitional countries.

MATERIALS AND METHODS

National Epidemiological Information System (NEIS)

As a starting point, the main components of NEIS were defined: the nomenclatures, the planning of the AO, the structure of the national flock and the main sources of animal disease information. The nomenclatures were practically lists of commonly used attributes necessary for data entry and analysis. In this way, lists were made of villages, EUs, EAs, animal species, diseases etc. Afterwards, the necessary referential integrated links between these lists were established e.g. the link between an individual village and appropriate EU, as well as the link between the EU and the appropriate EA (**Figure 1**). Similar links have been established between the animals and the diseases, owners and the appropriate villages and users of the software and their user authorisations.

Using such linked nomenclatures, we were able to facilitate compact data entry for the activities performed and the existing predefined attributes.

The planning of the Annual Order (**Figure 2**) is usually made during the last quarter of the year for the upcoming year (e.g. between October and December 2008 for the year 2009). Creating the computer recorded plan could enable the HVO to follow up the performed activities in real time at the level of EU/village/owner on the one hand, and the VP/DVI on the other. Consequently, deviations from the planned activities can be immediately seen and corrective measures applied.

The analysis of the structure of the national flock is of great importance from many organisational reasons. Firstly, the distribution of animals inside the units of observation (EAs, EUs and villages) is important for organising human resources for the planned activities and for implementing consequent measures (easy to vaccinate or control a flock of 10 000 sheep, difficult to do the same in 2 000 flocks, each of 5 sheep!).

The structure of the national flock in the country is as follows:

The total area of the F.Y.R. of Macedonia is 25 713 km^2; divided into 30 EAs, within which 123 EUs are defined. There are 1 803 administrative villages, each of which belongs to a defined EU.

Approximately 87% of the cattle population is bred in farms (holdings) smaller than 30 animals. Sheep and goats are mainly kept in flocks of 50–100 animals/flock. Pigs and poultry are bred mainly in smaller (backyard) farms. The rest of the animals (approx. 13% of cattle, 20–30% of sheep and goats, 27% of pigs and 50% of poultry) are bred in professional farming facilities. This structure of the national flock results in the wide dissemination of large number of small flocks (holdings). Having these data, the HVO can easily estimate the number of official veterinarians, designated practices and intensity (timeframes) of activities for fulfillment of the AO.

The main sources of disease information were investigated because there was a need to match real sources of disease data and

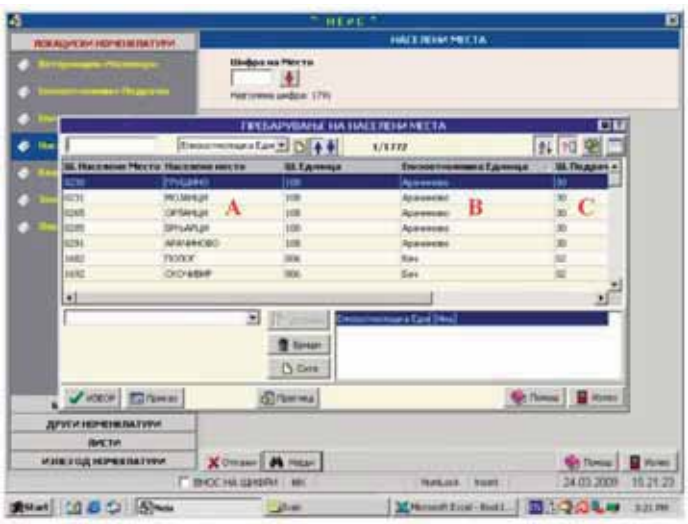

Figure 1. Nomenclatures: linking villages (column A) to appropriate EU (column B) and EA (column C).

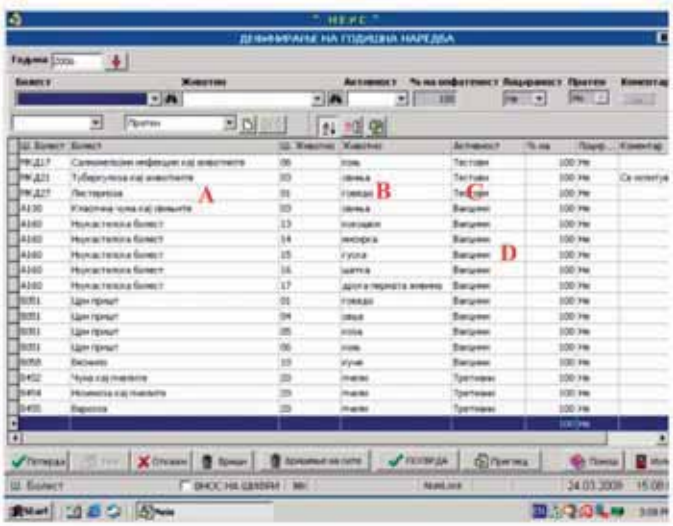

Figure 2. Planning of the AO by disease (column A), animal species (column B), type of activity (column C) and coverage (column D).

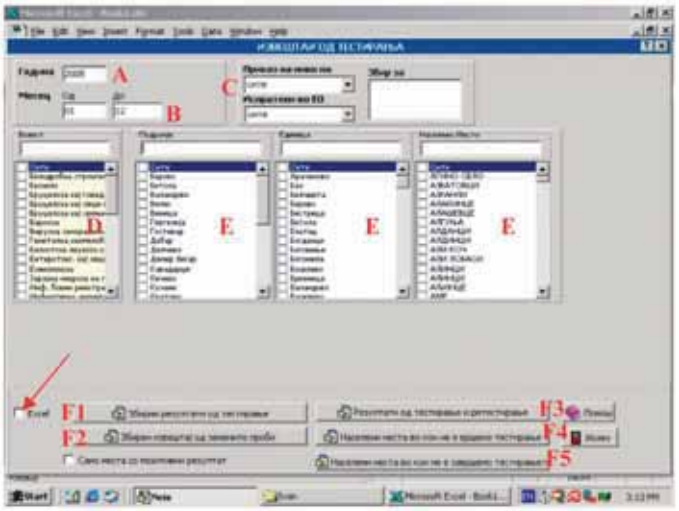

Figure 3. Example of a request for a report on performed testing (explanation in the text)

Figure 4. Example of a report for performed testings, after data filtering (explanation in the text)

entry tables/forms of the NEIS. These sources of information were divided into five classes, and for each of them a separate entry port (screen) was created. The classes of disease information are shown in **Table 1**.

Each of the five entry screens for the appropriate class of information is in compliance with the existing forms of DVPs and VIs, which enables a user-friendly entry by appropriate VIs. For each entered diagnosis, the source is also entered (clinical, post mortem, laboratory), to enable the HVO to establish its relevance. Once the data are entered into the local NEIS database, they are automatically transferred into the central database at HVO on a daily basis. However, if an outbreak occurs, the local VI can transfer the recorded data to the HVO immediately by pressing a single button. This tool is enabling the HVO to have an immediate overview of the situation in the field. Analysing entered data is enabled via the predefined reports. However, the system allows user-defined reports by selecting data the users want to see at any given moment. Additionally, the reports are generated using 'menu forms' where significant filtering of data is enabled, the aim being to select only critical information required during each request. An example of a typical report menu is shown in **Figure 3**. The report contains a selection of the parameters of interest, such as: selection of year (A), months from/to (B), selection of all testing performed or a defined testing (C), selection of disease (D), village within appropriate EU and EA or selection of certain EUs, EAs or villages (E).

Letters F1-F5 generate reports of filtered data in different ways e.g. cumulative results of all testing (F1): cumulative results of all sampled animals, showing pending results (F2): results of testing and retesting on same animals (F3); only villages where testing has not been performed (F4); and villages where testing is still not finished (F5). The menu offers a button (red arrow) which exports the data into Microsoft Excel, for eventual further analysis by the user. Such a pattern of filtering is used for all the reports on different queries. A typical report after data filtering is shown in **Figure 4**.

The filtering form has been adjusted to show summarised data for each EU and all diseases tested for the period between January 2004 and December 2004 (M). Simultaneously, the filter has been set up to show the name of the EA (A), disease tested (in the example: bovine brucellosis (B1), ovine/caprine brucellosis (B2) and bovine tuberculosis (B3), animals species (column C), number of villages covered during testing (column D), number of villages with positive animals (column

Table 1. Sources of entry data for the NEIS.

Type of information collected in the NEIS	Responsibility/method of collection
Obligatory disease control programmes (vaccinations, anthelmintic treatments and field diagnostics /TBC/ etc.)	VI/manually, in the local NEIS database
Endemic diseases (anthrax, clostridia etc.)	VI/manually, after laboratory confirmation, in the local NEIS database
Outbreaks (once an outbreak occurs /FMD/ the veterinarian must not test every single animal in the lab., but count the number of animals showing clinical signs in the village	VI/manually, in the local NEIS database
Slaughterhouses (findings during slaughtering e.g. *Echinococcus, Trichinella* etc.)	VI/manually, in the local NEIS database
Laboratory results of annual surveys	LABIS at FVMS/Automatically

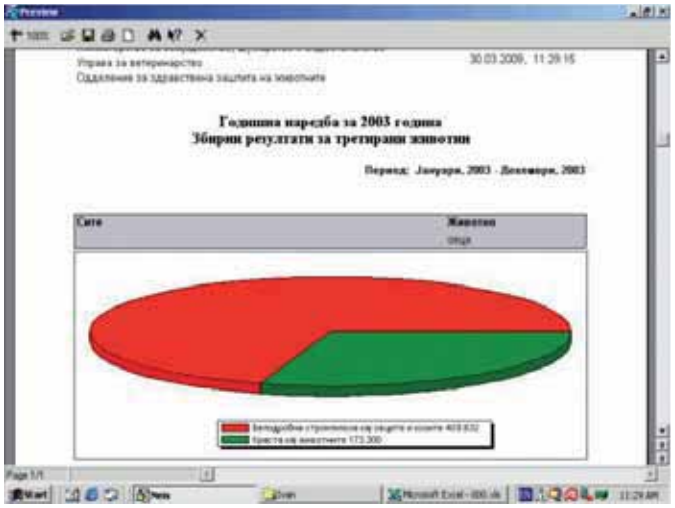

Figure 5. Example of a graph in a pie format (explanation in the text).

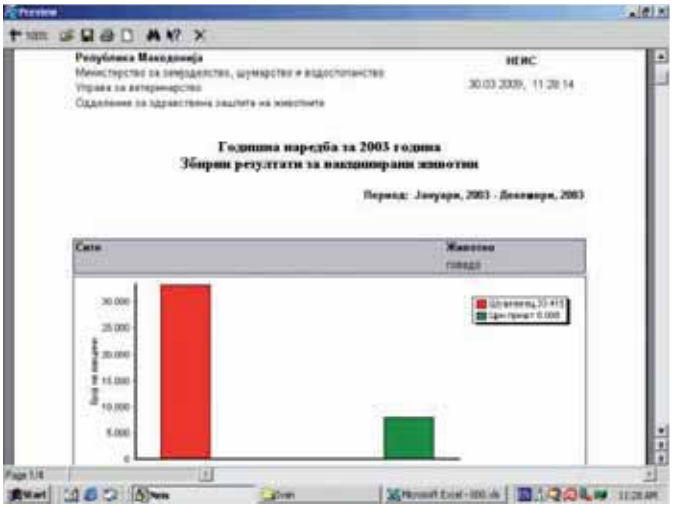

Figure 6. Example of geographical maps, upon different filtering criteria (explanation in the text).

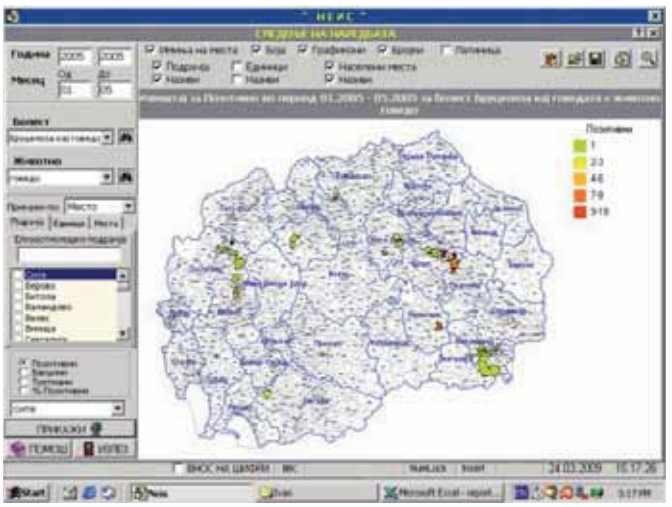

Figure 7. Example of a report as a geographical map, with appropriate filters over and left from the map (explanation in the text).

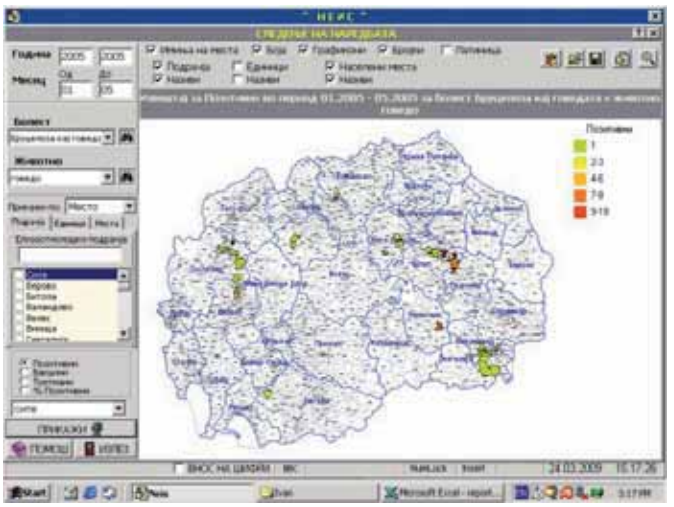

Figure 8. Entry form for the submission letter (description in the text).

E), percentage of villages with positive animals (column F), total number of tested animals (column G), total number of positive animals (column H), percentage MAFWE, 2002 of positive animals (column I), number of tests performed to obtain the final results (column J), number of slaughtered animal (column K) and number of destroyed animals (column L). Similar reports can be generated if the menu form is set up to filter the data to a lower level of observation (EU or village). An additional possibility for the user is to select columns he/she wants to see after the report is generated. This offers an opportunity to additionally avoid unnecessary data. This pattern of report is similar for all other reports generated by NEIS.

An additional potential of NEIS is to transfer the tabular data into graphs and maps. Two formats of graphs can be generated, the pies (**Figure 5**) and columns (**Figure 6**). The maps (**Figure 7**) are generated using mapping software. The maps show the data by linking the geographical borders of the administrative units (EAs, EUs and villages) to the tabular data from the reports. Consequently, the maps can show cumulative data based on a village, EUs or EAs. The same filtering principles are available for the maps as for the classical reports. The difference is only that the maps show coloured surfaces of the required geographical unit, which indicate the number of the required parameter or (upon request) a percentage of it.

Figure 5 is a pie chart where the occurrence of strongilosis (red colour) is compared to scabies (green color), for all the country. The coloured labels indicate the required parameter and the absolute number of variables for each parameter. **Figure 6** (just an example), shows a column graph, where the occurrence of blackleg (red colour) is compared with the occurrence of anthrax (green colour), cumulative at the level of the whole country. The coloured labels (lower part of the graph) indicate the required parameter and the absolute number of variables for each parameter.

Similar graphical presentations can be generated for the level of EA, EU or village.

Laboratory Information System (LABIS)

LABIS is designed to work in conjunction with the NEIS. For that reason, the nomenclatures are identical and picked up from one

Table 2. Steps for entering data in the LABIS.

Step	Activity	Comment
1.	Reception of samples	Samples are received, data of the main letter entered into LABIS and 'Certificate of Reception' issued to submitters.
2.	Entering the main submission letter with the corresponding forms with details	Required component for further processing of the samples in the laboratory.
3.	Electronic sending of the samples through the laboratory/ies.	Records on by whom, where and when the samples are processed.
4.	Definition of tests which are required.	Activity performed by request (HVO) or by decision of the head of the laboratory (outbreak/mortality?)
5.	Creating results from different tests.	If necessary, the head of the laboratory can send the sample to another laboratory for additional testing.
6.	Creating of the final result, based on one or more analyses for a certain disease.	The system will not allow generating the final result unless all requested testing and diseases are finished!
7.	Reports on tests performed, final results issued and epidemiological information based on the laboratory result.	These records are important as information support for the laboratory and communication with the HVO.

Figure 9. Entry form for sending of the samples to the laboratory.

Figure 10. Entry form for sending of the samples to the laboratory.

source. This enables a synchronised transfer of data between the two systems.

The data flow in LABIS is designed to follow several steps which are in accordance with the former procedures involving manual sample submission and processing through the laboratory/ies. Briefly, the samples are submitted with the submission letter (obligatory document for submission) at the reception desk of FVMS. The submission letter is comprised from a main letter and the corresponding forms with details for the samples. The main letter contains general data about the village, owner, farm, the DVP and the aim for the sampling. The corresponding forms with details contain details about the sampled animals. The steps required for data entering into the LABIS are shown in **Table 2**.

The same pattern is used also in LABIS (**Figure 8**), with a remark that all forms with details are obligatory for entering in the database. Using this way of data entry, systematic data are stored in the database and the manual miss-filling of the forms omitted.

After entering the main letter (**Figure 8**, area A), the system generates a unique ID of the submission letter and a Certificate for Reception, which is given to the submitter.

The detailed forms (**Figure 8**, area B) can be entered into the system immediately or if needed, later, but in any case before sending the samples to the laboratory.

A special command button is available to enter the sample ID numbers in the system (**Figure 8**, area C). Entering of sample IDs into the system is not obligatory. They all can be added into the system, or upon request of the submitter (HVO), only positive samples will be added.

Upon entering the basic data into the system, the staff of the reception desk enter the name of the targeted laboratory/ies for testing (**Figure 9**). This process records who has sent the samples (A), when (date and time), (B), which is the recipient laboratory (C), which person should receive the samples (D) and the details on the submission letter to which the samples belong (E). This entry is important to follow up the activities on the samples through the responsible persons for them.

Once the responsible person in the first recipient laboratory opens the form with sent samples to his lab., the system will show him/her what has been sent and what kind of tests are required. If needed, the responsible person from the first recipient laboratory can resend

Figure 11. Form showing all details performed on the samples from a single submission letter.

the samples to an additional laboratory for which the same data from **Figure 9** are entered.

Different laboratories can test the samples using one or more tests (methods), (**Figure 10**). The results of all these tests are recorded for each sample individually (column B), showing the type of sample (column C), proposed final result (column D), and the performed tests to achieve the result (T1-T2). Separate columns with the computer generated unique ID of the sample is shown as is a column for free text for each sample (column A and remarks, column E respectively).

The proposed result (column D) is generated by the computer, based on the majority of the test results. The diagnostician is allowed to deliver his/her own final result based on knowledge related to the diagnostic performances of the tests performed. However, the system keeps all data, according to which the final status of the sample has been delivered, together with the name of the person who has generated it. Once the final result for a certain disease is confirmed by pressing the confirmation button (red arrow), it is stored in the database and cannot be edited anymore.

The same principle is used to confirm the diagnosis if multiple diseases are required for testing. The final result can be issued only after the status for all requested diseases has been confirmed.

After issuing (closure of the submission letter) of the final result, the authorised person can have a detailed look it using a special form (**Figure 11**). This form offers basic details related to the required submission letter (A), its forms and details (B and C), movement of the sample/es.

These reports are linked only to the laboratory results obtained in the designated labs. They are aimed to support the organisation of the laboratory, to gain epidemiological information from laboratory results and to support the HVO in the process of planning.

Since 2004, a Law for I & R of Animals has been issued (Official Bulletin of R.M., 2004). This law defines precisely the registers of owners, holdings, animals tagged with unique IDs and their relationships (actions to be taken when changing the owner, holding movement etc). As some of the species are identified at an individual level (cattle, sheep, goats) and some on a flock level (feeding lambs, farming pigs), the transfer of data from LABIS to I & R may cause difficulties. Additionally, some species are not subject to I & R, but will be of epidemiological interest (migratory birds, wild boars, wild carnivores). For this reason, a intermediate module is planned, aimed on enabling communication between LABIS and I & R.

RESULTS AND DISCUSSION

I & Rs are based on a strict link between the animal ID and the computer database. This requires almost perfect performance of the veterinary service, farmers and supporting staff (database users and managers). However, from a technical standpoint, while a good information system can be easily created, the difficult task is to build up a continuously supportive veterinary service. Such support should cover registration of any activity related to the animals (newborns, slaughtered/destroyed, animal movements etc.), as well as registration of epidemiological events, all of which are quite dynamic. Additionally, veterinary services should keep a history of all these events, which should be available for reviewing at any time.

If such a system is not working continuously and properly, early discrepancies will occur (experiences from Macedonia) in terms of missing young animals (unregistered calves), missing previously existing animals during sampling (unreported slaughtered/sold animals), or sampling animals that exist in the national flock occurring in totally different places (unregistered animal movement-trade).

Having this in mind, the concept of NEIS and LABIS was based on continuous collection of field information (NEIS) or from the annual obligatory testing campaigns (LABIS), simulating a 'snapshot' of the epidemiological situation.

Several major advantages have resulted from the two databases. Firstly, the traditional paperwork was largely replaced by computerised data collection, although some phases are still based on paper recording. Additionally, manipulation and interpretation of epidemiological data were significantly improved as all the data are permanently stored in the database. Finally, prompt reporting of data was enabled, either as tables, graphs or maps.

Using this concept, we have succeeded to link the animal to the owner, village, EP and EA which has resulted in the possibility of linking the epidemiological event related to the animal with its geographical origin, defined by the date of the event. Also, since these events were linked to the DVPs, the same events could be reviewed through them. When showing these data in a cumulative manner (quarterly, annually), we could generate an ongoing 'snapshot' of the epidemiological situation, although the I & R of animals is still not implemented. Using these 'snapshots' the HVO can have precise idea on the epidemiological situation in the country, focus activities (human and financial resources) towards the most actual problems and monitor the results of control/eradication campaigns. Reporting and monitoring of outbreaks by prompt transmission of field data has also improved the capacity of the HVO to monitor up-to-date activities.

In case of LABIS, we have succeeded to systemise multiple events connected with the sample from the moment of submission through to issuing the final result. These events include testing of one or more samples (blood, tissues, liquids) from one or more animals against multiple diseases using multiple diagnostic methods. As described above, all the events related to a single disease are interpreted via the 'final result', which can be transferred to NEIS. This concept of data recording enabled us to link the final result to its history (performed tests) and offer evidence on the relevance of the established diagnosis. Additionally, it has improved many pre-existing organisational problems such as sample flow through the laboratory and communication between the designated laboratory/ies and the HVO.

In terms of sample flow through the laboratory, where the samples are analyzed, using which methods, which technicians are producing more results and which protocols have been used for

individual samples as well as which submission letters (samples) are pending can be easily monitored. This information is directly improving the organisational capacities of laboratory management, such as engagement of people, planning of purchasing and supporting ISO 17025 requirements by systematic data recording.

In terms of the communication between the designated laboratory and the HVO, the system is promptly reporting both the final results obtained to the HVO and the history (performed analyses). After transfer of the data to NEIS, the laboratory results can also be presented in tabular, graphical and/or mapping formats.

Based on current experiences, both systems have some problems to be solved in the field. In the case of NEIS, during the start-up phase there were many older VIs who were not familiar with using computers at all, although the entering forms were practically identical to the old, paper forms. This caused problems in entering of data into the system and was generally overcome by employments of younger (computer friendly) VIs. An additional and remaining actual problem is the lack of sufficient numbers of VIs in the field which results in an accumulation of duties to the existing once.

In case of NEIS, there are two major problems. The first problem is recording of sample IDs in the system during large scale surveys. The recording is still manual, and the temporary solution agreed with the HVO is to record only the IDs of positive samples, leaving negatives only with the number of tested samples linked to the submission letter.

The second problem is recording of results according to the ISO 17025:2005 standard (page 20, chapter 5.10.1), which requires the laboratory to keep the records even when the customer agrees to simplified reporting. This problem is temporarily solved by keeping the hard copies of submission letters and linking them to the recorded results from the database.

Development of the I & R has created a demand to synchronise the work of NEIS/LABIS according to the I & R. The I & R can record data using individual animal numbers or flock (holding) number. Both numbers are coded according to a certain algorithm which requires recognition by LABIS. However, animals having flock (holding) numbers can be either retagged using temporary individual numbers, or the flock can be given a 'flock' result. An additional problem is the animals which are not covered by I & R (wild/ free-living animals etc.) which will have to be tagged using uncoded numbers. Having these two types of numbers, LABIS should be reprogrammed to recognise and distinguish them.

As a solution to these problems, we are planning to build a 'between module' which will represent a communication between LABIS and I & R. Additionally, we plan to redefine the preparation of the submission letter to be filled at the DVPs, the idea being that after sample collection, the DVPs will have to submit the submission letter directly in a local LABIS database. These data will be transferred to the LABIS via the 'between module'. The veterinarians will print their own 'Certificate of Reception' and will bring the formatted racks directly to the reception desk at the FVMS. The employees of the FVMS will find the filled submission letter in the computer according to the unique ID of the Certificate of Reception and recheck the submitted samples using a barcode reader. Each record from the barcode reader should find its match in the database belonging to the defined unique letter ID. Correct matching will be proof that the samples are correctly submitted. This action will also solve the problems occurring during large scale surveys and fulfill the requirements of ISO 17025.

Finally, it should be mentioned that after development of the two softwares, a period of 1–2 years is required to find gaps and 'fine tune' the systems with user-defined queries and reports, which should be a subject of guarantee for the programmers.

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Novel and Rapid Technologies for the Early Diagnosis and Molecular Epidemiology of Viral Diseases

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ABSTRACT

Early and rapid identification of disease-causing pathogens, particularly those responsible for serious epidemic diseases, is a key element in the prevention of outbreaks and protection of susceptible populations. The detection of pathogen-specific nucleic acids has proven to be an invaluable tool in the diagnostic field. The advancement of technology involving the integration of amplification and signal detection systems has increased diagnostic capability, enabled development of robust, standard quantification techniques, as well as sequence analysis, by exposing products of the polymerase chain reaction (PCR) to a thermal gradient, i.e. melting and hybridisation curves. The most recent molecular technologies provide precision or broad detection facility, which is crucial to finding low level viraemia, distinct subtypes of interest, or mutants. In order for the development of new technologies and assays to proceed, a detailed knowledge of the diagnostic requirements is needed to create fit-for-purpose tools for the detection and discrimination of present and emerging diseases. The development of fit-for-purpose tools only makes sense if they can be transferred to, and applied in, appropriate laboratories and the field to provide reliable results using the most efficient methods for recognition of pathogens thereby allowing effective control measures to be employed as soon as possible. The prime objective in using these diagnostic tools should be to detect the pathogen at the earliest stage of the disease to prevent its spread.

INTRODUCTION

Currently, there are a number of factors that create an increased threat from emerging and re-emerging animal diseases (including 'unknown diseases'), and those that affect humans and seriously impact on a secure and safe food supply. Those factors include increased and more concentrated human and domestic animal populations (urbanisation and intensive husbandry practices), increased human and animal movement (both in frequency and geographically), including the globalised trade of live animals and animal products, bedding and feeds. Perhaps the biggest concern for food security is the outbreak of a pandemic event. Despite considerable efforts -— such as programmes for disease control, diagnostics, vaccination (conventional and marker) (Lubroth et al., 2007), regulation of animal transfer or movements and even stamping-out policies — animal diseases still continue to have a high bearing on animal health and human welfare.

Highly pathogenic H5N1 avian influenza (HPAI), and the pandemic H1N1 influenza ('swine flu') viruses are the most recent pathogens to present a global zoonotic hazard in terms of human health, with pandemic potential. These two pathogens each represent one half of a nightmare scenario. With a mortality rate, which may be greater than 50%, highly pathogenic H5N1 is truly a killer virus, while the 'pandemic H1N1', as the name and status given by the World Health Organization (WHO) indicates, possesses features of transmissibility that allowed it to spread around the world rapidly, with much lower mortality rate, as recorded so far. In addition to influenza, other diseases such as those caused by Hantaviruses, Japanese encephalitis virus, Human immunodeficiency virus (HIV), Dengue viruses, Menangle virus, Australian bat lyssavirus, Ebola virus, severe acute respiratory syndrome (SARS) coronavirus, Nipah virus and Hendra virus, also raise real concerns for human health, often in terms of their potential for global spread.

The globalisation of many aspects of animal production, from 'farm to fork', and the opening of borders between countries such as the European trading block, have created new risks and challenges for maintaining safe and stable food supplies. Even in the developed world, major disease epidemics have caused immense losses in the animal production sector. Foot-and-mouth disease (FMD), bluetongue (BT), and classical swine fever (CSF) have caused major problems throughout the EU. Rift Valley fever (RVF) moving into the Arabian Peninsula is another example of the significant economic and social consequences that result from the spread of highly contagious transboundary animal diseases (TADs).

Climate change is another factor with a global impact that is likely to affect the incidence, appearance and proliferation of viral pathogens, particularly arboviruses, whose lifecycle involves arthropods as vectors. Some examples that appear to support this predicted trend is the spread of BT virus and African horse sickness (AHS) virus into more northern regions, particularly in Europe (Schwartz-Cornil et al.,

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2008). Another vector-borne viral disease, African swine fever (ASF) has also spread outside of Africa to various regions of the world, including the recent spread into the Caucasus regions and beyond.

In addition to TADs, endemic viruses also have very high socio-economic impact. Pathogens such as bovine herpesviruses (BHV 1–5), bovine viral diarrhoea virus (BVDV), bovine respiratory syncitial virus (BRSV), porcine reproductive and respiratory syndrome virus (PRRSV), swine vesicular disease virus (SVDV), porcine circovirus type 2 (PCV2), among others, are found in cattle and swine populations around the world and activities designed to eradicate them require considerable inputs of resources. In fact, while the terms TAD and endemic are useful for classifying diseases they are not mutually exclusive. For example, CSF can be termed both as a TAD and as an endemic disease; it has been eradicated from domestic pigs in the EU, but wild boar populations are still infected in several countries of Europe. Therefore, this virus now poses a permanent danger to domestic herds with the likelihood of re-infecting them with a serious TAD. Another example is the arterivirus PRRSV; which was first detected and described in the Netherlands in 1991 and today is found around the globe.

The above examples clearly indicate that there is a need for reliable detection of targeted pathogens in the earliest phase possible of infection or in low copy contamination of various animal products or fomites. Although there are now well established techniques in molecular biology, nucleic acid amplification platforms such as PCR are relatively new compared with other diagnostic techniques. However, such amplification methods have proven to be unmatched in providing rapid and sensitive diagnostic tests, therefore the focus of this review is on thermal amplification platforms, their associated technology and the various detection chemistries employed. This review is intended to provide information on the current state-of-the-art and looks to future technological and molecular developments that can improve diagnostic capabilities for both human and animal populations.

One issue that is crucial in reaching the goal of producing effective diagnostic tools is the harmonisation and validation of new assays and technologies. In the environment of continual assay development and technological advancement (both simple and sophisticated), new diagnostic tools can only be widely adopted if they have been definitively shown to be effective and fit for their intended purpose. Furthermore, for early warning against many important livestock diseases, inexpensive and robust methods and devices are needed to cover the entire analytical process from sample extraction to reporting results to appropriate authorities. This review looks primarily at the field of biotechnology-based molecular diagnostic approaches.

TECHNOLOGY REVIEW

Application of Molecular Biology for the Diagnosis of Viral Diseases

Since the advent of nucleic acid amplification with the invention of the PCR in the mid-1980's, the development of diagnostic tools using molecular biology has exploded. This has been accompanied by a concomitant, parallel development in hardware technology, both simple and sophisticated. The most important driver of these advancements is the increased demand for secure and safe food, which includes the need to detect and control plant and animal pests and diseases. This technology review concentrates on the molecular diagnostic and characterisation technologies that are showing promise for providing diagnostic tools that deliver early and rapid detection of pathogens that threaten both human and animal health, as well as the food supply chain, under a range of circumstances from penside diagnosis in the field, through various levels of sophistication to well equipped diagnostic laboratories with skilled human resources.

PCR Platforms

Since the late 1980s, starting with gel-based PCR assays, a large variety of hardware technology and molecular chemistries have been applied to the diagnosis of pathogens including TADs and zoonoses in food, feed and fomites (Viljoen et al., 2005a). In gel-based systems, sensitivity and specificity may be increased by performing a nested PCR. This approach involves amplifying a region of the genome and then an inner region within the original amplified PCR product for another round of PCR. Real-time PCR has revolutionised molecular diagnostics, offering platforms that can readily be used in routine diagnostic laboratories as well as in research (Belák and Thorén, 2001; Belák et al., 2009). The list of real-time PCR platforms and their respective chemistries is extensive. Some examples include TaqMan[1] (the most widely used chemistry), molecular beacons (MB), primer-probe energy transfer (PriProET), linear-after-the-exponential PCR (LATE PCR), Scorpion primers, and SYBR Green. All these chemistries have been used to create highly sensitive and specific assays for the detection of pathogens (Belák, 2007). Some real-time PCR platforms are able to detect fewer than ten genome copies of the targeted viruses. Along with this very high analytical sensitivity, the majority of these platforms are able to amplify and detect exclusively the selected target nucleic acids. The chemistries all detect nucleic acids but the manner in which they do so differs at the molecular level. Some chemistries function by hybridising to a unique string of nucleic acids in the target genome, e.g. TaqMan and MB. The TaqMan assays rely on enzymatic degradation of a probe while in MBs there is a conformational change. SYBR Green chemistry functions as an intercalating dye which binds to any double-stranded DNA (Viljoen et al., 2005a). Sometimes co-development of assays makes sense in order to produce the best possible detection method (Gyarmati et al., 2007).

Real-time PCR provides sensitivity and specificity close to or equal to traditional nested PCR. Its value in the field of diagnostics comes from a number of important, well-proven advantages. For routine diagnostics, such as monitoring programmes, the main advantage is minimising the contamination risk posed by post-amplification products by measuring the amplified products in the reaction vessel, thereby reducing exposure to other samples or any other part of the outside environment. There are other significant advantages as well. In addition to providing a positive or negative result, by using a standard curve of known copy number the tests can give a quantitative estimation of the target nucleic acid. Pathogen load is often of interest but for certain pathogens it is crucial to the onset of disease as is the case in the diagnosis of PMWS in swine, where the viral load of porcine circovirus type 2 (PCV2) has to be determined (Segalés et al., 2005). As there is no requirement for gel preparation and staining, manual efforts are reduced. Automation of most of the process (extraction, preparation, amplification) is possible and with the use of a microtitre plate format, high throughput can be achieved.

A number of probes can be labelled with several different detection molecules, (i.e. fluorophores), so that multiplex PCR systems can be created. Multiplex PCR platforms use multiple primers to allow amplification of several different templates within a single reaction. This approach can be used to analyse a single nasal swab for a respiratory disease or a rectal swab collected from an animal suffering enteritis/diarrhoea syndrome. By performing multiplex PCR,

1 TaqMan is a registered trademark of Roche Molecular Sytems, Inc.

the detection specificity is broadened to detect all possible pathogens present that can be considered as agents for this particular disease syndrome. This is useful for detecting multiple targets as well as providing various internal controls that verify the assay is functioning properly. Compared with single-target PCR platforms, construction of multiplex platforms is more complex as the number of primer pairs increases (Viljoen et al., 2005a). Potential competition between the oligonucleotides can also be a hindrance to the development process, but with expertise and the use of bioinformatics and design software; sensitive and specific multiplex PCRs using dozens of primer pairs have been developed (Viljoen et al., 2005a). This level of multiplexing may not often be useful in real-time PCR platforms, but does allow microarray platforms to become a powerful tool at the diagnostic front-line (see below). That is not to say that real-time PCR platforms cannot effectively use multiple primers. For instance, LATE-PCR employs MBs read at different temperatures, allowing for double-digit primer pairs to be used effectively. Another multiplex technology, that uses an advanced gel-based system for detection has been put forward by Seegene Inc (Rockville, MD and Seoul, Korea). It has 16 capture elution (CE) marked products most of which include viral detection. One such assay is the Seeplex® RV12 affinity capture elution (ACE) detection assay designed to detect 12 major respiratory viruses (11 RNA and one DNA virus). For the veterinary field, the Seeplex® Porcine Diarr-V detection kit and Seeplex® Porcine Diarr-B detection kit are designed to detect five viruses and bacteria causing swine gastrointestinal diseases, including porcine epidemic diarrhoea. The general system used for these assays combines a novel chemistry using bipartite primers connected with a poly-inosine linker and conventional gel-based detection using amplicon size (Jun et al., 2008; Kim et al., 2008). Finally, although real-time systems have a higher up-front cost, when large sample numbers are to be tested, it provides lower costs per detected agent (Belák and Thorén, 2001).

The specification of the real-time PCR platform with regards to both hardware and chemistry is important, considering that the various equipment and chemistries have different strengths and weaknesses. For hardware, cost, reliability, maintenance requirements, flexibility, format (e.g. 96-well plate), speed, as well as other technical specifications like laser channels and filters, all have to be considered in deciding which platform is most suitable. For different chemistries, detection range, sensitivity, specificity, sensitivity to mismatch etc., can all affect the platform's results. It should be noted that at times, unexplained variation can occur within and between laboratories using the same platform (Belák and Thorén, 2001). It is crucial to be exact when describing a real-time PCR platform, chemistry, and assay in order to produce sound longitudinal and/or comparative data for routine or research purposes.

In reality, most molecular diagnostic laboratories generally employ a number of assays and it is not practical if all assays used are of different design or utilise different real-time PCR machines. Therefore, often it is not feasible for these laboratories to set up the recommended assays exactly as prescribed or to choose what may be the absolute best assay. As an example, the routine molecular diagnostic laboratory at the National Veterinary Institute (SVA) in Sweden is relatively small, running less than 20 000 assays/year (the Animal Health Service, GD, in the Netherlands runs at least ten times as many); however, SVA still has dozens of assays in use. Since the pathogen threat list is essentially decoupled from throughput, smaller laboratories must still be capable of testing for many pathogens. For the purposes of monitoring and early warning, it is often not wise to set up assays *ad-hoc*, based on perceived risks. Generally, diagnostics must be in place for all pathogens that are likely to present a risk. Recently at SVA, the molecular diagnostic laboratory has adapted virtually all of its assays to identical reaction conditions. This allows several different assays to be run simultaneously, thus creating a very efficient and economical work scenario. This is possible due to the combination of the robust TaqMan chemistry and a new molecular diagnostic kit that enables all PCR assays to be run using one thermal cycling routine.

Another strategy employed by smaller laboratories is to split up responsibilities for less common tests among laboratories. This can compromise the strategy of providing the earliest diagnosis possible but may be the most viable option when considering the economics of maintaining reagents for assays that may only be needed a few times in a year within a large region. Clearly therefore, in the real world, the ideal will usually not be possible. Adaptation to different nucleic acid extraction methods, real-time PCR machines and kits will always be the case. This illustrates the importance of reference laboratories for aiding proficiency testing, coordinating ring trials, and maintaining reference sample banks. Laboratories will often not be using the exact same testing procedures but it is essential that the various modifications still give comparable assay characteristics.

Automated Sample Preparation

Effective molecular diagnostic assays are dependent on the efficient processing of samples to extract nucleic acids (Viljoen et al., 2005a). This process can be difficult and varies depending upon the nature of the sample. Many sample matrices contain elements that can inhibit PCR to varying degrees and this in turn affects the effectiveness of the assay. When there are many samples, the extraction of nucleic acids can delay the throughput of samples and the analysis process. The use of nucleic acid extraction robots is common in both routine and research laboratories. Molecular diagnostics has evolved in a similar way to ELISA-based diagnostics, using automated systems for most of the procedures from sample processing to analysis. This allows laboratories to provide rapid, robust, low-cost diagnostics with improved reliability and less chance of contamination. Extraction robots generally function on two basic premises, using either filter- or magnetic bead-based extraction protocols. Examples of the latter include the Magnatrix 8000[2] and the more recent Nordiag Bullet[3] and the QIASymphony SP[4]. Larger robotic platforms can typically purify nucleic acids from 96 samples within 3–4 h (Belák and Thorén, 2001; Belák, 2007). Pipetting robots can prepare the PCR mixture and add the sample; other automated components can feed assays into the analytic machines.

Alternative Platforms to Real-Time PCR

Microarray technologies allow many specific sequences to be analysed simultaneously. They exist in several formats and they all allow many nucleic acid sequences to be identified through the use of complementary probes. The first microarray-based kit approved by the Food and Drug Administration (FDA) of the USA for use in the EU was the AmpliChip Cytochrome P450 Genotyping Test (Roche Molecular Systems Inc. Pleasanton, Calif.) in 2008. The assay uses a solid phase, planar microarray format and was designed to identify dozens of variants of the genes cytochrome P450 2D6 and cytochrome P450 2C19 in patients to help determine their proficiency in drug metabolism. Microarray-based diagnostics has now expanded into the field of viral diagnostics, aided by new array technology which is more suitable for use in diagnostic laboratories. The traditional microarray

2 NorDiag AB, Sweden

3 NorDiag AB, Sweden

4 QIAGEN Inc., Gaithersburg, MD, USA

Figure 1. Clondiag Chip technolgies ArrayTube (AT) platform. From left to right: array tube (with probe array chip on the bottom of the tube), the ATR 03 reader (connected to a PC or laptop to run image acquisition and analysis), magnification of probe array chip (detection reaction consists of horseradish peroxidase and HRP-substrate tetramethylbenzidine which reacts to form a blue precipitate.

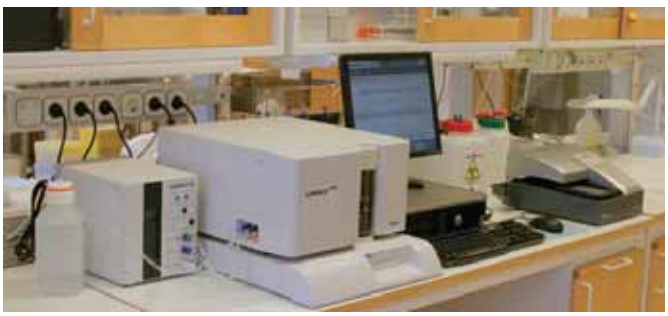

Figure 2. Luminex 200 system including from left to right: waste bottle, the Luminex SD sheath fluid delivery system the Luminex 200 instrument (above), the Luminex XY platehandling platform (below), Luminex xPONENT 3.0 software and a PC.
The microsphere-based detection system allows for 100 different analytes to de detected in each sample (100-plex). Also pictured (far right), is the Tecan Hydroflex wash station with buffer and waste bottles. This instrument performs automated microplate washing and vacuum filtration for use with both bead types (magnetic and polystyrene) used in the Luminex system.

format uses spotted glass slides and is not particularly user friendly. The company, CLONDIAG chip technologies GmbH (Jena, Germany) has produced the ArrayTube and ArrayStrips systems which use a planar array printed on the bottom of custom sample tubes or strips and read in their specialised workstations (**Figure 1**). The UK's Veterinary Laboratories Agency has developed a test based on the microarray technology or analysing antimicrobial resistance or virulence in a range of different bacteria (www.identibac.com). Examples of this technology applied to viral diagnostics include the detection and subtyping of avian influenza (Gall et al., 2008) and the detection of herpesvirus and adenovirus co-infections (Müller et al., 2009).

Another multiplex system, which has entered the field of viral diagnostics uses nucleic acid probes which are covalently linked to microspheres instead of attached to a solid surface, thus the microarray is in liquid suspension. This technology was brought to the market by the Luminex Corporation (Austin, Texas, **Figure 2**). In this system, nucleic acids are coupled to coloured microspheres creating up to a 100-plex assay on the Luminex® 200™. A 500-plex instrument is currently in the final stages of commercial development. The technology is an open platform with 'naked beads' available to be coupled to custom oligonucleotides (Dunbar, 2006). This technology can also be used to detect proteins (de Jager and Rijkers, 2006). The xTAG Respiratory Viral Panel (RVP) (Mahony et al., 2007) received 510(k) clearance from the U.S FDA in 2008 and is also CE marked for use in EU. This test was FDA cleared for 12 viruses and subtypes and CE marked for 19. The xTAG RVP is the first multiplexed nucleic acid test for respiratory viruses cleared for *in vitro* diagnostic use by FDA, the first test the FDA cleared to detect human metapneumovirus, to subtype influenza A, and to detect adenovirus.

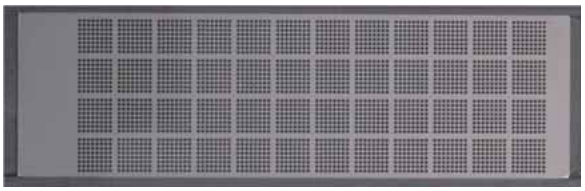

Figure 3. BioTrove OpenArray platform plate. A plate consists of 48 sub-arrays each containing 8 × 8 through-holes (wells) for a total of 3 072 reaction chambers per plate. Three plates can be read in the NT instrument (not shown) for a total of 9 216 simultaneous real-time qPCR results.

Besides probe-based methods, there are other technologies being employed in viral diagnostics including surface plasmon resonance (SPR) and mass spectrometry. The IBIS T5000 Universal Biosensor system uses mass spectrometry on PCR products to derive base compositions (Ecker et al., 2008; Blyn et al., 2008). Microfluidic platforms such as droplet-based PCR are a recent technology for high-throughput parallel PCR analysis, which allows for millions of discrete picoL reactions (Williams et al., 2006). The BioTrove OpenArray system[5] is a low volume, multi-well, PCR based system using nano-volume reaction mixes. Using a standard PCR cycling protocol, it produces 3 072 simultaneous PCRs in 33 nL reaction volumes in one plate (approx. dimensions of conventional microarray slide). The plate composition consists of 48 sub-arrays each with 64 through-holes or wells (**Figure 3**). The OpenArray plate can be composed in two ways: the wells can be spotted with primers or they can be empty with primers being added as part of the reaction mix. The cycling instrument (NT cycler) can run up to three plates simultaneously (9 216 individual PCR assays) and monitors the reaction progress in real-time, using either SYBR Green or TaqMan chemistry.

Alternative Chemistries to Polymerase Chain Reaction (PCR)

Methods such as Invader[6], nucleic acid sequence based amplification (NASBA) or loop-mediated isothermal amplification (LAMP) technologies, which use isothermal conditions in the amplification process instead of varying temperature amplification methods (as in PCR) are becoming more accepted (Hjertner et al., 2005; Blomström et al., 2008). For example, the LAMP method is simple and rapid, being performed in less than one hour.

A one-step reverse transcriptase LAMP (RT-LAMP) assay was developed recently to improve the detection of swine vesicular disease virus (SVDV). In this method, a set of six specially designed primers targeted eight distinct sequences of a target gene. The assay detected all 28 isolates tested in the developmental phase. Clinical

5 Life Technologies Corporation, Carlsbad, California

6 Invader is a registered trademark of Third Wave Technologies, Inc

Figure 4. BioSeeq Portable Veterinary Diagnostic System. Pictured left: portable PCR instrument with five independent thermocyclers (running assay in far left thermocycler) with built-in communications including GPS, WiFi and Bluetooth technology. Picture right: disposable automated sample preparation unit (SPR) processes raw sample into PCR mixture allowing test to be conducted at pen-side.

samples from nasal swabs, serum and faeces were used to evaluate the performance of the RT-LAMP compared with real-time PCR assays. The results from nasal swabs and serum were not significantly different from the TaqMan assay but with faecal samples the RT-LAMP assay performed significantly better than real-time PCR (Blomström et al., 2008).

A novel system for nucleic acid detection using Zinc finger proteins has been developed (Osawa et al., 2008). Zinc finger proteins are DNA-binding proteins that can bind to double stranded (ds) DNA with high affinity and specificity. They directly detect PCR products and check for specific PCR amplification. This has been used to detect simultaneously three pathogens: *Legionella pneumophila, Salmonella spp*. and Influenza A virus (Osawa et al., 2008).

Technology in the Field

Portable PCR technology is now coming to the marketplace. Companies are producing battery-powered machines that are simple to operate, work under field conditions, can be disinfected and have simple sample preparation methods. They are designed to bring laboratory facilities and the ability to make diagnosis closer to field cases and disease outbreaks (Viljoen et al., 2005b). For example, several companies (Smiths Detection[7], DxNA[8], Qiagen[9]) have produced portable devices and technology platforms specifically for field veterinarians. The system consists of a briefcase-sized PCR instrument, a disposable sample preparation unit and LATE PCR chemistry. It provides on-site identification under a wide range of weather conditions and the operator requires no technical training in PCR methodologies (**Figure 4**). Another company that has entered the high-tech side of portable diagnostics is UK-based Enigmadiagnostics (Salisbury, UK) whose Enigma FL instrument has been tested successfully by the UK Veterinary Laboratory Agency.

Some isothermal methods, such as LAMP assays have several features that support their use for penside diagnosis as they can be performed in modestly equipped field laboratories. Isothermal amplification requires a simple thermal block, can be obtained within 30–60 min, is highly specific and sensitive and the result can be assessed either by gel electrophoresis or directly, visually through the addition of SYBR Green. There are also several companies that produce low tech portable PCR lateral flow devices, which are designed to detect amplified nucleic acids using simple and cheap dipsticks, similar to pregnancy tests. These companies generally do not provide the entire system but there are firms which sell the devices e.g. BESt cassettes, Biohelix Corp (Beverly, MA, USA); production equipment, BioDot Ltd. (Chichester, UK) and even aid in the construction of portable systems such as Diagnostic Consulting Network Inc. (Carlsbad, CA, USA).

Multiplex methods are also being developed for use in field laboratories. A novel assay has been developed for the detection and discrimination of pestiviruses, i.e. BVDV types 1 and 2, CSFV and BDV using magnetic bead detection of PCR products in microarrays. In this method the PCR products are hybridised onto an array, followed by visualisation with streptavidin-coated magnetic beads for visual inspection or microscope examination. This assay was evaluated using a panel of pestiviruses comprising members of all four accepted species and performed as well in the ring trial as real-time PCR. This magnetic bead-based assay offers a novel technology for multiplex molecular diagnostics in virology (LeBlanc et al., 2009).

Molecular Methods for Monitoring Disease Epidemiology

Specific DNA products yielded by different chemistries can be analysed, sequenced and characterised. This means that exact information can be derived and stored in databases to enable comparison among samples. Large international databases such as GenBank, are powerful tools that aid in the phylogenetic identification, classification, and tracing of pathogen evolution and spread. For example, it is now hypothesised that EU and US genotypes of the porcine respiratory and reproductive syndrome virus (PRRSV) evolved from a common ancestor that was suspected to have originated from Eastern Europe (Stadejek et al., 2002; Ståhl et al., 2005 and 2007). In terms of early warning, molecular epidemiology can be combined with simple detection methods to better monitor potential health risks. For instance, influenza is a virus where in-depth sequence data could be very useful. If swine populations were better monitored with detailed analysis performed at routine intervals, then perhaps the current Influenza A H1N1 ('swine flu') pandemic could have been avoided.

Standardization and Validation according to OIE Standards

The increasing tendency to place molecular diagnostic assays at the forefront of routine analysis, combined with the tendency towards 'in-house' assay development and modification, have made it imperative to ensure international standardization and validation of developed assays. It is crucial to the success of these technologies that validated techniques exist to ensure they are fit-for-purpose and that results can be compared among laboratories. This approach provides the best prospect for the successful control of TADs and endemic diseases on a broad scale, while following the 'One world, one health' principle. Authorities, both national and international, require proof that assays used in laboratories are as reliable as possible (Belák and Thorén, 2004). International agencies such as the OIE and FAO, national diagnostic laboratories and research institutions as well as commercial companies have their own requirements and it is important that all of them work together on the international standardisation of protocols. The OIE has the lead role in this activity by publishing standards for the validation of diagnostic assays

7 Smiths Detection, Watford, UK

8 DxNA LLC. Saint George, UT, USA

9 QIAGEN Inc, Valencia, CA, USA

(http://www.oie.int/vcda/eng/en_background_VCDA.htm?e1d9) and publishing these in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2008 (http://www.oie.int/eng/normes/manual/A_summry.htm).

Whole-genome Sequencing

The design of suitable molecular assays will rely increasingly on available bioinformatics since sequence data are required for suitable probe and primer design. Whole-genome shotgun sequencing together with powerful computational algorithms to facilitate sequence data assembly, gene prediction and functional annotation have played important roles in this process. Primers designed to target conserved sequences of bacteria e.g. rRNA have allowed for the development of broad spectrum PCR for detecting bacteria (Picard and Bergeron, 2002). The variable internal rRNA gene regions can be sequenced and the data obtained compared with sequences from databases to make potential identifications at the species level, for contributing to epidemiological studies or for strain typing purposes, so as to determine traits such as antimicrobial resistance and virulence. Sequence data from 140 viruses have been used to design long oligonucleotide DNA microarrays with the potential of simultaneously detecting hundreds of viruses (Wang et al., 2002).

Bioinformatics, Comparative Analysis of Sequence Data

Many challenges also exist regarding the storage, analysis, management and integration of generated data. In the case of microarrays, pattern recognition tools are becoming increasingly important not only for direct pathogen detection, but also for analysing expression profiles from infected cells (Stenger et al., 2002). Methods of improving internet access to vast amounts of biomedical literature, using improved search engines and data mining programmes will also make important contributions to bioinformatics.

Internet and wireless communications technologies are also being harnessed to facilitate diagnostic systems (Cranfield Centre for Analytical Science Institute of BioScience and Technology, Cranfield University). Data that have been collected remotely can now be readily transferred to a central application for processing. Broadband internet, mobile phones and wireless communications technology among others, will allow the development of fully integrated, distributed applications across the internet.

Detection of Emerging, Re-emerging or 'Unknown' New Viruses

Various methodologies including random amplification followed by partial or full-genome sequencing, have been developed and are under development with the intention of discovering emerging and/or new viruses in humans and animals (Ambrose and Clewley, 2006; Delwart, 2007). The increasing availability and use of new sequencing technologies such as the 454 (Roche), SOLID (Applied Biosystems) and Solexa (Illumina) are accelerating this approach, which is becoming a frequently used tool to study different metagenomic issues (Shendure and Ji, 2008; Bosch and Grody, 2008).

In the development of 'discovery' technologies to identify pathogen genomes that no-one has identified before the group of Ian Lipkin, has played a pioneer role at the Columbia University's Mailman School of Public Health. This group has been working on technologies focusing on viral discovery since the late 1980s, being among the first researchers to identify microbes using only molecular tools. The team has identified close to 200 new viruses so far (see details and future ideas in recent summaries and discussions [Lipkin, 2009; Westley, 2009]). Other groups are following suite - Blomstrom et al. (2009) recently reported the detection of a novel porcine bocalike virus in the background of porcine circovirus type 2 viruses that induced a postweaning multisystemic wasting syndrome.

Biosensors

Biosensors consist of a biological recognition element in intimate contact with a transducer. The latter may include amperometric and potentiometric electrodes, field-effect transistors, magnetoresistive sensors, piezoelectric crystals, optical and optoelectronic devices, as well as miniature cantilevers. Developments in DNA-based biosensors are one of the fastest growing areas in nucleic acid analysis. Nucleic acid probes are used as the biological recognition element although ion channels have also been proposed for detecting base pair composition of DNA, with changes in electron current monitored according to the degree of pore blockage by different bases as single-stranded (ss) DNA is pulled through (Meller et al., 2000; Kristensen et al., 2001; Scheller et al., 2001). Electrochemical methods include the voltametric detection of redox intercalators and the mediated oxidation of guanine within the DNA. One format utilises signalling probes labelled with the redox agent ferrocene which bind to the target DNA previously immobilised on a capture probe embedded in a self assembling monolayer coating a gold electrode (Umek et al., 2001). Alternatively, the hybridisation event can be amplified using an enzyme label followed by monitoring of impedance. The conductive properties of a DNA duplex for electrons or holes can allow for monitoring using a redox probe at one end and the electrode on the other. Charge transfer is influenced by distortions with mismatches during hybridisation. In the future, ultraminiaturised electrochemical DNA sensors should allow for online monitoring and analysis. Electrochemical methods in combination with microfabrication techniques are likely to play important roles in providing highly sensitive assays.

The Bead ARray Counter (BARC) uses DNA hybridisation, magnetic microbeads, and giant magnetoresistive (GMR) sensors to detect and identify biological warfare agents. The current prototype is a table-top instrument consisting of a micro-fabricated chip (solid substrate) with an array of GMR sensors, a chip carrier board with electronics for lock-in detection, a fluidics cell and cartridge, and an electromagnet. DNA probes are patterned onto the solid substrate chip directly above the GMR sensors, and sample analyte containing complementary DNA hybridises with the probes on the surface. Labelled, micron-sized magnetic beads that specifically bind to the sample DNA are then injected. A magnetic field is applied, removing any beads that are not bound to the surface. The beads remaining on the surface are detected by the GMR sensors, and the intensity and location of the signal indicate the concentration and identity of pathogens present in the sample. The current BARC chip contains a 64-element sensor array. With recent advances in magnetoresistive technology, however, chips with millions of these GMR sensors will soon be commercially available, allowing simultaneous detection of thousands of analytes (Edelstein et al., 2000).

Piezoelectrical sensors are quartz crystal acoustic sensors that detect changes in mass on the crystal surface and would thus detect DNA-DNA hybridisations. The thickness shear mode type acoustic model is now being used especially for biomedical analysis (Pavey, 2002). Surface plasmon resonance (SPR) allows for real-time monitoring of binding between nucleic acid target and probe on the surface of a gold-coated prism. SPR monitors accumulating changes in surface mass following DNA-DNA hybridisation and can be used to confirm the specificity of a PCR reaction (Bier et al., 1997; Caruso et al., 1997). Single-stranded DNA obtained following asymmetric

PCR has been used to hybridise with a biotinylated probe attached to the sensor surface (Bianchi et al., 1997). Cantilevers coated with receptor layers act as force transducers and are being used in micro-fabricated biosensor devices (Oak Ridge National Laboratory [http://www.ornl.gov]; Graviton Inc. [http://www.graviton.co.jp]; Protiveris Inc. [http://www.protiveris.com]; Cantion A/S [http://www.cantion.com IBM, http://www.ibm.com]). Changes in surface stress, temperature and magnetisation can be monitored following receptor-target binding, such as DNA hybridisation. Monitoring is done using optical lever, interferometry or beam-bounce techniques. In the case of inter-digitated cantilevers, a diffraction pattern is monitored in accordance with cantilever deflection. Capacitor plates or peizoelectrical cantilevers can be used to monitor changes in capacitance or conductivity in response to surface stresses (Bianchi et al., 1997). Silicon nanowires (Nanosys, CA, http://www.nanosysinc.com) and carbon nanotubes (Molecular Nanosystems, CA, http://www.monano.com) have been described which can monitor changes in conductance during binding of biological molecules to the surface (Alivasatos, 2001). DNA labelled with gold nanoparticles can be used as probes on chips that can then be monitored electrically. Carbon nanotubes, molecular transistors and switches including allosteric and ribozymal nucleic acids, have exciting contributions to make as components of biosensors (Soukup and Breaker, 1999).

Microfabrication, Microfluidics and Integrated Systems

The integration of sample processing, amplification and detection systems is an important goal in achieving suitable point-of-care with on-site devices. Several partly integrated systems have been developed for the detection of biowarfare agents. The Lawrence Livermore National Laboratory and University of California, Davis (LLNL-UC Davis) consortium have developed a self-contained system that continuously monitors air samples and automatically reports the presence of specific biological agents. The LLNL Autonomous Pathogen Detection System (https://ldrd.llnl.gov)is an integrated aerosol collector, sample preparation and detection module that detects and identifies pathogens and/or toxins by a combination of an immunoassay and PCR. Nanofabrication and molecular electronics are being used by Nanogen (http://www.epochbio.com) to develop various sample-to-answer devices.

Micro-fabricated devices are being used to perform PCRs for faster cycling using small chambers and integrated heaters. A variation of this approach uses the well of a microchip as the PCR chamber, with the entire chip undergoing thermal cycling. Electrophoretic separation at the level of the wells of the microchips can be achieved by transferring samples between a PCR chamber and a well. The PCR has also been integrated with detection using fluorogenic DNA probes. Most integrated systems combine the processing and detection phases using a microfluidic platform. Microfluidic systems consist of microchannels and tiny volume reservoirs and utilise electrokinetic or pneumatic mechanisms to transport fluids. The flow rates are in the nL/sec range through flow channels with cross-sectional dimensions in the tens of μ meters. Advantages include improved speed of analysis, reproducibility, reduced reagent consumption and the ability to perform multiple operations in an integrated fashion. Further development of this technology is expected to yield higher levels of functionality of sample throughput on a single microfluidic analysis chip.

Integrated systems using microfluidics are usually termed 'lab-on-a-chip' technologies. Although still in the developmental phase, they are likely to make dramatic impacts on molecular diagnostics in the future, especially as point-of-care devices with important contributions from nanotechnology.

Cepheid (Sunnyvale, CA, USA) has developed a device that consists of disposable cartridge-containing reagents and chambers for bacterial cell lysis and test sample preparation for fluorescent-based nucleic acid detection in a device called GeneXpertTM. Infection Diagnostic developed a rapid sample preparation method for both Gram positive and negative bacteria in different sample specimens. Lin et al. (2003) constructed a micro-fabricated device for separating and extracting ds DNA fragments using an array of microelectrodes and a cross-linked polyacrylamide gel matrix that is amenable to integration with reaction chambers into a single device for portable genetic-based analysis.

Microfluidic-based laboratory card devices have resulted in credit card-sized designs suited for processing whole blood for haematological applications, and involves flows of sample reagents and control solutions in microchannels using capillary flow, hydrostatic pressure and fluid adsorption (Micronics Inc. WA, http://www.micronicsinc.com) (Bousse et al., 2000; Cronin and Mansfield, 2001). Optical microchips have also been devised whereby a microarray of optical scanning elements is integrated with microfluidic circuits (Ruano et al., 2003). These allow for fluorescence detection and have the potential to provide other optical biosensor platforms such as SPR, evanescent field technology and interferometry.

DISCUSSION AND CONCLUSIONS

Given the socio-economic impact of infectious diseases in human and animal health today, there is clearly a demand for highly sensitive and robust diagnostic techniques which facilitate the early detection of the dangerous pathogens and assist in the immediate implementation of disease control measures. Globalisation and extensive international trade have created a scenario where infectious agents have the potential for global spread within hours or days, causing serious epidemics. It is extremely important to apply techniques, which can detect pathogens rapidly and effectively. Advances in real-time PCR methods, such as PriProET and LATE PCR are providing new opportunities to provide the best validated, fit-for-purpose assays. Portable PCR machines and isothermal amplification techniques such as NASBA and LAMP, and LFDs allow the application of PCR for 'point-of-care' or 'on-site' detection of pathogens. 'On-site' tests provide powerful early warning tools to the authorities responsible for prevention and control of infectious diseases; however, in general and especially in the case of important pathogens such as TADs, the results from on-site tests have to be confirmed in the laboratory. Microarrays allow a more complex investigation of pathogens. By combining a wide range of highly specific target sequences, a detailed investigation of the pathogen responsible for the disease outbreak is possible. Microarrays can simultaneously detect several agents or even a high number of pathogens, co-infections, subtypes and pathotypes, providing information rapidly so that appropriate actions can be taken based on a detailed understanding of the disease status in affected population(s). Microarrays, especially liquid-phase systems, are also amenable to automation and the use of high-throughput platforms.

Other detection methods, which are still at the more developmental stages include surface plasmon resonance and matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (Sampath et al., 2007; Jores et al., 2009). Advances in biosensors, the development of integrated systems such as lab-on-a-chip devices and enhanced communications systems are all promising. No doubt, some of these newer technologies will provide valuable early warning tools in the future.

In addition to the control of disease outbreaks, the need for safe tracing, identification and testing of various pathogens in food and feed products is also increasing. Furthermore, for zoonotic diseases, transmission of pathogens between various hosts in animal and human populations necessitates extensive monitoring programmes. The appearance of 'unknown' or 'completely new' pathogens creates new epidemiological situations that rapidly cover large regions of the globe (e.g. causative agents of SARS, H5N1, H1N1 'swine flu', pandemic simian immunodeficiency virus [SIV]). In this area, the most advanced technologies available will be required to provide the most complete analysis possible (e.g. full-genome sequencing).

With the continual risks from transboundary and endemic diseases which are compromising food safety, and also the risk posed by zoonoses, the 'One World, One Health' approach is imperative; particularly today, in the current environment of intense globalisation, international trade, global tourism, and climatic changes as well as financial, energy and political instability. Diagnostic research laboratories will be expected to improve detection capabilities by developing novel biotechnology-based diagnostic techniques, some of which have been outlined in this article. Finally, although not a subject of this article, the need to complement new techniques with the maintenance of classical ones is vital. The devices and technologies described in this article enable ever more efficient and speedy diagnosis without the user needing to process live pathogens in the laboratory, but it is in danger of creating a generation of scientists whose knowledge of the actual organism is seriously deficient. Classical methods in virology and bacteriology, with special regards to virus isolation in cell culture, and *in vitro* and *in vivo* characterisation are in jeopardy of being lost as laboratories replace them with modern technology. This is very short-sighted considering the likely impact on science if laboratories lose the ability to isolate and cultivate different strains of pathogenic micro-organisms, particularly those causing newly emerging diseases, where it is necessary to understand their pathogenesis, develop diagnostics, perform repeatable experiments, produce vaccines as well as study many other aspects of infection biology.

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Molecular Detection Technologies for Arboviruses

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ABSTRACT

Arthropod-borne animal viruses (arboviruses) cause significant livestock and economic losses to world agriculture. This paper discusses the current and potential impact of these viruses, as well as the current and developing molecular diagnostic tools for those emerging and re-emerging insect transmitted viruses affecting livestock and wildlife. The emphasis is on viruses for which there have been significant recent outbreaks in livestock including: bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), vesicular stomatitis virus (VSV), and Rift Valley fever virus (RVFV). The current readiness for rapid detection of arboviruses is fairly high, but there is a need for global harmonisation and continued evaluation due to the genetic variation of these unique pathogens. The tool chest for molecular detection contains a range of assays from low technology to high-throughput sophisticated devices.

Key words: *Arbovirus, bluetongue, Rift Valley fever, detection.*

INTRODUCTION

Arthropod-borne animal viruses (arboviruses) are of increasing concern to both veterinary and public health due to the recent expansion of these viruses into new areas. The introduction of West Nile virus into the USA initiated this growing concern (Lanciotti et al., 1999). The danger of the spread of arboviruses into new geographic locations was also unfortunately realised by the introduction of bluetongue virus (BTV) serotype 8 into northern Europe (Thiry et al., 2006; Toussaint et al., 2006). This has resulted in tremendous efforts to understand the epidemiology of the disease in Europe as well as detect and control the spread of the virus. Biting midges of the genus *Culicoides* transmit BTV and the related epizootic haemorragic disease virus (EHDV). These viruses cause sub-acute to lethal disease in cattle, sheep, goats and/or wild ungulates, the resulting worldwide losses attributable to BTV alone being estimated at $3 billion annually. There was a fairly good understanding of the epidemiology of BTV until the recent introduction of BTV into Europe. Of particular concern is the economic and unique disease impact that BTV–8 has had in Europe and the fact that there have been multiple isolations of exotic BTV serotypes in the USA over the past 3 years. In Europe, killed BTV–8 vaccines are being used to control and potentially eradicate the disease (Mintiens et al., 2008). In the USA, there is only one commercial vaccine available, and it is specific to BTV type 10. There is limited or no cross-protection between serotypes, thus complicating the control of the disease. The related orbivirus, EHDV, is of considerable interest to the captive cervid industry, and EHDV serotype 7 has been associated with clinical disease in Israeli cattle (Yadin et al., 2008) A number of assays are available for detection of viral RNA using reverse transcriptase-polymerase chain reaction (RT-PCR) genome amplification for BTV and EHDV (Wilson 1994; Shad et al., 1997; Aradaib et al., 1998). Additionally, real-time RT-PCR (rRT-PCR) assays are available to detect all BTV serotypes (Shaw et al., 2007; Toussaint et al., 2007) and all EHDV serotypes (Wilson et al., 2009b). This report summarises a multiplex assay developed to detect BTV and EHDV and distinguish between the two viruses in a single closed tube (Wilson et al., 2009c).

There are periodic outbreaks of vescicular stomatitis virus (VSV) in the USA presumably introduced by insect vectors from enzootic regions of Mexico (Rodriguez et al., 2000). *Culicoides*, black flies, and sand flies transmit VSV to cattle and horses (Drolet et al., 2005). Insects are believed to play an essential role in transmitting the virus to domestic livestock. Once initial infection has occurred, direct contact transmission is believed to be another route of infection. Humans associated with infected livestock can become infected resulting in mild to severe febrile illness. The clinical severity of VSV, and its similarity to clinical foot-and-mouth disease (FMD), results in quarantines, sale barn closures, and restrictions on the movement of livestock and animal products. As with BTV, there are a number of standard diagnostic tools available for detection of VSV, including a recently developed multiplex rRT-PCR for detecting and distinguishing VSV Indiana from VSV New Jersey (Rodriguez et al., 2009; Wilson et al., 2009a).

Recent outbreaks of Rift Valley fever (RVF) in East Africa have raised worldwide concerns of the potential spread and disease impact of this virus, which can cause high mortality in young small and large ruminants and abortions in older animals. This is a zoonotic virus resulting in mild to lethal disease in humans. Retinal degeneration has been reported in up to 10% of infected humans. Few veterinary diagnosticians are immunised with the expensive investigational vaccine to allow them to work safely with this virus. Therefore a rapid, sensitive, and specific diagnostic tool such as a molecular amplification technology that quickly inactivates the virus during sample processing is ideal. In addition, diagnostic assays are being developed to differentiate infected from vaccinated animals (DIVA). A multiplex

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real-time RT-PCR for all three virus segments is in development to provide a robust and DIVA compatible assay for RVF.

MATERIALS AND METHODS

The general approach to real-time PCR (rRT-PCR) design was to select specific gene target(s) based on what is known about the molecular biology of the arbovirus. If insufficient sequence information was available, then sequencing of appropriate diverse virus populations was done. The sequence information was submitted to various bioinformatic analysis tools to select potential real-time RT-PCR signatures (primer and probe combinations). General descriptions of the assay compents are provided below, but specifics can be found in the reference cited for each virus.

RNA Extractions

Three RNA extraction systems were applied to the various arboviruses. The first systems used phenol-based extractions such as Trizol (Invitrogen Corporation, Carlsbad, CA), TriReagent (Applied Biosystems/Ambion, Austin, TX) and TriPure (Roche Applied Diagnostics, Indianapolis, IND). The second method used was a spin column system (Qiagen Inc. CA). The final system used was magnetic bead-based (Applied Biosystems/Ambion, Austin).

PCR Procedures

Amplification was first confirmed using standard SYBR green RT-PCR reagents (Applied Biosystems/Ambion, Austin). Once amplification was confirmed then dual-labeled probes were obtained (Biosearch Technologies, Inc., Novato, CA; Integrated DNA Technologies, Inc., Coralville, IA). Standard one-step real-time RT-PCR reagents were used for the TaqMan® assays (Applied Biosystems/Ambion, Austin, TX).

RESULTS AND DISCUSSION

Bluetongue virus continues to be an important arbovirus of livestock and the recent introduction of BTV–8 demonstrates the need for continued diagnostic advances. The sequence analysis performed to develop the BTV/EHDV multiplex real-time RT-PCR demonstrated a greater sequence variability in the target genes than previous studies predicted (Wilson, 1994). The target genes were selected based on previous phylogeny studies (Wilson, 1994), but it was found that the sequence information at the time was insufficient to design universal primer sets for BTV (Wilson et al., 2004). Additional sequence data from the prototype strains was generated and a bioinformatics approach was used to design a more robust rRT-PCR assay that rapidly detects and distinguishes between BTV and EHDV strains. All of the RNA extraction methods commercially available worked with the assay, but the magnetic bead extraction afforded the best sensitivity. This was estimated using spiked blood and found to be 10 $CCID_{50/ml}$ for BTV and 1 $CCID_{50/ml}$ for EHDV (Wilson et al., 2009c).

Vesicular stomatitis virus is important not only because of the severe, but usually non-lethal disease it can cause, but because the clinical signs in cattle and swine resemble that of FMD. As with BTV/EHDV, a bioinformatic approach based on phylogenetic studies was used to develop a robust rRT-PCR design (Rodriguez et al., 2009). Since there are multiple serotypes of VSV; the assay was designed to detect and distinguish between VSV New Jersey and VSV Indiana, the most prevalent serotypes. The VSV multiplex assay performed very well using both phenolic and column-based RNA extraction methods in the laboratory. Field evaluation was performed using the column-based RNA extraction method to be consistent with the FMD assay developed previously (King et al., 2006). The field evaluation was performed in endemic countries and a distinct genetic VSV population in Costa Rica was found not to be detectable by the assay. Additional sequence analysis was performed and a new primer/probe design developed that resulted in near 100% sensitivity and specificity. This assay has also been successfully applied to experimental VSV infection studies of insects (Mead et al., 2009).

The 2006–2007 outbreak of RVF in Kenya, Tanzania and Somalia has resulted in increased research efforts for early detection and control of this deadly and zoonotic virus that is associated with high abortion rates in livestock. Three RT-PCR assays have been developed for RVF (Garcia et al., 2001; Drosten et al., 2002; Bird et al., 2007). These assays all target different segments of the tripartite genome. Two of the assays were shown to be effective with clinical samples (Bird et al., 2007; Njenga et al., 2009). We have applied these assays to samples generated in experimental virulent and vaccine RVF strain infections of sheep, cattle and mosquitoes. The three available commercial extraction methods worked well with these assays. The choice of extraction method is primarily based on throughput and approved institutional safety protocols. The three rRT-PCR protocols were combined to generate a robust single tube multiplex RT-PCR assay which detects all of the three genome segments using different reporter dyes for each segment for confirmation purposes. During optimisation, the L-gene primer set (Bird et al., 2007) had low sensitivity and the S-gene primer set (Garcia et al., 2001) was consistently less sensitive in our single step format as compared with the original protocols. The newly designed L and S primer sets worked well as individual primer/probe sets and their incorporation into the multiplex rRT-PCR is currently in progress. There are two real-time reverse transcription-loop-mediated isothermal amplification assays (LAMP) available that provide alternative amplification detection tools for RVF viral RNA (Peyrefitte et al., 2008; Le Roux et al., 2009).

CONCLUSIONS

Standard RT-PCR viral gene amplification assays are being discarded by many diagnostic laboratories because they are prone to cross-contamination problems. The real-time RT-PCR assays such as those described here for the arboviruses, can be performed in a closed tube environment. This decreases the cross-contamination that occurs more frequently with gel-based assays and the instrumentation required is becoming more comon even in developing countries. These assay are specifically designed to be robust and flexible. On routine analysis, small numbers of samples can be run, but in outbreak situations the real-time RT-PCR assays can be automated to allow high throughput. In addition, the assay can be rapidily modified to detect new genetic variants. Additonal modifications including multiplexing for differential pathogen disease targets, and inclusion of an internal or positive RNA control such as in the multiplex vesicular disease panel (Lenhoff et al., 2008), could provide future improvements. New technologies such as linear-after-the-exponential (LATE)-PCR (Sanchez et al., 2004), LAMP (Peyrefitte et al., 2008; Le Roux et al., 2009) and surface enhanced raman spectroscopy (SERS, (Harpster et al., 2009) are taking advantage of the existing amplification detection knowledge base and providing both low and high technology solutions for virus genome detection. The development of validated diagnostics tools is needed for effective control strategies and the formulation of reasonable animal regulatory statutes to reduce the economic impact of these arboviruses.

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A Recombinant Nucleocapsid-based Indirect ELISA for Serodiagnosis of Rift Valley Fever in African Wildlife

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ABSTRACT

An indirect ELISA (I-ELISA) based on the recombinant nucleocapsid protein (rNp) of Rift Valley fever virus (RVFV) was evaluated for the detection of specific serum IgG antibody in African wildlife. Data sets derived from field-collected sera (n = 918) in Africa (antelopes = 570, black rhinoceros = 43, common zebra = 24, elephant = 73, giraffe = 81, grevy zebra = 78, warthog = 49) were categorised according to the results of a virus neutralisation test (VNT). At cut-offs optimised by the two-graph receiver operating characteristics analysis, the diagnostic sensitivity of the I-ELISA was 100% and diagnostic specificity ranged from 99.8% to 100% while estimates for the Youden's index (J) and efficiency (Ef) ranged from 0.99 to 1 and from 99.7% to 100%, respectively. The rNp-based I-ELISA is highly accurate, safe, and offers a single assay format for rapid detection of IgG antibody to RVFV in sera of different wildlife species.

Key words: *Rift Valley fever virus, recombinant nucleocapsid protein, IgG antibody, indirect ELISA, African wildlife.*

INTRODUCTION

The recent occurrence of the first confirmed outbreaks of Rift Valley fever (RVF) outside Africa (Jupp et al., 2002), together with the ability of RVF virus to replicate in a wide range of mosquito vectors (Turrel et al., 2008) and the effects of global warming which facilitate spread of arthropod-borne viruses (Purse et al., 2005) into non-endemic regions of the world are of medical and veterinary concern. Antibodies to RVF virus have been found in many wildlife species (Davies, 1975; Anderson and Rowe, 1998; Fischer-Tenhagen et al., 2000; Paweska et al., 2005; Evans et al., 2008; Paweska et al., 2008) but their importance in the epidemiology of the disease during the inter-epidemic and epidemic periods has yet to be elucidated.

Various forms of enzyme-linked immunoassays (ELISA) for serodiagnosis of RVF in different host vertebrates have recently been validated. Whilst these assays were shown to be highly sensitive and specific, they are based on β-propiolactone inactivated and/or gamma-irradiated, sucrose-acetone-extracted whole antigens (Paweska et al., 2003a; Paweska et al., 2003b; Paweska et al., 1995). The production of such antigens requires high bio-containment facilities to limit the risk of exposure of laboratory personnel while culturing the virus prior to inactivation. Other disadvantages include high production costs and the risk of incomplete inactivation. An indirect ELISA (I-ELISA) based on recombinant nucleocapsid protein (rNp) of RVF virus was reported to have high analytical accuracy for the detection of IgG antibody in experimentally infected and vaccinated sheep (Jansen van Vuren et al., 2007; Fafetine et al., 2007). The test was also shown to have high diagnostic performance characteristics in testing African buffalo sera (Evans et al., 2008; Paweska et al., 2008).

This paper describes evaluation of the rNp I-ELISA as a single test format for rapid detection of IgG antibody to RVF virus in different wildlife species.

Table 1. Number of field-collected wildlife sera tested in the VNT.

Species	Total Tested	VNT-[a]	VNT+[b]
Black rhinoceros	43	29	14
Common zebra	24	24	0
Elephant	73	69	4
Giraffe	81	81	0
Grevy zebra	78	77	1
Warthog	49	47	2
Eland	66	63	3
Gerenuk	6	1	5
Hartebeest	10	10	0
Impala	339	330	9
Kudu	73	66	7
Waterbuck	42	40	2
Thomson gazelle	8	1	7
Grand Total	918	864	54

[a] = number of sera tested negative in VNT; [b] = number of sera tested positive in VNT.

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MATERIALS AND METHODS

Serum Specimens

A total of 918 wildlife sera collected between 1978 and 2008 in Kenya, South Africa and Zimbabwe were used. Sera which tested negative in the virus neutralisation test (VNT) were regarded as reference panel from non-infected animals, and sera which tested positive as reference panel from animals infected with RVF virus (**Table 1**).

Virus Neutralisation Test

Duplicates of serial two-fold dilutions of sera inactivated at 56°C for 30 min were tested as previously described (Paweska et al., 2003b). Titres were expressed as the reciprocal of the serum dilution that inhibited ≥ 75% of viral cytopathic effect. A serum sample was considered positive when it had a titre of ≥ $\log_{10}$ 1.0, equivalent to a serum dilution ≥ 1:10.

ELISA Antigen and Procedure

The assay procedure was carried out as previously described (Paweska et al., 2008), and ELISA results were expressed as a percentage of the high-positive control serum (PP) (Paweska et al., 2003b). The assay runs were accepted within the upper and lower control limits for the internal controls as previously statistically determined (Paweska et al., 2008).

Selection of Cut-off Values and Determination of ELISA Diagnostic Accuracy

Cut-off values at 95% accuracy level were optimised using the misclassification cost term option of the two-graph receiver operating characteristics (TG-ROC) analysis (Greiner, 1996). In addition, cut-off values were determined by mean + 2 standard deviations (SD). and by mean + 3SDs derived from PP values in uninfected animals. Estimates of diagnostic sensitivity and specificity and other measures of combined diagnostic accuracy were calculated as previously described (Paweska et al., 2008).

RESULTS

Antibody Dilution Curves

Dose response curves using different dilutions of sera known to be positive or negative in the VNT had the expected analytical slope and the I-ELISA clearly differentiated between different levels of specific IgG antibody against RVFV in African wildlife (**Figure 1**).

Cut-off Values and Diagnostic Accuracy

Threshold values for IgG I-ELISA were derived from data sets dichotomised according to the results of the VNT (**Table 1**). The effect of differently determined cut-off values on distinguishing between sera which tested negative or positive in this assay, and consequently on estimates of sensitivity, specificity, and other estimates of diagnostic accuracy is given in **Table 2**. Optimisation of cut-off values using the misclassification cost term option of the TG-ROC analysis was based on the non-parametric programme option (Greiner, 1996) due to departure from a normal distribution of data sets analysed. Graphical presentation of the TG-ROC analysis is shown in **Figure 2**.

At a cut-off value of 20.4 PP the overall misclassification costs become minimal under the assumption of a 50% disease prevalence and equal costs of false-positive and false-negative test results. The two curves represent MCT values based on non-parametric (smooth line) or parametric (dashed line) estimates of sensitivity and specificity derived from data sets in field-collected sera. Optimisation of cut-off values was based on the non-parametric program option due to departure from a normal distribution of data sets analysed.

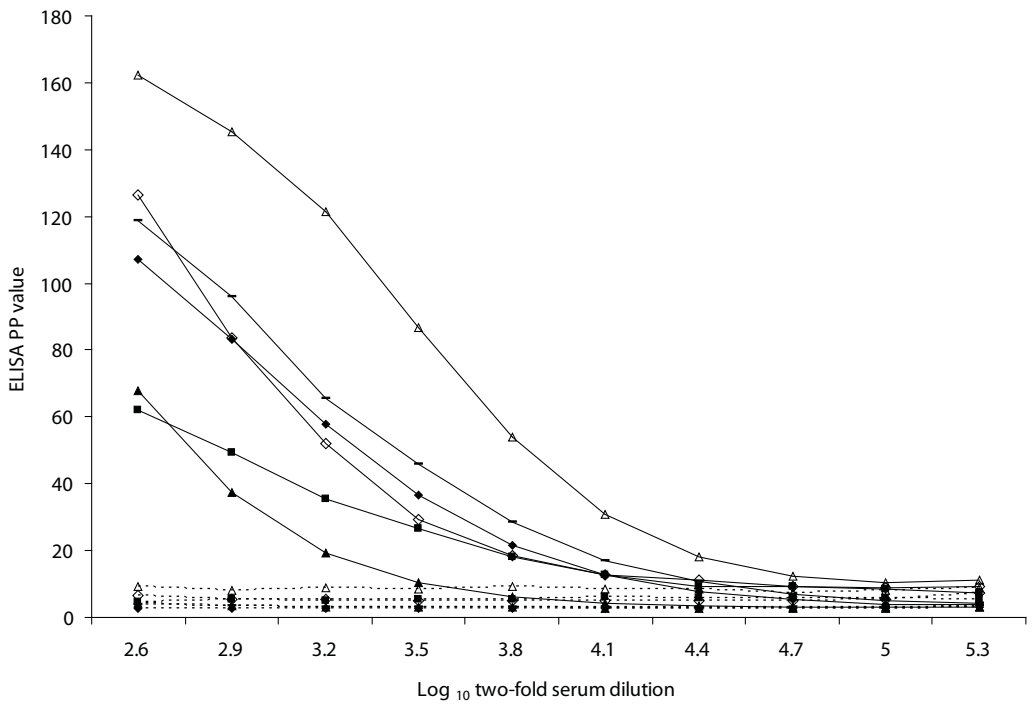

Figure 1. Dose response curves of wildlife sera in IgG I-ELISA tested positive (□) or negative (---) in a VNT: black rhinoceros (♦), eland (▲), gerenuk (■), kudu (◊), impala (Δ), Thomson gazelle (—). VNT titres in positive sera ranging from $\log_{10}10^{1.9}$ (■) to $\log_{10}10^{3.1}$ (Δ).

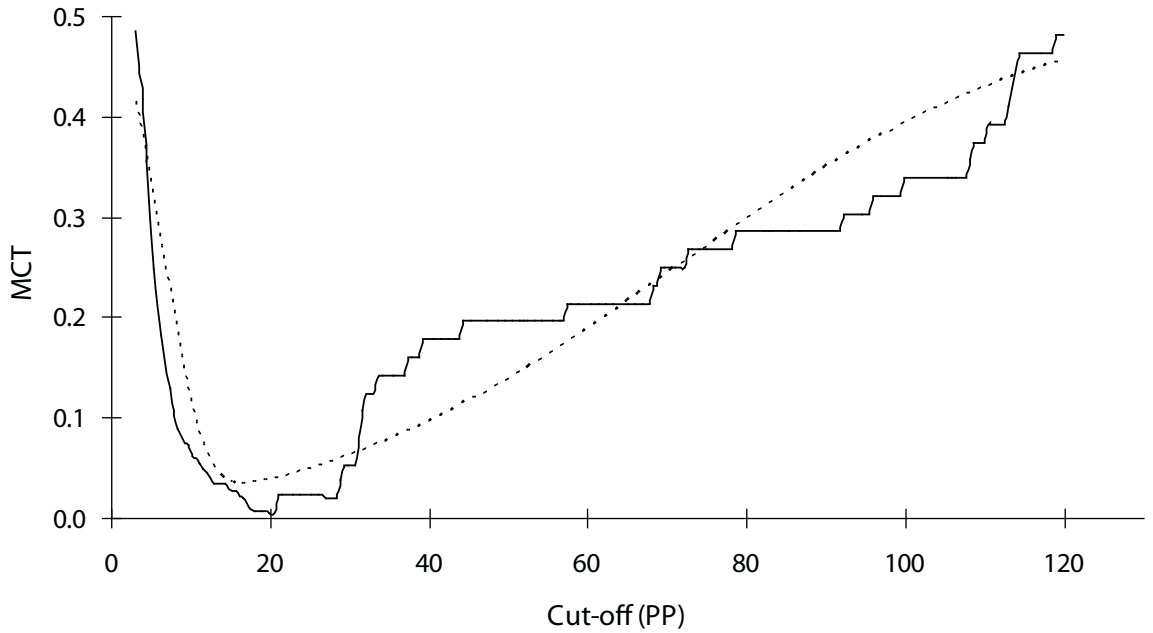

Figure 2. Optimisation of cut-off value for Rift Valley fever nucleocapsid-based I-ELISA in African antelopes using the misclassification cost term (MCT) option of the two-graph receiver operating characteristic analysis.

Table 2. Diagnostic accuracy of Rift Valley fever recombinant nucleocapsid–based I–ELISA in African wildlife.

Species	Cut–off[a]	D–Sn[b]	D–Sp[c]	Y[d]	Ef[e]	PPV[f]	NPV[g]
Black rhinoceros	33.6[h]	100	100	1	100	100	00
	27.5[i]	100	91.3	0.91	93.7	81.5	00
	35.5[j]	100	100	1	100	100	00
Common zebra	–	–	–	–	–	–	–
	13.9	–	100	–	–	–	–
	17.9	–	100	–	–	–	–
Elephant	28	100	100	1	100	100	100
	10.6	100	95.8	0.96	99.7	99.7	100
	13.6	100	97.2	0.97	99.8	99.8	100
Giraffe	–	–	–	–	–	–	–
	11.7	–	100	–	–	–	–
	14.3	–	100	–	–	–	–
Grevy zebra	–	–	100	–	–	–	–
	17.3	–	100	–	–	–	–
	22.5	–	100	–	–	–	–
Warthog	27.7	100	100	1	100	100	100
	13.5	100	95.9	0.96	96	49.7	100
	17.5	100	97.9	0.98	98	66.4	100
Antelopes[l]	20.4	100	99.8	0.99	99.7	95.6	100
	8.4	100	88.1	0.88	88.7	32.4	100
	14.4	100	97.0	0.97	97.1	67.9	100

Animals were categorised according to the results of the VNT.

[a] — cut–off value expressed as a percentage positivity (PP) of an internal high–positive serum control; [b] — diagnostic sensitivity (%); [c] —diagnostic specificity (%); [d] —Youden's index; [e] — efficiency (%); [f] — positive predictive value (%); [g] — negative predictive value (%); [h] — cut–off value optimised by TG–ROC analysis; [i] — cut–off value based on mean + 2 SD of ELISA PP values in VNT–negative population; [j] — cut–off value based on mean + 3 SD of ELISA PP values in VNT–negative population; [k] — not determined due to unavailability or very limited number of VNT–positive sera; [l] — eland, gerenuk, hartebeest, impala, kudu, Thomson gazelle, waterbuck.

DISCUSSION

Traditional methods for detecting antibodies to RVF virus include haemagglutination-inhibition, complement fixation, indirect immunofluorescence, and virus neutralisation assays. The last of these is regarded as a gold standard but is laborious, expensive and dependent on the availability of live virus and tissue cultures. Therefore, it is only used in specialised reference laboratories housing high biocontainment facilities. Laboratory safety and other advantages of the rNp-based I-ELISA compared with the VNT have been discussed recently (Paweska et al., 2008).

Antigenic cross-reactivity studies in animals (Swanepoel et al., 1986) and recent results in the I-ELISA failed to provide any evidence that other African phleboviruses could hamper reliable serodiagnosis of RVF (Paweska et al., 2007). However, to account for possible cross-reactivity with unknown phleboviruses, sera in this study were tested at relatively high dilution. The I-ELISA had high estimates of diagnostic sensitivity when cut-offs determined by traditional statistical approach were used but the diagnostic specificity and combined measures of assay accuracy were lower compared with those which were based on the cut-offs derived from the TG-ROC analysis. A cut-off determined as two or three SDs above the mean in uninfected individuals which is still commonly used for interpretation of serodiagnostic assays, assumes a normal distribution of test values in targeted populations, and therefore provides only an estimate of diagnostic specificity but not sensitivity (Jacobson, 1998). Deviations from normality are often observed in serological data and should be addressed in the selection of threshold values (Vizard et al., 1990). The prevalence assumed in the study sample may not be representative of the prevalence in the target populations and this should be borne in mind in applying the estimates of diagnostic accuracies reported for the rNp-based I-ELISA in the present work.

This study confirm previous findings (Paweska et al., 2008) that the rNp-based I-ELISA accurately identifies sera with different concentrations of specific IgG antibodies to RVF virus, and compared with the VNT it has very high diagnostic performance in various wildlife animal species. As a single and safe test format, it provides a useful tool for seroepidemiological studies of RVF virus infections in African wildlife species. Such investigations might help to elucidate their specific role in the epidemiology of the disease during the inter-epidemic and epidemic periods, and including enigmatic mechanisms of the virus cryptic maintenance within the host-vector natural cycle.

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Variability of the IFN-β Promoter Repressing Activity of NSs Proteins Derived from Field Isolates of Rift Valley Fever Virus

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ABSTRACT

Variability of viral and host genetic factors may be involved in the pathogenesis of Rift Valley fever virus (RVFV) infections and may explain the wide range of clinical outcomes in susceptible vertebrate hosts. The differences in virulence among RVFV isolates may be due to the interference with either the innate and/or adaptive immunity. In this work, the interferon-β antagonistic function of NSs of RVFV isolates from different sources (animals, humans, and insects) was assessed after cloning and sequencing the non-structural S segment gene (NSs). The NSs clones were monitored for their immune modulatory effects by analysing their ability to suppress the activation of the IFN-b promoter using a reporter assay system. Additionally, expression of NSs in Vero E6 cells was monitored by immunofluorescence staining. Two RVFV NSs proteins (derived from isolates R7 and R18) failed to inhibit IFN-β promoter activation whereas the remaining 24 showed efficient suppression of IFN-β promoter activity. Additionally R7-NSs and R18-Nss were unable to form nuclear filaments which are a typical feature of wild-type RVFV-NSs. Sequencing of R18-NSs revealed a large internal in-frame deletion identical to the mutation described for the naturally occurring RVFV mutant clone 13, which leads to a non-functional NSs-protein. Indeed, R18 was later identified as a RVFV clone 13 isolate. In contrast, R7-NSs contains a point mutation in the NSs gene, which results in the replacement of a leucine by proline. Interestingly, this unique point mutation has effects comparable to the large in-frame deletion of clone 13 NSs.

Key words: *interferon-β, Rift Valley fever virus, NSs protein.*

INTRODUCTION

A Rift Valley fever (RVF) outbreak leading to heavy mortality in newly-born lambs on a farm in Kenya was first described in 1931 (Daubney et al., 1931). RVF virus (RVFV) mainly causes disease in domestic ruminants inflicting high rates of abortion and mortality. Recurrent enzootic and epizootic outbreaks have been documented in eastern, southern and western Africa, Madagascar and Egypt. In 2000 RVFV even spread to the Arabian Peninsula, thereby emerging outside of Africa for the first time. The zoonotic disease can also cause epidemics in humans as recorded in Egypt in 1977, in Saudi Arabia and Yemen in 2000, and in Kenya and Tanzania in 2007 (Imam and Darwish, 1977; Jouan et al., 1990; Thiongane et al., 1996; Abd el-Rahim et al., 1999; Madani et al., 2003) RVFV survives dry periods in vertically infected eggs of different mosquito species and disease outbreaks are often linked to preceding heavy rainfalls.

RVF outbreaks usually begin in livestock with elevated abortion counts. In humans the symptoms range from mild fever to encephalitis, retinitis and fatal hepatitis with haemorrhages. The more severe forms occur in less than 1% of patients of which up to 50% may die (Woods et al., 2002). Although RVFV is mainly transmitted by mosquitoes (Hoogstraal et al., 1979), transmission to humans can also occur by contact with infected tissues e.g. from abortions from livestock (Hoch et al., 1985; Logan et al., 1992) or by aerosols from slaughtered animals (Chambers & Swanepoel, 1980) and consequently livestock workers and wildlife rangers in Nigeria (LaBeaud et al., 2007; LaBeaud et al., 2008) but also nomadic tribes in Kenya (Olaleye et al., 1996) show a very high seroprevalence towards RVFV.

RVFV is a member of the genus *Phlebovirus* in the *Bunyaviridae* family. The segmented negative single strand RNA genome of the virus codes for the polymerase (L-segment), the glycoproteins G1 and G2 and two non-structural proteins NSm14 and NSm78 (M-segment) and for the nucleocapsid (S-segment). The S-segment also codes for a non-structural protein (NSs) and a nucleoprotein (N). The NSs of RVFV is a 31-kDa protein composed of 265 amino acids which is phosphorylated by casein kinase II at two serine residues located in the C-terminus. It accumulates in the nuclei of infected cells, where it forms filamentous structures. A carboxy-terminal domain mediates oligomerisation and is responsible for filament formation (Yadani et al., 1999). Non-U.S. Gov't</keyword><keyword>Viral Nonstructural Proteins/*chemistry/physiology</keyword></keywords><dates><year>1999</year></dates><accession-num>10233964</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=m&form=6&dopt=r&uid=10233964</url><url>http://www.ncbi.nlm.nih.gov/pmc/articles/PMC112546/pdf/jv005018.pdf</url></related-urls></urls></record></Cite></EndNote>. The nuclear localisation of NSs is intriguing because all steps of the viral life cycle are known to occur in the cytoplasm (Billecocq et al., 2004).

The naturally attenuated RVFV strain clone 13 originally isolated from a non-fatal human case in Bangui, Central African Republic

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(Muller et al., 1995) carries a large in-frame deletion in the NSs gene. It is not virulent *in vivo* (in mice and hamsters) but grows as well as wild-type RVFV in cell cultures (Muller et al., 1995).

Various cells in the body are capable of sensing infectious viruses and initiating reactions collectively known as antiviral innate responses. These responses include the production of antiviral cytokines such as type I interferon (IFN) and subsequent synthesis of antiviral factors, which are responsible for impairing viral replication and promote adaptive immune responses (Samuel, 2001). IFN-**α/β** is induced by pathogen-associated molecular patterns like double-stranded RNA (dsRNA) or single-stranded RNA with a non-modified triphosphate residue at the 5′ end. It activates the Jak/Stat signaling pathway which leads to the expression of antiviral active factors. To overcome these antiviral responses, viruses have evolved various strategies to inhibit IFN production, IFN signaling, or IFN action. The efficiency by which a virus antagonises the IFN system is critical for its pathogenicity and its ability to infect and spread in a host (Billecocq et al., 2004).

A comparative analysis in mice showed that the RVFV clone 13 (carrying a mutant NSs gene) though avirulent in wild type mice can kill IFN-α/β receptor knockout mice, which cannot mount an antiviral response. In contrast to RVFV clone 13, virulent RVFV strains (carrying an intact NSs gene) like the strain ZH548 do not induce an IFN-response in wild type mice. This observation led to the conclusion that NSs is an IFN antagonist (Bouloy et al., 2001; Billecocq et al., 2004). Further analysis revealed that NSs is a potent repressor of the IFN-b gene expression. In addition, clone 13 proved to be an excellent inducer of early IFN-α/β production *in vivo*. In contrast, the virulent strain ZH548 failed to induce detectable amounts of IFN-α/β and replicated extensively in both IFN-competent and IFN-defective mice.

NSs is located exclusively in the nucleus of RVFV-infected cells which is rather surprising since this virus, like all the members of the family *Bunyaviridae*, utilises only the cytoplasm as its site for replication. Intranuclear inclusions were first detected in the hepatocytes of RVFV-infected animals (Daubney et al., 1931). Later, Swanepoel and his group (Swanepoel and Blackburn, 1977) detected nuclear filaments in cells infected with various virulent RVFV strains and showed that the nuclear filament is composed of bundles of 50-nm-thick fibrils, which occupy half the length of the nucleus and are confined exclusively to the nuclei but not associated with nucleoli.

The immune compromising effect of wild type RVFV strains through NSs-mediated interference with the innate immune system is an important virulence factor. Variations in the NSs sequence may influence the NSs function and might therefore contribute to the wide range of clinical outcomes of RVFV infections in man and cattle. In this work the ability of NSs of wild type RVFV isolates to block type I IFN induction (i.e. activation of the innate immune system) was assessed after cloning and sequencing the NSs gene. Additionally NSs expression, nuclear localisation and filament formation was monitored by immunofluorescence analysis.

MATERIAL AND METHODS

cDNA Preparation

Total RNA was prepared from virus culture supernatants of strains previously described (Weidmann et al., 2008). The strains were blinded and coded R1-R33. After RNA extraction, the complementary DNA (cDNA) was prepared for each strain. The first strand cDNA synthesis was performed using Superscript III (Invitrogen, Leek, The Netherlands) according to the manufacturer's instructions. The cDNAs obtained were stored at –80 °C until used.

The cDNAs were used as templates in PCRs using Pfx DNA polymerase (Invitrogen) with RVFV-NSs specific forward primer which adds a 5′ BglII restriction site (5′GACAGAAGATCTATGGATTACTTTCCTGTGATATCTG3′) and reverse primer (5′GTCGACTCACTTGTCATCGTCGTCCTTGTAGTCATCAACCTCAACAAATCCATC3′) which adds an immunogenic FLAG tag as well as a 3′ SalI restriction site. The addition of the FLAG tag at the C-terminus of RVFV-NSs neither affects the IFN-antagonistic function of NSs nor the ability to form nuclear filaments (Billecocq et al., 2004). A touch down program for PCR was used: after the denaturation step at 95 °C for 120 s, ten cycles were performed with denaturation at 95 °C for 30 s, an annealing step for 30 s with an initial temperature of 57 °C which was decreased by 0.5 °C for each cycle and an extension step at 68 °C for 60 s. Then, 25 cycles were performed with a constant annealing temperature of 52 °C while the other parameters remained unchanged. After the final extension step at 68 °C for 300 s and subsequent cooling, the amplification products were separated by electrophoresis in a 0.8% TAE agarose gel and the PCR products were recovered using peqGOLD Gel Extraction kit (PEQLAB Biotechnologie GmbH, Nürnberg, Germany).

Cloning Experiments

TA Cloning of Purified PCR Products

TA cloning of the purified PCR products into the vector pCRII was performed according to the manufacturer's instructions (Invitrogen). Positive clones were pre-screened by blue/white staining. White colonies were picked and amplified in LB-Amp selection medium. Subsequently, plasmid DNA was isolated using PeqGold Plasmid Miniprep Kit (PEQLAB Biotechnologie GmbH). A restriction digest using EcoRI enzyme was done to identify plasmids carrying the NSs cDNA and a 1% agarose gel electrophoresis was run to visualise and differentiate the digestion products. Finally, positive plasmids were sequenced using the primers M13 forward (5′GTAAAACGACGGCCAG3′) and M13 reverse (5′CAGGAAACAGCTATGAC3′) to determine orientation and sequence integrity of the cloned NSs cDNAs as well as the presence of the C-terminal added FLAG tag (Seqlab Laboratories, Göttingen, Germany).

Subcloning of NSs cDNAs into the Eukaryotic Expression Vector pI.18

The 26 different pCRII-RVFV-NSs plasmids obtained were digested with BglII and SalI (Fermentas, St. Leon-Rot, Germany) to cut out the NSs cDNA fragments. Following agarose gel electrophoresis and gel purification using Zymoclean gel DNA recovery kit (HISS Diagnostics, Freiburg, Germany), the NSs cDNAs were subcloned into BglII/SalI-digested eukaryotic expression vector pI.18. Ligation products were transformed into Z-competent E. coli XL1blue and transformed bacteria were grown on LB-amp agar plates. Subsequently clones were picked and amplified in LB-Amp medium followed by plasmid DNA isolation. Isolated plasmids were screened for NSs cDNA insertion by BglII/SalI digest. Positive clones were amplified in 50 ml LB-Amp medium and plasmid DNA was isolated using the Nucleobond 100 kit according to the manufacturer's instructions (Macherey-Nagel, Düren, Germany).

Monitoring the Repression of IFN-β Promoter Activation by RVFV-NSs Proteins

The inhibitory effect of RVFV-NSs on VSV-RNA-mediated IFN-β promoter activation was tested in a luciferase reporter assay. With this aim $1x10^5$ Vero E6 cells were cotransfected with p125-luc (firefly luciferase cDNA expression driven by the human IFN-β promoter),

pRL-SV40 (renilla luciferase cDNA expression driven by the $SV40_{early}$ promoter, Promega, Mannheim, Germany) and pI.18-RVFV-NSs (NSs cDNA expression driven by the hCMV-IE promoter linked to the hCMV intron A; plasmid pI.18 was kindly provided by J. Robertson, Hertfordshire, UK) using FuGene HD (Roche Biochemica, Mannheim, Germany) according to the manufacturer's instructions. After 24 h the transfected cells were either stimulated with RNA from VSV-infected Vero E6 cells or with RNA from uninfected Vero E6 cells as a control. RNA from VSV-infected cells contains high amounts of viral RNA with an unmodified triphosphate 5' end which is a potent inducer of IFN-β expression (Pichlmair et al., 2006). For this purpose, cells were transfected with 1 µg RNA using FuGene HD. Cells were lysed 16 h post stimulation and a dual-luciferase assay performed according to the manufacturer's instructions (Promega, Mannheim, Germany) using a Fluostar Optima reader (BMG Labtech, Offenburg, Germany).

Monitoring the Formation of Filaments in the Nucleus of Vero E6 Cells Transfected with RVFV-NSs Expression Plasmids using an Indirect Immunofluoresence Assay

Vero E6 cells were cultured on coverslips and transfected with RVFV-NSs Flag expression plasmids using FuGene HD (Roche) as transfection reagent. At 24 h post transfection, the cells were fixed with 3% paraformaldehyde and permeabilised with 0.5% TritonX100 (Sigma, Deisenhofen, Germany). The permeabilised cells were then incubated with a monoclonal mouse anti-FLAG antibody (Sigma) as primary antibody (diluted 1:200 in PBS containing 1% FCS), followed by incubation with an Alexa Fluor 555 labelled anti-mouse IgG (Invitrogen) as secondary antibody. Then coverslips were mounted in Fluosave mounting medium (Calbiochem, Bad Soden, Germany). After solidification of the mounting medium, the slides were examined by fluorescence microscopy using a Nikon TE2000-S inverted microscope.

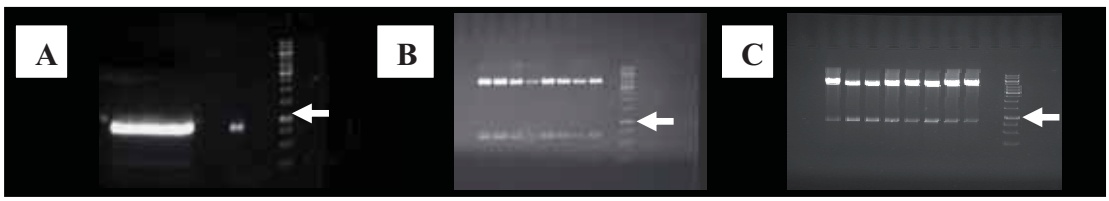

Figure 1. (A) PCR product from cDNA of RVFV-NSs R17 in 0.8% agarose gel and 0.05% ethidium bromide, (B) Eight clones of the pCRII-RVFV-NSs R17 construct after restriction digest using EcoRI in 1% agarose gel and 0.05% ethidium bromide. The 200 bp fragment of the 5'NSs fragment is hardly visible, (C) Eight clones of the pI.18RVFV-NSs R17 construct after restriction digest using SalI and BglII in 1% agarose gel and 0.05% ethidium bromide. The arrows indicate the 1 000 bp band of the DNA marker.

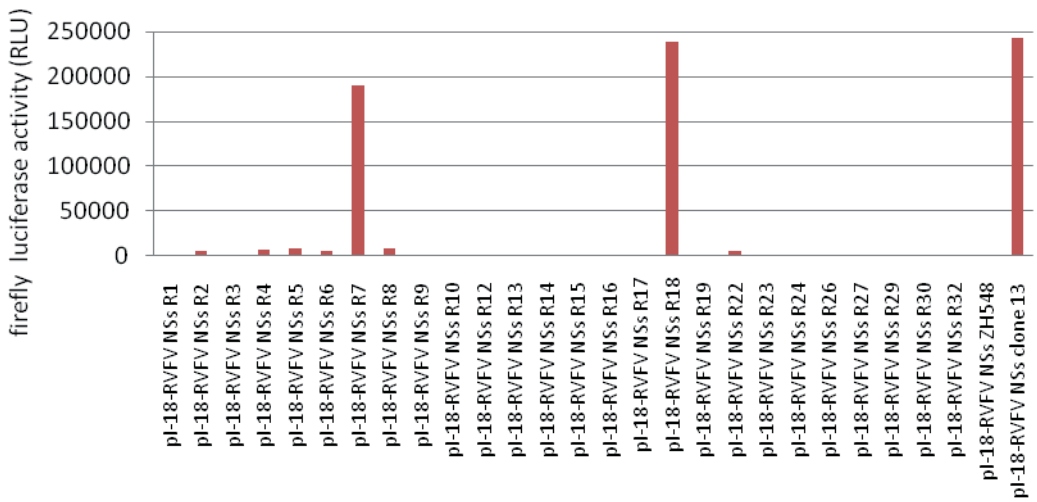

Figure 2. Effect of NSs on IFN-β promoter activity after stimulation with RNA from VSV-infected cells for the 26 RVFV-NSs clones, RVFV-NSs ZH548, and RVFV-NSs clone13 in a luciferase reporter assay. Relative light units (RLU) represent the readout for firefly luciferase activity which indicates the activity of the IFN-β promoter. The diagram represents the mean values of five independent experiments for each NSs clone.

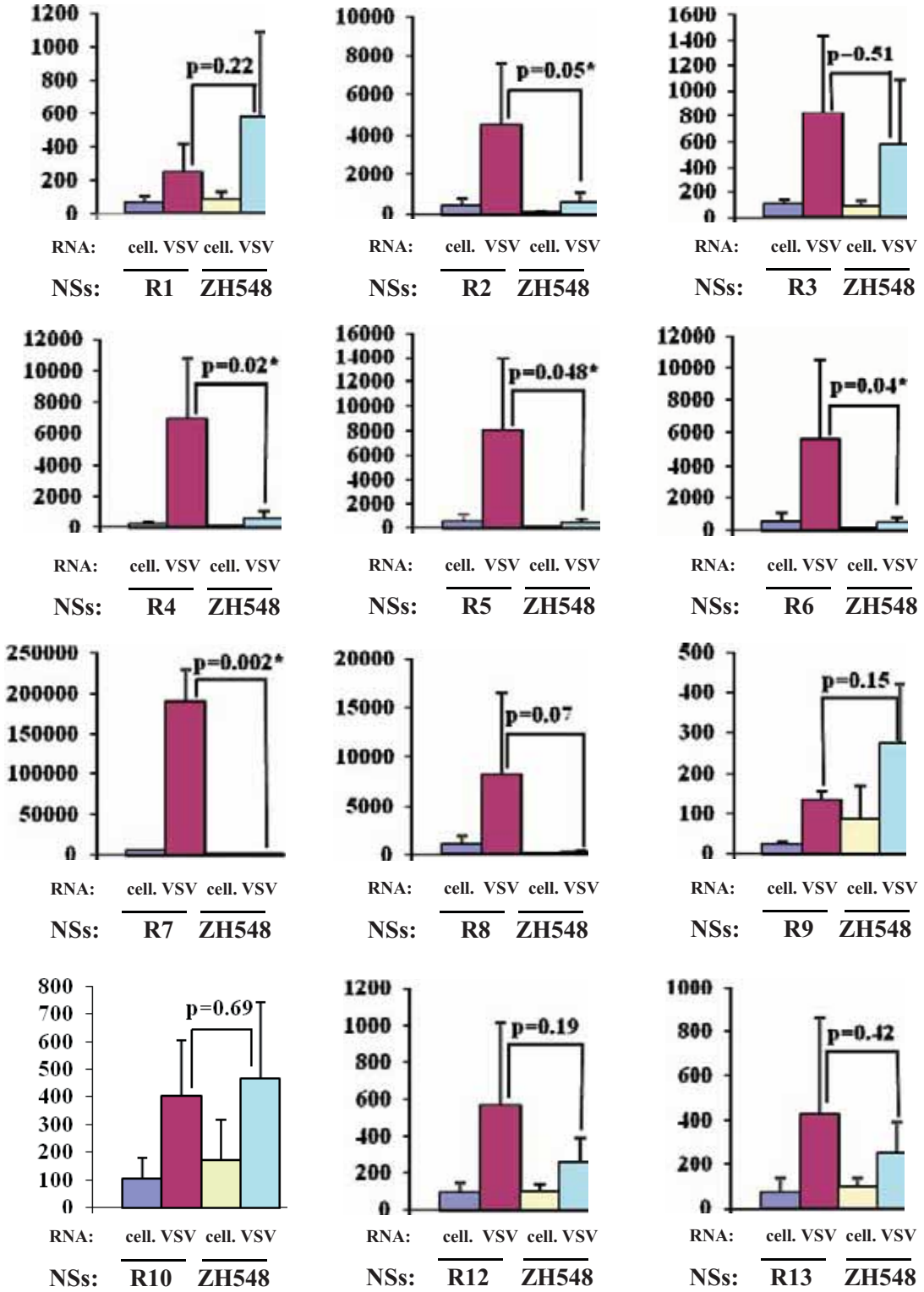

Figure 3. IFN-β promoter activity measured by firefly luciferase activity after stimulation with RNA of VSV-infected Vero cells (VSV) or with RNA of uninfected cells (cell.) for RVFV-NSs R1, 2, 3, 4, 5, 6,7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19, 22, 23, 24, 26, 27, 29, 30, 32 in comparison to wt RVFV-NSs ZH548. The diagrams represent the mean values of five independent experiments for each NSs clone; p values were calculated by Student's t-testtest. Significant differences (p<=0.05) are marked with an asterisk.

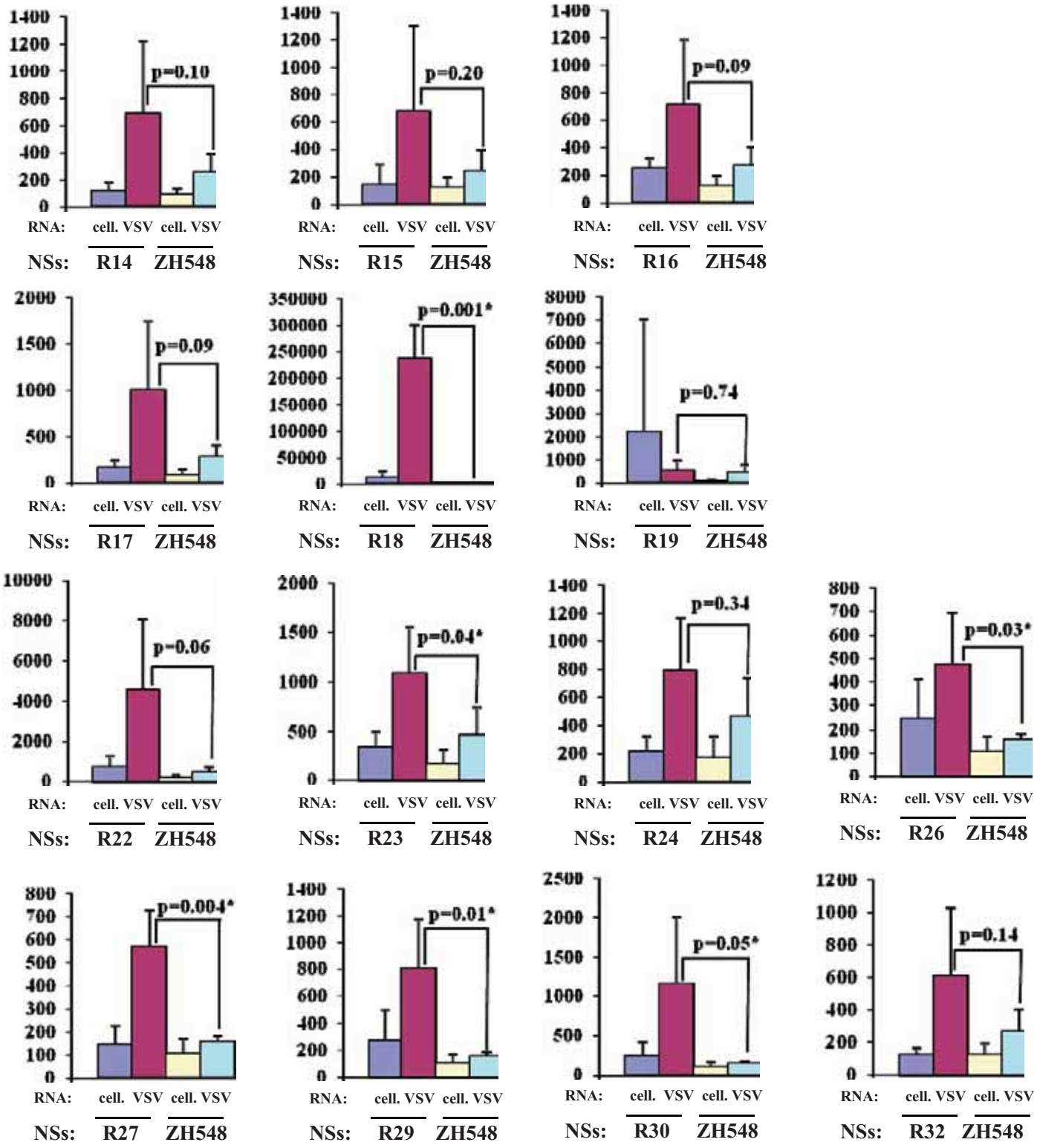

Figure 3. (continued).

RESULTS

Cloning of the NSs of RVFV Isolates into the Eukaryotic Expression Vector pI.18

First, NSs cDNAs were obtained by reverse transcription of viral RNA followed by PCR amplification and cloning into the TA-vector pCRII. Subsequently, the cDNAs were subcloned into the eukaryotic expression vector pI.18.

An example of the successful cloning procedure is shown for RVFV-NSsR17 in **Figure 1**. The expected band size for RVFV-NSs after PCR amplification is around 800 base pairs which indeed was observed for R17-NSs (**Figure 1A**). Ligation of the NSs cDNA into pCRII was monitored by EcoRI digest which results in a 4 000 bp band (vector backbone), a 600 bp band (3' fragment of NSs cDNA) and 200 bp band (5' fragment of NSs cDNA) (**Figure 1B**). After subcloning the NSs cDNA into the eukaryotic expression vector pI.18, successful ligation was confirmed by BglII/SalI digest which results in a 4 300 bp band (vector backbone) and a 800 bp band (NSs cDNA) (**Figure 1C**).

Inhibition of IFN-β Promoter Activation by RVFV-NSs

From the 33 RVFV isolates available, 26 RVFV NSs cDNAs were successfully cloned and ligated in pI.18. For the remaining isolates we were not able to amplify the NSs cDNA most probably due to minor quality of the RNA used as template for reverse transcription. To test whether expression of the NSs cDNAs inhibits the IFN-β promoter activation, Vero E6 cells were cotransfected with expression plasmids for (i) NSs of wild type virus ZH548 as a positive control, (ii) NSs of the attenuated RVFV clone 13 as a negative control, and (iii) NSs of the 26 RVFV isolates together with the reporter plasmids p125-luc and pRL-SV40. After stimulation with RNA of VSV-infected Vero E6 cells or uninfected cells IFN-β promoter activities were determined by luciferase assay.

The results of the luciferase assays (**Figure 2**) show that two RVFV-NSs clones (R7 and R18) failed to suppress IFN-β promoter activation after stimulation with RNA of VSV-infected cells as indicated by the high firefly luciferase activities when compared to RVFV ZH548- NSs (reference virulent wild-type strain). In contrast, the other RVFV-NSs clones showed efficient suppression of IFN-β promoter activity since only low firefly luciferase activities were detected. While RVFV-NSs R7 may have some residual activity, RVFV-NSs R18 has no inhibitory effect at all which is very similar to the NSs from the avirulent RVFV strain clone 13. Furthermore, the NSs clones with inhibitory effect on IFN-β promoter activation as well as RVFV ZH548-NSs were able to decrease the activity of the constitutively active SV40 promoter as indicated by low renilla luciferase acitivities when compared with RVFV-NSs clone 13 (data not shown). This is in line with previous observations that RVFV-NSs affects RNA polymerase II mediated transcription (Billecocq et al., 2004; Le May et al., 2004).

Comparison of the Activity of the RVFV-NSs Clones with the Reference Wild-type RVFV ZH548-NSs

The results show significant differences ($P <= 0.05$) in the ability to inhibit IFN-β promoter activation between the wild type RVFV ZH548-NSs and R2, R4, R5, R6, R7, R18, R23, R26, R27, R29, R30 RVFV-NSs (**Figure 3**). Although significant, the observed differences are small with the marked exception of R7- and R18-NSs. There is no significant difference in blocking IFN-β promoter activation between the ZH548-NSs and the remaining RVFV-NSs clones.

Comparison of Filament Formation in the Nucleus of Vero E6 Cells Transfected with 26 RVFV-NSs Expression Plasmids

The immunofluorescence staining allowed detection of filamentous structures in the nucleus of transiently transfected Vero E6 cells for most of the RVFV-NSs isolates as well as for the wild-type strain ZH548-Nss (**Figure 4z**), however for the NSs of three RVFV isolates (R7, R10, and R18) nuclear filament formation was not observed.

Interestingly, R7-NSs and R18-NSs which failed to inhibit IFN-β promoter activation weren't detectable in immunofluorescence tests but had an intact FLAG-tag (**Figures 4g** and **4p**). For unknown reasons we were unable to detect filament formation for NSs-R10 (**Figure 4j**) which showed efficient inhibition of IFN-β promoter activation as well as an intact FLAG-tag. However, we cannot rule out the possibility, that the FLAG-tag was destroyed during subcloning of the NSs-cDNA into pI.18 and resequencing is required to check this hypothesis.

A Point Mutation in the NSs Sequence of RVFV-NSs R7 Affects NSs Function

All cloned NSs genes were sequenced and compared to the NSs sequence of the reference strain RVFV ZH548. We found nucleotide exchanges in 71 positions of the NSs ORF which has a length of 798 nt, however the vast majority of these mutations are silent. In the NSs sequence of RVFV isolates R1, R2, R3, R4, R5, R6, R7, R8 and R32 a G to A exchange at position 71 of the NSs ORF was detected which leads to the replacement of arginine by lysine on amino acid level. With the exception of R7-NSs these NSs clones were efficient inhibitors of IFN-β promoter activation (**Figures 2** and **3**) and formed nuclear filaments (**Figure 4**) indicating that this mutation has no effect on NSs function and stability. Frequent mutations were observed at positions 724–726. While the RVFV ZH548-NSs sequence contains the triplet ATT at these positions which corresponds to isoleucine, NSs clones R1 and R3 contain GTC (corresponding to valine), NSs of RVFV isolates R4, R5, R6, R7, R8, R22, R23, R24, R26, R27, R28, R29, R30 and R32 contain GTT (also corresponding to valine) and the NSs of the isolates R12, R13 and R14 contain ATC (which is a silent mutation). The remaining NSs sequences of isolates R9, R10, R15, R16 and R17 carry no mutations at these positions. Similar to the replacement of arginine by lysine in the N-terminal part of NSs, the isoleucin to valine exchange in the C-terminal part of NSs had no consequences with respect to suppression of IFN-β promoter activation (Fig 2 and 3) and nuclear filament formation (**Figure 4**). R7-NSs contains an additional mutation at position 344 (T to C) which leads to the replacement of leucine by proline. This mutation seems to have a strong effect on the NSs function since NSs R7 no longer inhibits activation of the IFN-β promoter (**Figures 2** and **3**). Additionally, this mutation might be detrimental for the stability of R7-NSs since no nuclear filament formation was observed (**Figure 4**) although the C-terminal domain (amino acids 249 to 265 of the NSs protein) which is required for oligomerisation and filament formation (Yadani et al., 1999) is present in R7-NSs. In contrast, R8-NSs which is — apart from the leucin to proline exchange — otherwise identical to R7-NSs (on nucleotide as well as amino acid level) is an efficient inhibitor of IFN-β promoter activation (**Figures 2** and **3**) and forms nuclear filaments (**Figure 4j**).

Sequencing of RVFV R18-NSs revealed a large in-frame deletion of 549 nucleotides starting at nucleotide position 46 of the NSs ORF which is identical to the deletion observed in RVFV clone 13-NSs. Interestingly, the point mutation observed in R7-NSs and the in-frame deletion observed in R18-NSs (which includes position 344

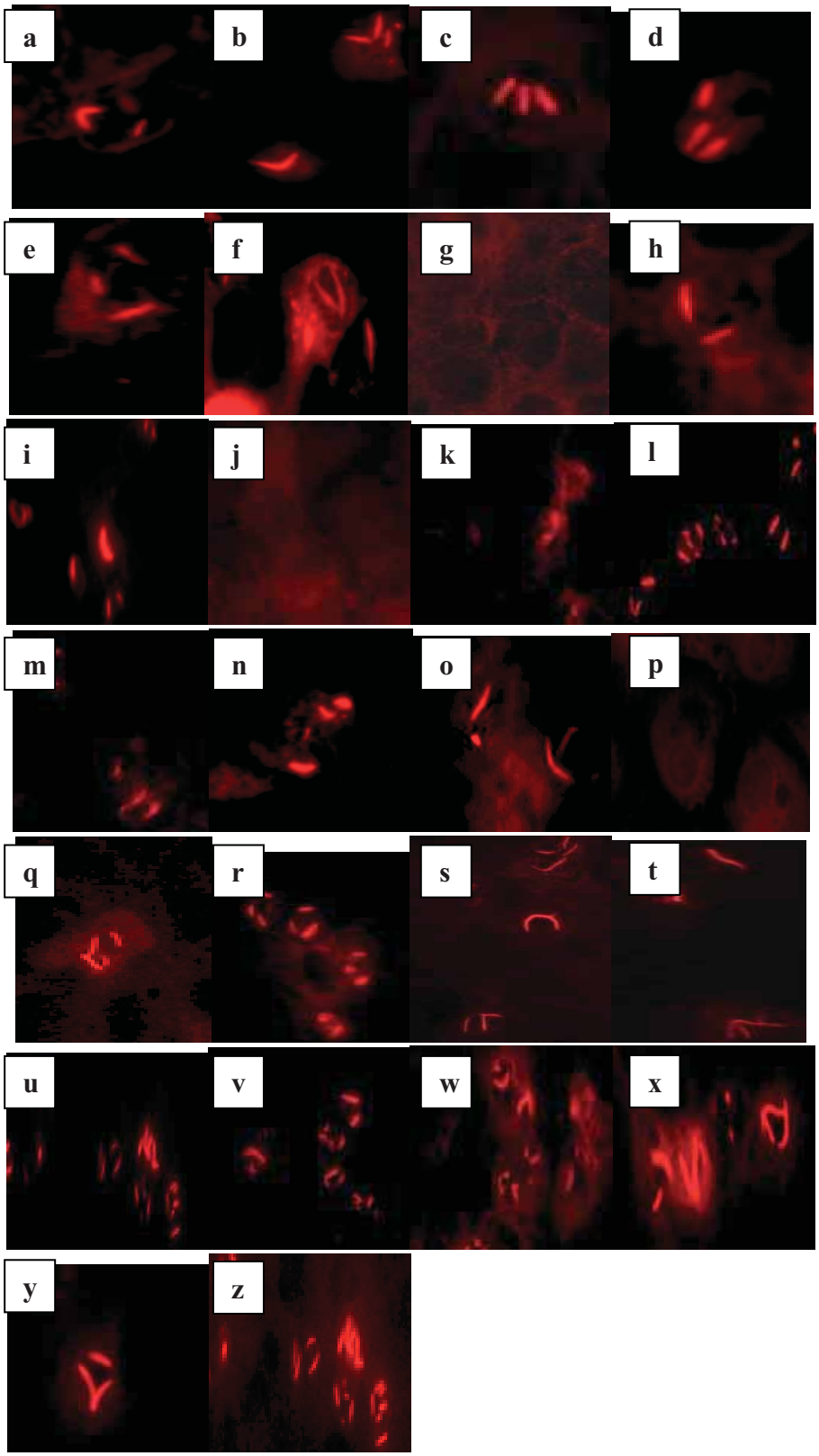

Figure 4. Filamentous structure formation of RVFV-NSs in transfected cells. (a) R1, (b) R2, (c) R3, (d) R4, (e) R5, (f) R6, (g) R7, (h) R8, (i) R9, (j) R10, (k) R12, (l) R14, (m) R15, (n) R16, (o) R17, (p) R18, (q) R19, (r) R22, (s) R23, (t) R24, (u) R6, (v) R27, (w) R29, (x) R30, (y) R32, (z) ZH548. No filament formation was observed for R7 (g), R10 (j) and R18 (p).

of the R7-NSs mutation) have similar effects: Both NSs clones fail to inhibit IFN-β promoter activation and do not form nuclear filaments.

DISCUSSION AND CONCLUSIONS

Significant differences in IFN-β promoter activation in the presence of NSs from RVFV isolates R2, R4, R5, R6, R7, R18, R23, R26, R27, R29, R30 were observed in this study when compared with the wild type RVFV ZH548-NSs. However, with the exception of R7- and R18-NSs all these NSs clones were still efficient inhibitors of IFN-β promoter activation. R7-NSs and R18-NSs were unable to suppress IFN-β promoter activation and did not form nuclear filaments. A follow up of viral RNA samples revealed that strain R18 is indeed a RVFV clone 13 isolate. The follow up has not yet revealed the identity of the strain from which R7-NSs was amplified. Consequently the results for R18-NSs simply confirm the loss of function of clone 13-NSs (Vialat et al., 2000) whereas R7-NSs truly shows a significant inability to suppress IFN-β promoter activation induced by RNA from VSV-infected cells.

Our reporter assay results are consistent with previous findings that RVFV clone 13 (lacking a functional NSs), but not wild type virus induces IFN-β gene expression (Billecocq et al., 2004). Previous work also demonstrated that recombinant NSs of wild-type strain ZH548 forms filamentous structures in the nuclei of transiently transfected cells; these structures were identical to those observed in ZH548-infected cells (Billecocq et al., 2004). In contrast, the truncated NSs of RVFV clone 13 was barely detectable and located mainly in the cytoplasm (Billecocq et al., 2004). It was reported that — except for RVFV clone 13 — the NSs proteins of all the RVFV strains analyzed so far form filamentous structures in the nuclei of infected cells (Muller et al., 1995). Interestingly, we showed here that R7-NSs, which failed to inhibit IFN-β promoter activation, wasn't detectable in IFA tests although sequencing results revealed an intact C-terminal domain required for oligomerisation and filament formation as well as an intact FLAG-tag. Therefore, the observed absence of filament formation as well as the loss of function can be attributed to the point mutation found in the R7-NSs sequence which results in a replacement of leucine by proline. This mutation probably leads to an unstable conformation which might result in proteasomal degradation as it is the case for RVFV clone 13 NSs (Vialat et al., 2000). RVFV-NSs is normally quite stable, and the situation observed for RVFV clone 13-NSs is exceptional. Obviously, the unique point mutation leading to a leucine to proline exchange at amino acid position 115 in R7-Nss has effects comparable to the large in-frame deletion of clone 13 NSs and is critical for NSs function and stability. Further experiments have to be done to test whether strain R7 is less virulent than the reference strain ZH548 *in vivo* or even non-pathogenic like RVFV clone 13.

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Phylogenetic Analysis of the Capripoxvirus RPO30 Gene and its Use in a PCR Test for Differentiating Sheep Poxvirus from Goat Poxvirus

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ABSTRACT

The Genus Capripoxvirus (CaPV) of the Poxviridae family comprises sheep poxvirus (SPPV), goat poxvirus (GTPV) and lumpy skin disease virus (LSDV) which are responsible for economically important diseases affecting sheep, goats and cattle respectively. To date, there have been no molecular criteria upon which to base strain designation. The complexity of CaPVs host specificity shows the need to develop more reliable tools for CaPVs identification than the current method which is based on the host origin. Previous reports, based on partial or full genome sequencing indicated that CaP viruses are genetically distinct from each other and can be grouped as three different species: SPPV, GTPV and LSDV.

In contributing to the creation of more stringent data for genotyping CaPVs, we have analysed the RPO30 gene of several isolates. The phylogenetic reconstructions have shown that the viruses can be segregated into three different lineages according to their host origins: the SPPV, the GTPV and the LSDV lineages. In addition, a 21-nucleotides deletion found in all individuals within only the SPPV group was exploited to design a classical PCR method to differentiate SPPV from GTPV. This test allows the rapid differential diagnosis of diseases caused by either SPPV or GTPV strains.

Key words: *Capripoxvirus, RPO30 gene, lineages, sequencing, differential diagnosis, polymerase chain reaction.*

INTRODUCTION

The genus *Capripoxvirus* (CaPV) of the *Poxviridae* family comprises sheep poxvirus (SPPV), goat poxvirus (GTPV) and lumpy skin disease virus (LSDV) which are responsible for economically important diseases affecting sheep, goats and cattle respectively. The criteria upon which CaPVs are named remain the host origin. This tends to suggest that they are strictly host specific. However there are several reports indicating the involvement of both sheep and goats in different outbreaks and therefore, this way of naming CaPV cannot be fully reliable (Diallo and Viljoen, 2007; Babiuk et al., 2008). In the same way, the fact that CaPV infections cannot be distinguished clinically or serologically, emphasises the need to establish more reliable tools such as those based on molecular methods for strain identification. Previous reports based on partial or full genome sequences, have shown that CaPVs are genetically heterogeneous and can be grouped as three different species SPPV, GTPV and LSDV (Tulman et al., 2002; Hosamani et al., 2004; Le Goff et al., 2009).

Here we describe the suitability of the CaP viruses' RPO 30 gene, the orthologue of the *Vaccinia* virus E4L gene encoding the 30 kD RNA polymerase subunit for virus-animal origin discrimination, and the design of a polymerase chain reaction (PCR) method to differentiate SPPV from GTPV based on the presence of a 21 nucleotide deletion found exclusively in the SPPV sequences.

MATERIALS AND METHODS

Genomic DNA from CAP viruses (**Table 1**) was extracted with AllPrep DNA/RNA Mini Kit (Qiagen) and a region containing the full RPO30 gene was amplified, cloned into pGEM-T plasmid (Promega) and sequenced. The sequencing data were analysed with Vector NTI.10 and the BioEdit software was used to perform the multiple alignments of the nucleotides and amino acid (aa) sequences. The Mega 4 software was used to generate the phylogenetic tree.

The PCR primers SpGpRNAPol F (tctatgtcttgatatgtggtggtag) and SpGpRNAPol R (agtgattaggtggtgtattattttcc) were designed (with Allele ID 6 software) on both sides of the region containing a deletion in SPPV sequences so that the PCR products from SPPV would be shorter in comparison with GTPV and LSDV.

RESULTS AND DISCUSSION

Phylogenetic and Sequence Analyses

The phylogenetic analyses using the Neighbor-Joining method showed three lineages: the LSDV lineage, the GTPV lineage and the SPPV lineage (**Figure 1**). A few discrepancies were found: isolate

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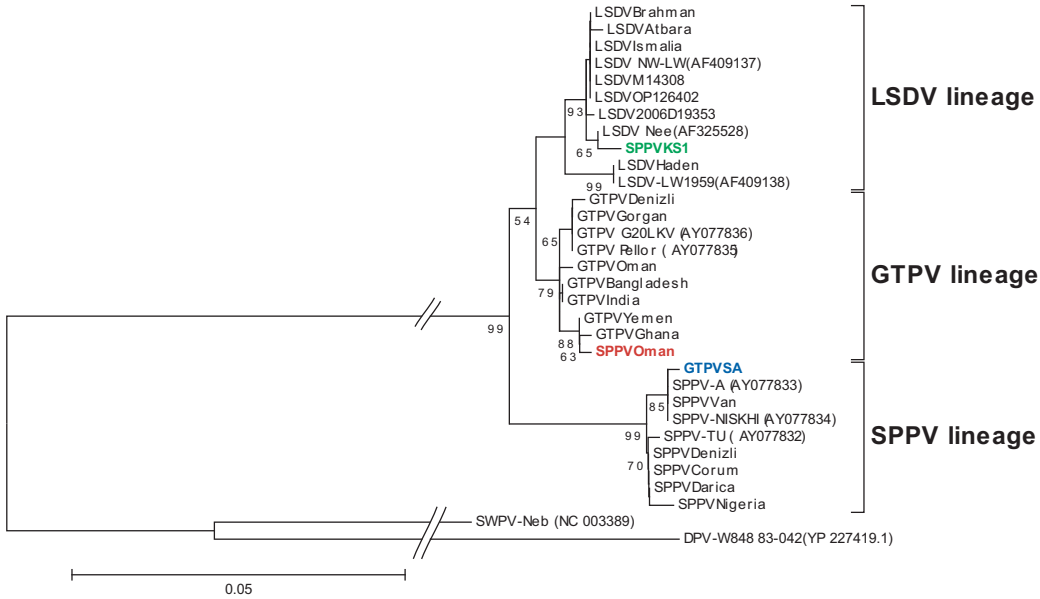

Figure 1. Phylogenetic tree derived from the nucleotide sequences of 30 CaPVs RPO30 genes by the Neighbor-Joining method Distance analysis based on Kimura 2-parameter distance. Deer poxvirus (DPV) and swine poxvirus (SWPV) retrieved from the gene bank were used as out-groups. The numbers at the nodes are the bootstrap confidence levels (in percentages) obtained for 1 000 replicates. Only bootstrap > 50 are shown.

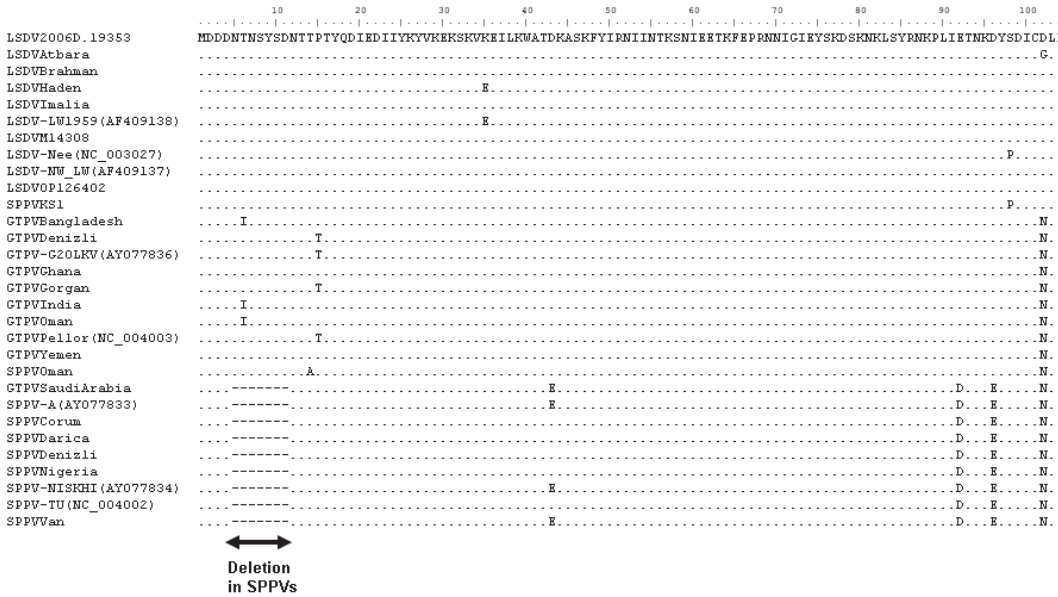

Figure 2. Alignment of the amino acid sequences of 30 CaPVs RPO30 genes (Partial representation) showing a 21 nucleotide deletion in SPPV sequences.

SPPV Oman was found to be located in the GTPV lineage, while isolate SPPV KS1 was in the LSDV group and GTPV Saudi Arabia was present in the SPPV group. Interestingly, these results are fully in agreement with our previous study using the CaPVs' GPCR gene (Le Goff et al., 2009). In addition, the SPPV KS1 and SPPV Oman have been shown to be related to LSDV and to GTPV respectively (Black et al., 1986). The alignment of the aa sequences showed seven aa deletion (corresponding to a 21-nucleotide deletion) in all and only the members of the SPPV lineage at positions 5 to 11 (**Figure 2**).

PCR for Differentiating SPPV from GTPV

Because a 21 bp deletion was found in all individuals belonging to the SPPV lineage (**Figure 2**), we have used it as a marker for differentiating SPPV from GTPV in a single PCR step. Primers were designed for both side of this region so that the PCR products from GTPV/LSDV would differ in length from those produced with SPPV. After the migration of the PCR products on a 3% high resolution agarose gel, all individuals in the SPPV group were found to produce a shorter amplicon (151 bp) compared with GTPV/LSDV (172 bp) as

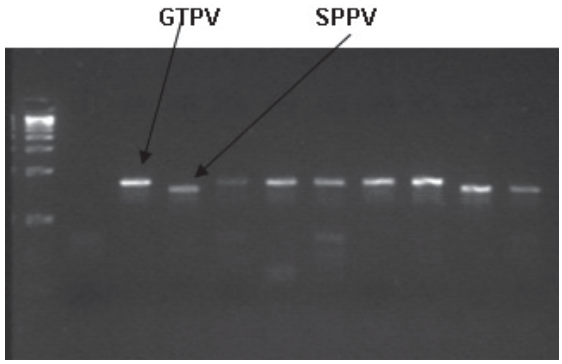

Figure 3. Classical PCR for differentiating GTPV from SPPV
SPPV and GTPV PCR products have different lengths due to a deletion in the target region within SPPV RPO30 gene.

shown in **Figure 3**. This system can therefore be used to differentiate GTPV from SPPV, assuming that the samples are coming from domestic small ruminants since with the exception of KS1, no strain of LSDV has been reported in small ruminants (Diallo and Viljoen, 2007; Babiuk et al., 2008). Results of the genotyping using this PCR method are similar to those obtained by sequencing the full RPO30 gene and also in agreement with our previous phylogenetic studies using the CaPVs' GPCR gene (Le Goff et al., 2009).

CONCLUSIONS

This study shows that the animal species origin of CaPVs can be determined by sequencing their RPO30 gene. Furthermore, using the 21 nucleotides deletion found only in all members of the SPPV lineage, a simple and quick PCR method was developed to differentiate CaPVs in small ruminants without the need for sequencing. Also, since it does not require the use of any genotype-specific primers or any multiplexing, this method is suitable for use in large scale screening and genotyping during disease outbreaks.

Table 1. List of Capripoxviruses used in this study.

	Strain name	Species of origin	Source/Accession number
1	LSDV Brahman	Cattle	OVI/South Africa
2	LSDV Atbara	Cattle	OVI/South Africa
3	LSDV Ismalia	Cattle	AGES/Austria
4	LSDV NW-LW	Cattle	AF409137
5	LSDV M14308	Cattle	OVI/South Africa
6	LSDV OP126402	Springbok	OVI/South Africa
7	LSDV 2006D19359	Cattle	OVI/South Africa
8	LSDV Nee	Cattle	AF325528
9	LSDV Haden	Cattle	OVI/South Africa
10	LSDV LW1959	Cattle	AF409138
11	GTPV Denizli	Goat	VCRI-Pendik/Turkey
12	GTPV Gorgan	Goat	Pirbright/UK
13	GTPV G20LKV	Goat	AY077836
14	GTPV Pellor	Goat	AY077835
15	GTPV Oman	Goat	Pirbright/UK
16	GTPV Bangladesh	Goat	Pirbright/UK
17	GTPV India	Goat	Pirbright/UK
18	GTPV Yemen	Goat	Pirbright/UK
19	GTPV Ghana	Goat	Pirbright/UK
20	GTPV Saudi Arabia	Goat	Pirbright/UK
21	SPPV A	Sheep	AY077833
22	SPPV Van	Sheep	VCRI-Pendik/Turkey
23	SPPV Niskhi	Sheep	AY077834
24	SPPV TU	Sheep	AY077832
25	SPPV Denizli	Sheep	VCRI-Pendik/Turkey
26	SPPV Çorum	Sheep	VCRI-Pendik/Turkey
27	SPPV Darica	Sheep	VCRI-Pendik/Turkey
28	SPPV Nigeria	Sheep	Pirbright/UK
29	SPPV KS1	Sheep	AGES/Austria
30	SPPV OMAN	Sheep	Pirbright/UK

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Rift Valley Fever, Disease Ecology and Early Warning

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ABSTRACT

Rift Valley fever (RVF) once again dramatically affected the Horn of Africa (Kenya, Somalia, and Tanzania) in 2006–2007. This outbreak was linked to unusual rainfall associated with climatic events (El Niño) which affected the populations of the mosquitoes acting as vectors and reservoirs of the disease. The disease also reappeared in Sudan in the autumn of 2007, following excessive rainfall driven by a post-El Niño and unusually warm sea temperatures in the Indian Ocean. In 2008, the disease affected southern African countries (Swaziland, South Africa) and islands in the Indian Ocean (Comoros, Mayotte Island, Madagascar). Based on near real-time climatic data, forecasting models and early warning systems were available at the continental level and proved to be efficient in raising the alert before the onset of the epidemic, at least for the coastal countries of eastern Africa. These recent events provided an opportunity to review the natural history of RVF, especially in some places where its ecology was poorly documented. FAO and WHO officers used outcomes from the different models and then identified gaps or needs that could be filled in order to improve the use of these predictions. A brainstorming meeting was organised in Rome in September 2008 to discuss adjustments and complementarities to the existing models, as forecasting and early warning systems are the keys that may provide a time window for preventive measures before amplification of the virus is out of control.

Key words: *Rift Valley fever, zoonotic disease, arthropod vectors, ecology, areas of risk, early warning systems.*

INTRODUCTION

Rift Valley fever (RVF) is a per acute or acute viral disease of domestic and wild ruminants, caused by a mosquito-borne virus and characterised by necrotic hepatitis and a hemorrhagic state, but infections are frequently unapparent or mild. The disease is considered more severe in sheep, cattle and goats, producing high mortality rates in new-born animals and abortion in pregnant animals. However, there are within- and between-species variations in the manifestation of disease, depending on several factors including herd immunity levels (FAO, 2003).

RVF is a zoonotic disease and the vast majority of human infections result from direct or indirect contact with the blood or organs of infected animals while human infections have also resulted from the bites of infected mosquitoes. Infection in humans is usually associated with mild to moderately severe influenza-like illness. While most human cases are relatively mild, a small percentage of patients develop a much more severe form of the disease. This usually appears as one or more of three distinct syndromes: ocular disease (0.5%–2% of patients), meningo-encephalitis (less than 1%) or hemorrhagic fever (less than 1%). The case-fatality ratio for patients developing the hemorrhagic form of the disease is high at approximately 50% (WHO, 2007).

RVF was first identified by Daubney in 1931 after heavy seasonal rains in the Rift Valley in Kenya between lakes Naivasha and Elementaita (Daubney et al., 1931). It has been reported in most sub-Saharan countries, as well as in Mauritania, Egypt and the Indian Ocean islands (Comores, Madagascar) and has also spread in the Middle East (Saudi Arabia, Yemen).

The virus may be transmitted by a very large number of arthropods. Thirty-eight species of mosquito have been found infected in nature, of which at least 35 have proved their vector competence in controlled conditions (European Food Safety Agency, 2005). For mosquitoes only, six genera are represented in the first list: *Mansonia*, *Anopheles*, *Coquillettidia*, *Eretmapodites*, *Culex, Aedes* inc. *Ochlerotatus.* Some species of the latter two are considered to be the main vectors (McIntosh et al., 1980; Meegan et al., 1988). In addition, mechanical transmission has been demonstrated with other haematophagous insects, including *Stomoxes*, phlebotomies or *Culicoides* midges. The wide diversity of arthropods from which the virus has been isolated emphasises the difficulty of understanding the epidemic/endemic cycle of the virus.

ECOLOGY OF RVF

Endemic versus Epidemic

RVF endemic cycles occur in temperate, tropical and sub-tropical zones of Africa. The virus is capable of inhabiting a variety of different bioclimatic conditions, including: wet and tropical areas, e.g. Ivory Coast and Congo; hot and arid areas, e.g. Yemen or Chad; and irrigated regions, e.g. the Senegal River valley and the Nile Delta (Chevalier et al., 2004). Most RVF viral activity is cryptic, at a low level, and not associated with detectable disease in humans and animals. Many African countries have found significant sero-prevalence in sheep, goats and cattle for the RVF virus throughout various agro-climatic zones in their country, without clinical signs being reported in humans or in animals (Davies, 2006). Most countries are not really

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aware of the circulation of the virus because of a lack of systematic surveillance activities.

At irregular intervals of about 5–12 y, large epidemics of RVF have occurred in southern and eastern Africa and these epidemics have been associated with above average rainfall, huge activity of the vectors and the presence of susceptible livestock. The 1997–1998 epidemic in the Horn of Africa, which is considered as one of the most devastating RVF epidemics in East Africa, was associated with torrential rains (60–100 times the seasonal average) that resulted in the worst flooding in the Horn of Africa since 1961 (WHO, 1998). Flood plain zones are particularly affected, as are any areas where the water table has been raised by irrigation or other water conservation practices and serves as breeding habitats for mosquito vectors of RVF. Because climatic events tend to occur over large areas, there is a tendency for outbreaks to occur simultaneously in adjacent territories.

Throughout its wide ecological range, the pattern of RVF activity is much influenced by the ecological characteristics of the particular biotope under consideration. The agro-climatic zones of Africa can give a good guide as to the expectations from RVF virus activity (Davies, 1998) and can be schematically summarised as follows:

- enzootic activities (+/-cryptic) in high rainfall forests and forest edges;
- periodic increased virus activity to epizootic proportions in bushed and wooded grasslands;
- rare but explosive epizootic RVF activities in dry grassland and semi-arid zones associated with flood plains;
- very rare but long lasting RVF outbreaks in animals and humans following introduction of viraemic animals in irrigation scheme areas (**Table 1**).

Persistence and Spread of RVF Virus

Current evidence suggests that the RVF virus in sub-Saharan Africa is maintained in inter-epidemic periods, primarily by transovarial transmission in *Aedes* mosquitoes (Linthicum et al., 1985). *Aedes* eggs can resist desiccation for long periods and hatch in water inundation, such as occurs following prolonged rainfall or flooding. *Aedes* mosquitoes are thought to be the reservoir of the RVFV between those epidemics triggered by above-average rainfall that leads to a rapid increase in vector numbers. Once infection has been amplified in livestock, secondary epidemic vectors such as *Culex* or, *Anopheles* mosquitoes that breed in semi-permanent pools of water and get infected by biting infected vertebrates can become involved in transmission and some, like *Culex*, serve as excellent secondary vectors if immature mosquito habitats remain flooded for long enough (Linthicum et al., 1985).

It is not always clear if the RVFV is maintained between epidemics or is re-introduced. In some sub-Saharan countries, it has been speculated that limited *Aedes* populations may still play a role in maintaining a low level of viral transmission to livestock every year (Chevalier et al., 2005). Recent studies conducted in the Ferlo region of Senegal demonstrated that several generations of *Aedes vexans* can emerge during the same rainy season (Mondet et al., 2005), depending on the succession of rains and dry periods and consecutive changes in the water levels of temporary ponds. This mechanism could be the way the disease persists at low incidence in livestock.

The persistence of the activity of the virus is also modulated by the immunity of the affected host populations. Classical epidemic curves show a few weeks/months decrease in incidence after the peak. Depending also on the natural resistance of the animals, and subsequent level and duration of viraemia, the outbreak picture can significantly differ. In the irrigated highly cultivated area of Gazeera in Sudan, the abundance of so-called secondary vectors and a significant proportion of naive exotic dairy herds were favourable for an outbreak in 2007 that lasted for several months.

THE 2006–2009 OUTBREAK IN EAST AND SOUTHERN AFRICA

An updated map of the distribution of RVF outbreaks is given in **Figure 1**.

Kenya

By mid-December 2006, the disease emerged in its epidemic form in the North-Eastern Province of Kenya, where the 1997–98 outbreak was also centred (WHO, 1998). By the middle of March 2007, 684 human cases were reported by the Ministry of Health, including 155 deaths. The disease started in the Garissa District and half of the cases were reported in the North-Eastern Province (also in the Ijara and Wajir Districts). It then appeared in one district of the Rift Valley Province (Kajiado); five districts of the Coastal province (Kilifi, Tana River, Malindi, Isiolo, Taita Taveta); two districts

Table 1. RVF related characteristics of different ecological systems potentially suitable for RVF occurrence.

Ecological Zone	II	III	IV	V	VI
Vegetation	Forest & derived grasslands	Bushed & wooded grasslands	Dry grassland	Semi-arid lands	Irrigation scheme
Virus activity in inter-epizootic period	Yes	Possible every 3–5 y	Unlikely, unless floods	No-except in riverine flood zone	No-except if viraemic animals are imported
Mosquitoes population	Constant and diverse	Seasonal	Explosive	Explosive	Constant: *Culex, Anopheles*
Herd Immunity	Stable	Stable	Low	Very Low	Very Low
Epizootic virus activity	Yes	Yes	Usually	Not usually	Not usually
Duration of Epizootics	Peak 3–6 months; Cases 1–3 y	Peak 3–6 months Some foci one y	3–6 months	3–4 months	6–10 months
Periodicity of Epidemics	3–15 y	5–15 y	5–25 y	25–35 y	10–25 y

Adapted from (Davies, 1998). The ecological zones have been defined by Pratt et al. (1966).

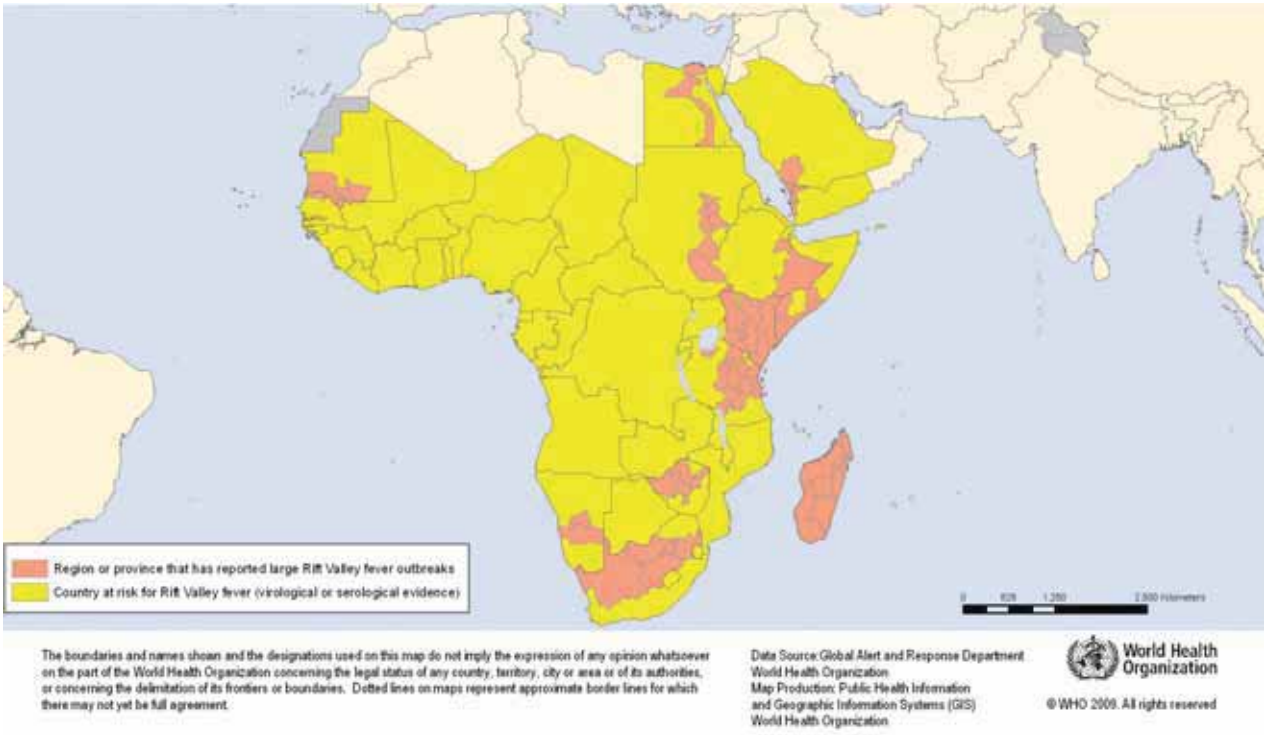

Figure 1. Location of large Rift Valley fever outbreaks and infected areas (the map includes historical and recent reports of RVF in human and/or animals).

of the Central province (Kirinyanga, Marangua), two districts of the Eastern Province (Banngo, Nakuru); and one district in the Nairobi area (WHO, weekly epidemiological record, 18/05/2007). Bans on livestock slaughtering and vaccination of livestock with live attenuated vaccine were put in place.

Somalia

In mid-December 2006, suspected cases were also reported to WHO from the Lower Juba Region. Investigations were limited by the civil unrest, but a final total of 114 human cases were reported, including 51 deaths. The geographical distribution of cases was mainly in the Lower Juba Region, followed by the Gedo Region, the Hiran Region, the Middle Juba Region, the Middle Shabelle Region and the lower Shabelle Region (WHO, weekly epidemiological record, 18/05/2007).

Tanzania

In mid-January 2007, RVF was suspected in Tanzania in both animals and humans in the Arusha Region in the north of the country. The disease was then reported in the central part of the country, including the regions of Dodoma, Iringa, Manyara, Morogoro, Mwanza, Pwani, Singida and Tanga. At the beginning of May, there were 290 reported human cases, including 117 deaths. Clinical suspicions in livestock also concerned southern provinces bordering Mozambique, Malawi and Zambia. It should be noted that the previous epidemic in 1997–98 also started in the northern provinces, but did not affect the central region of the country. However, field evidence of high prevalence of antibodies in livestock in 2007 in the south-west of the province suggests that the disease is probably widely endemic in Tanzania and not a recent introduction from Kenya, as was initially believed (B. Swanepoel, pers. comm.).

Sudan

WHO reported an RVF outbreak in humans in Sudan at the beginning of November 2007. A total of 738 human cases, including 230 deaths, were officially reported in White Nile, Sinnar and Gesira States. Some serological and virological analysis conducted on animal samples revealed the presence of specific antibodies and RVF virus, confirming, if it were necessary, the circulation of the virus in livestock.

Neighbouring Countries

Animal cases were reported from Burundi and Swaziland in June 2008 while, surprisingly, no cases were reported from Ethiopia, Zambia, or Mozambique. During the first few months of 2008, small focal outbreaks of RVF affecting mostly captive buffalo, cattle and small ruminants were also reported in South Africa, as well as about 20 human cases.

Comoros and Mayotte Islands

In July 2007, a 12 year-old child originating from Moroni, Comoros, was hospitalised in Mayotte and found positive for RVF. Clinical suspicions in small ruminants were also reported in Comores (unpublished data). Eight human cases were then reported on Mayotte between September 2007 and May 2008. A serological survey in cattle in 2008 confirmed the active transmission of the disease on Mayotte (23% of herds affected). The origins of the disease in these islands have never

been clearly established, but seem to involve illegal trade of livestock from Tanzania (Cêtre-Sossah et al., 2009).

Madagascar

Central Madagascar experienced events of cattle mortality in December 2007. By the end of January 2008, human cases were reported in Tolagnaro city in the south and almost simultaneously in the highland Anjozorobe District, 80 km north of Antananarivo and on the eastern coast of Madagascar. The outbreak lasted until June 2008, and a total of 476 suspected human cases were reported from 15 districts, with 19 deaths from the disease. The disease was then again detected in cattle in October 2008 in the Fianarantosoa Districts, central highlands, and 236 cases were suspected from four contiguous districts, where seven people died. The real incidence of RVF in Madagascar was difficult to establish over the course of the outbreak because of poor reporting channels and the limited capacities for active surveillance in livestock. Recently, a serological investigation carried out among cattle sampled after the 2008 RVF outbreak confirmed the wide distribution of recent antibodies on the island with high prevalence in Toliara, the traditional livestock breeding areas of Madagascar (E. Jeanmaire, in preparation). A national cross-sectional serological survey among people working in slaughterhouses confirmed that the virus had circulated in at least in 92 of the 111 districts of the country (Andriamandimby et al., in preparation).

AREAS AT RISK

Persistence and Emergence

Certain types of environment may be favourable to the persistence of the virus and this could explain why the disease re-emerges from the same sites. Studies in Kenya have shown that flooding of grasslands may be important in the generation of RVF epidemics (Davies, 1975). In this country, the typical dambo habitats are shallow depressions often located near the head of a drainage system, associated with tall grasses, sometimes bushes or wooded savannah. They form temporary ground pools, varying from tens of metres to several km in length and remain dry for prolonged periods (y). The previously cited hypotheses regarding the possible reservoir role species from the *Aedes* genus (i.e. *Ae. cumminsii*, *Ae. circumluteolus, Ae. mcintoshi*) have derived from studies of mosquito ecology within these ecotypes that are abundant in the Garissa area (Linthicum et al., 1984; Linthicum et al., 1985).

In Sudan, the Kosti region 200 km south of Khartoum in the White Nile Province consists partly of low lying areas with many swamps and small tributaries of the White Nile. A biotype particular to the Sudan is the toich, seasonally flooded grassland in the catchment areas of the White Nile and its tributaries, which form a large part of the southeast of the country. After 6–12 weeks of flooding, these areas are extensively grazed by cattle. The disease is endemic in most parts of the Sudan (ecological zones II, II and IV) and the last major epidemic in 1973 also started in the Kosti agricultural district, and then extended as a devastating epizootic to the Upper and Blue Nile provinces (Eisa et al., 1977). Additionally, in June 1976, clinical signs and serological evidence of RVF in cattle and humans were reported in a dairy farm in Khartoum North, following the introduction of new cattle from the White Nile province (Eisa et al., 1980).

In Madagascar, the epidemiology of RVF is still very poorly understood. The recent outbreak occurred during a low rainfall period. In the past, RVF had already affected Madagascar in 1990–91 (Morvan et al., 1991), and it was established that the virus circulating in 2007–2008 is similar to the one isolated in the Horn of Africa in 2006–2007 (Andriamandimby, in prep). Therefore, while the origin of the disease in Madagascar has not been clearly established, it may involve illegal trade of livestock from the Horn of Africa (Kenya, Tanzania or Comoros). No cases were reported during the long inter-epizootic period, but anecdotic serological investigations revealed contacts of humans and animals with the virus in Toliara and the south-western provinces (Zeller, 1998), feeding the hypothesis of a low-level circulation of the virus and/or the possible existence of endemic areas. This area contrasts with the rest of the island in terms of ecotype, with agro-ecoclimatic patterns similar to those of the endemic areas of Kenya (Le Houérou et al., 1989). In addition, investigations conducted during the course of the 2008 outbreaks revealed that herds in the area around Toliara experienced a high rate of unreported abortions the year before and a significant number of sero-positives confirmed that these animals suffered from RVF several months before any report of cases (FAO, unpublished data).

Primary versus Secondary Foci

From endemic areas, the disease may spread with livestock movements and the introduction of viraemic animals in suitable areas (e.g. irrigation schemes). At the primary focus site the virus is maintained in the vectors and/or in the hosts while in secondary foci the virus is imported and spread between naïve ruminants thanks to local mosquitoes that are competent vectors for RVF (*Culex, Anopheles*…). During the 2007–08 outbreak in Sudan, the disease reached the irrigated area of Gazeera through sheep, camels and livestock on their way to the capital and the northern coast, from where hundreds of thousands of sheep are exported to Mecca for the Hajj. The presence of high densities of vectors and the abundance of naïve ruminant populations including highly susceptible exotic dairy cattle, acted as highly favourable factors for the amplification of the virus, with high impact on humans. The same applied for the Ifakarra rice valley in Tanzania, 2007, and in Madagascar in 2007–08. In Madagascar the cases were initially detected in the highlands, but the infection may have been imported from the livestock breeding areas in the south and north-west when the animals were transported to meat markets around the capital or to be used as oxen in the rice fields, where the mosquitoes are abundant. **Figure 2** schematically illustrates these different epidemiological processes.

The differentiation between primary and secondary foci is made more complex by the usual absence of clear chronology in cases reporting and the poor quality of reports, especially on the veterinary side. In addition, no systematic system of traceability or identification exists in most of these countries, and the history of an event is often difficult to document in detail. Other economical, sociological or cultural factors may also make the investigation even more complex.

DISEASE FORECAST AND EARLY WARNING

Land tenure, vegetation activity and some bio-climatic parameters can be captured by high level resolution satellite observations Associated with other data, they have been tentatively used to determine weather events or areas at risk and are of particular interest for RVF forecasting

Map of Suitability for Presence of RVF

Data derived from the AVHRR-NOAA satellites, including land surface temperature and NDVI data are processed and associated with a Digital Elevation Model (DEM), then confronted with a set of disease presence and absence points. Non-linear discriminant analysis captures the characteristics of sites of disease presence and absence, and these are used to define the status within a multivariate space of

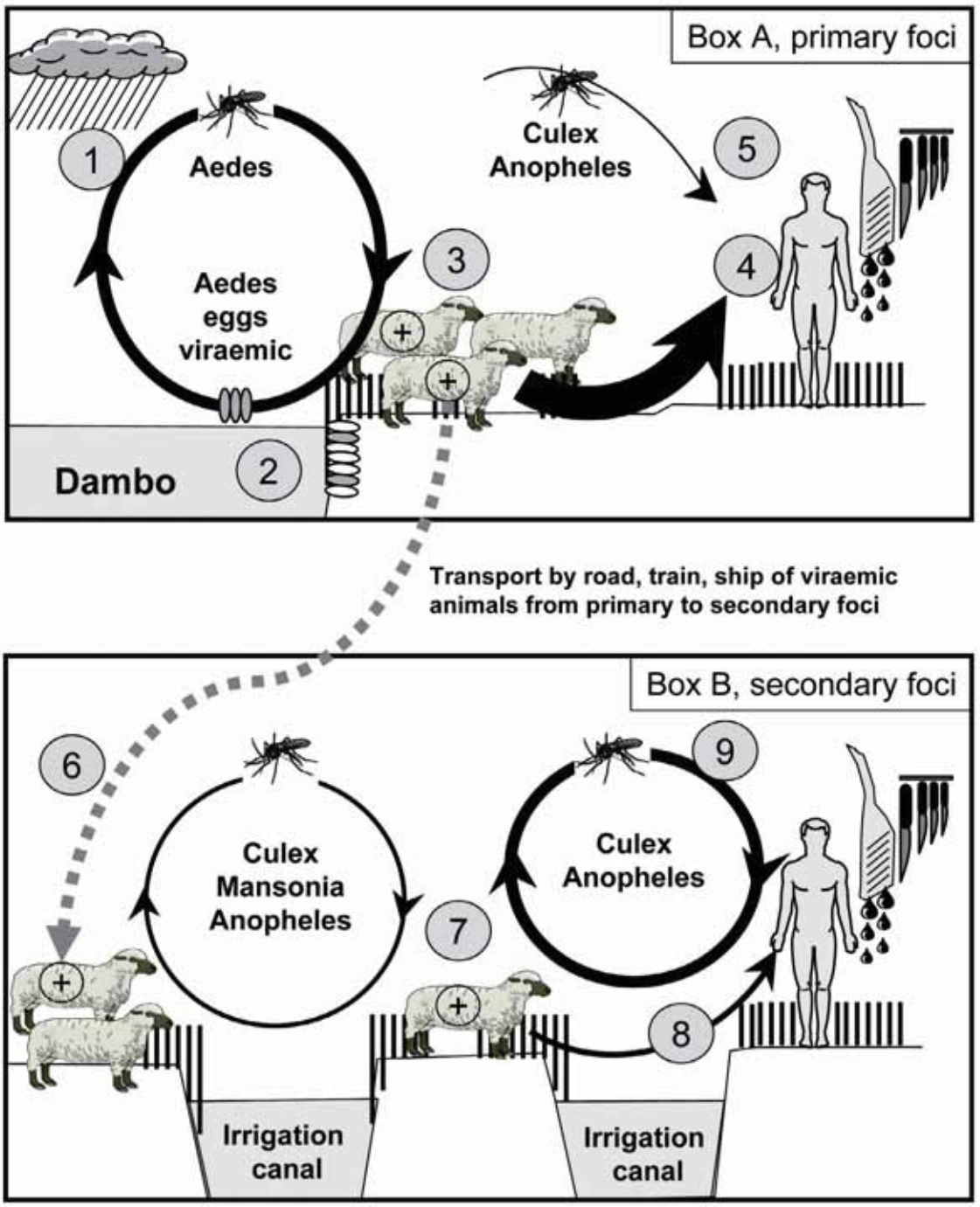

Figure 2. Schematic transmission of Rift Valley fever (Credits: P. Formenty, WHO).

Box A, primary foci — (1) Following heavy rainfall, dambos with *Aedes* breeding sites are flooded for at least a 10–15 days period of time. (2) A portion of the infected *Aedes* eggs leave dormancy and after hatching adults emerge infected with RVF virus. (3) *Aedes* females mosquitoes are aggressive mammophilic blood feeders and bite surrounding domestic ruminants (cattle, camels, sheep, goats) that serve as amplification host for the virus. RVF animal outbreaks are characterised by massive abortion of females and death of young ruminants (4). The large majority of human infections result from direct or indirect contact with the blood or organs of infected animals during slaughtering or butchering, assisting with animal births, delivering veterinary care or from the manipulation of fresh carcasses or foetuses. (5) Later in the course of the outbreak, secondary vectors such as *Culex* breeding in stagnated waters also contribute in the transmission of RVF virus to humans after acquiring the virus during a previous blood meal on an infected animal. The + within the circles indicates RVF infected sheep.

Box B, secondary foci — (6) Domestic animals infected with RVF at the primary site are imported to irrigation areas by route, train or ship. (7) Irrigation schemes are persistent habitats for *Culex, Mansonia* and *Anopheles* mosquitoes, whose abundant populations increase the circulation of the virus between amplifying hosts (cattle, camels, sheep, goats). (8) As in primary foci, human infections may result from direct or indirect contact with the blood or organs of infected animals. (9) However, most of the human infections result from bites from infected mosquitoes having acquired the virus during a previous blood meal on an infected animal.

any point within the risk-mapped area. On this basis, the probability with which the point belongs to the category of disease presence or absence is calculated and this probability is entered into the final risk map. This approach has been successfully used to draw maps of suitability for presence with various diseases or vectors, including Rift Valley fever (Rogers, 2006). However, the dataset used for the latter exercise is derived from a published literature search covering the period from 1968 to 2004 and therefore is far from exhaustive.

Monitoring Rainfall Anomalies

RVF outbreaks in East Africa have been associated with warm phases of the El Niño/Southern Oscillation (ENSO) that are usually a result of very heavy rainfall after a few weeks of drought. It has proved possible to predict periods of RVF epizootic activity in East Africa using data acquired by remote sensing, allowing national and regional monitoring of the rainfall and climate patterns (Linthicum et al., 1990). In operation since 1999, the monitoring and prediction system developed by NASA's Goddard Space Flight Center relies on interpretation of rainfall and analysis of NDVI anomalies to map areas showing conditions that would support RVF vector emergence, production and propagation (Linthicum et al., 1999). This system has a pre-epizootic predictive period of two to five months before virus activity occurs and has enabled the successful development of forecasting models and early warning systems for RVF. It processes in a Potential Epizootic Areas Mask (PEAM), based on RVF literature survey and climate variable (rainfall and NDVI) thresholds to derive what is referred to as potential epizootic area. These maps are made public on a monthly basis (http://www.geis.fhp.osd.mil/GEIS/SurveillanceActivities/RVFWeb/indexRVF.asp) but warnings are sent to key partners, including WHO and FAO in real time.

Early Warning in Real Life

In early autumn 2006, these models confirmed a sharp increase in the probability of observing heavy rainfall and floods in high-risk areas of Kenya, Somalia, Tanzania, Sudan and Ethiopia (Anyamba et al., 2009). The first warnings of a potentially serious outbreak were sent by the Nasa/Goddard Space Flight Center in mid-September. FAO and WHO forwarded this warning to their representatives in the at-risk areas and a consolidated warning was made public in November (FAO, 2006).

FAO and WHO, in collaboration with other national and international organisations (CDC, Pasteur Institute, OIE, UNICEF etc.) and national authorities in the countries were very involved in the emergency response. National action plans were reviewed, disease control activities were implemented and a significant amount of resources allocated to increase disease detection and surveillance. As a result, the existing models can now benefit from recent and unique data on both humans and animals, allowing adjustments and complementarities. At the same time, FAO and WHO officers had the opportunity to widely use outcomes from the different models to identify gaps or needs that could be filled in order to improve the use of these predictions in an integrated disease prevention and control perspective. In September 2008, experts on RVF modelling and forecasting were invited by FAO and WHO to a two-day workshop to share experience, identify gaps and explore potential improvement in the models, in order to further adapt outcomes to fit with the needs of the disease control strategies. The objective of this workshop was to review the natural history of RVF, review the forecasting models and risk distribution maps available and being developed, and propose a roadmap to improve these tools. The final goal was to define a roadmap for the development of tools for forecasting and real-time RVF outbreak management.

The conclusions of this meeting are available at: ftp://ftp.fao.org/docrep/fao/012/ak144e/ak144e00.pdf. Improvements and adjustments were proposed as follows:

- the model on suitability for presence could be improved significantly by completing the dataset and by distinguishing the primary foci (i.e. the area where the disease emerged) from secondary foci (e.g. where the disease was later introduced by animal movement). An updated dataset including human and animal outbreak data, and distinguishing primary from secondary foci, is currently being prepared by WHO and FAO;
- a MODIS-based mapping algorithm could be applied to capture the flood dynamics. With improved flooding maps (where, when and duration), statistical spatial analysis could be developed (using historical data on RVF outbreaks) and applied in order to better identify risk areas and risk conditions for the emergence of RVF;
- the explicit incorporation of rainfall into the climate-based risk mapping component, would enable improvement of the risk mapping through a ranking of risk based on accumulated rainfall;
- the map of suitability for presence could help in improving the Potential Epidemic Area Mask as some of the RVF outbreaks along coastal Kenya in 2006–2007, in South Africa in 2008, or in central Madagascar in 2008–2009 were outside the currently used PEAM;
- the use of existing climate forecasting models should be further explored.

In conclusion, RVF outbreak warning messages were sent in 2006, but the implementation of preventive measures and key control activities was difficult in this context, for various reasons, including a decrease in awareness and resources dedicated to RVF, lack of clear regional strategies and common contingency plans at the country and regional levels. FAO and WHO are currently preparing guidelines and options to improve the level of preparedness and the capacities of the countries for early response. Part of this effort is dedicated to early warning, as it is a key point that may provide a time window for preventive measures, before the amplification of the virus is out of control.

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Epidemiological Survey of Bovine Pleuropneumonia in Mali

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ABSTRACT

Now that rinderpest has been eradicated, contagious bovine pleuropneumonia (CBPP) remains the most important infectious disease of cattle in many tropical African countries including Mali. It is considered as a priority disease by the World Organization for Animal Health, the African Union /Inter-African Bureau for Animal Resources, the Pan-African Programme for the Control of Epizootics (PACE) and the FAO-Emergency Prevention System for Transboundary Animal Diseases (EMPRES). All these institutions and programmes have recognised that the lack of sufficient epidemiological data on this disease is an obstacle to implementing efficient plans for its control. Therefore, research must be conducted in order to generate the accurate data needed to prepare a technically appropriate and well coordinated control programme. The present epidemiological study was conducted in Mali with the aim of evaluating the seroprevalence of the disease in cattle and its geographical distribution. Serum samples collected from 7 628 cattle in different parts of the country were tested for the presence of specific antibody against CBPP by a c-ELISA. In parallel to this serological study, data on CBPP outbreaks and abattoir lung seizures over a 10-year period (1997–2006) were collected. Preliminary results indicate a national seroprevalence rate of 16.28% (1 242/7 628) and a herd seroprevalence rate of 85.18% (161/189). In general, both rates correlated and were relatively higher in the central and southern regions (17.77–28.33%; 89.29–100% respectively) than in the northern and western regions (4.63–11.89%; 60–83.33% respectively). The total number of outbreaks reported from the field was 121, while the number of suspected lung lesions detected in abattoirs was 12 470. The distribution of these figures varied between the country's regions but did not correlate with the observed seroprevalence rates.

Key words: *Contagious bovine pleuropneumonia, epidemiology, seroprevalence, Mali.*

INTRODUCTION

Cattle husbandry plays an important role in the economy of many tropical African countries including Mali. It is important for the rural populations as it contributes to their well-being through the production of milk, meat, hides, animals and draught power. In many of these countries cattle are considered as a primary source of wealth. Cattle can therefore be used for household consumption or to generate cash income. It is also important for the well-being of urban populations since demand for milk and milk products are increasing steadily. At the national level, cattle husbandry plays an important economic role through exports to regional markets. Therefore, improving cattle production in these countries is a major objective of the governments, veterinary services, international organizations and institutions involved in development. To achieve this objective, the control of animal diseases is a necessary requirement.

Among a panel of cattle tropical transboundary diseases present in African countries including Mali, our main interest and expertise concern contagious bovine pleuropneumonia (CBPP). The disease is caused by *Mycoplasma mycoides* subsp. *mycoides SC* (*MmmCS*) and characterised by a severe fibrinous exudative pleuropneumonia. With the successful achievement of the rinderpest eradication programme, it is now considered as a priority disease by the World Organization for Animal Health (OIE), the African Union /Inter-African Bureau for Animal Resources (UA/IBAR), the Pan-African Program for the Control of Epizootics (PACE) and the FAO-Emergency Prevention System for Transboundary Animal Diseases (EMPRES). In addition, the veterinary services in Africa all agree that the incidence of CBPP is increasing. Paradoxically, despite the recognition of the importance of CBPP, no significant studies have been conducted to evaluate its prevalence and economic impact. This is a major limiting factor for designing cost-effective control strategies.

The objective of this study was to evaluate the seroprevalence of CBPP among cattle in Mali and its geographical distribution.

MATERIALS AND METHODS

Sampling Methods

The cattle population in Mali, which is the subject of this investigation, is estimated to be approximately 8 385 703 heads (Direction Nationale des Services Vétérinaires, 2008) and is distributed as described in **Table 1**. Each region is subdivided into prefectures ('circles'), making a total of 49; each prefecture or 'circle' is divided into communes, making a total of 703 communes; and each commune consists of a number of villages, giving a total of 12 044 villages. The capital city of Bamako, called the District of Bamako comprises 6 urban communes (www.dgemali.net).

According to this distribution of cattle and administrative structure in Mali, seven out of the eight regions (excluding the region of

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Table 1. Sampling frame according to the administrative structure and distribution of cattle in Mali.

Region	Estimated cattle population*	Number of prefectures	Number of communes	Estimated total number of sampling units**	Number of units sampled	Number of serum samples collected
Kayes	893 077	7	129	1 623	28	1127
Koulikoro	1 203 348	7	108	2 032	28	1112
Sikasso	1 336 681	7	147	2 324	28	1120
Ségou	945 908	7	118	2 280	28	1160
Mopti	2 347 997	8	108	2 107	33	1346
Tombouctou	848 633	5	52	1 035	20	800
Gao	722 848	4	24	414	18	723
Kidal	59 538	4	11	150	ND	ND
Bamako	27 673	6	6	79	6	240
Total	8 385 703	55	703	12 044	189	7628

* DNSV Annual Report, 2008; ** village or herd; ND = not done.

Kidal, with the lowest number of cattle) were studied; the District of Bamako was also included in this study. Consequently, all the prefectures (45) representing the seven regions, and the six communes of the District of Bamako were involved.

The sampling unit was the herd. Animals in the same village or communal corral which share common grazing fields or water bores were considered as a single herd. This means that the sample selection was based on villages (in the regions) and on corrals (in the District of Bamako).

In collaboration with the veterinary services, a total of 189 villages and corrals were purposively selected from the 45 prefectures and the six communes of Bamako District, based on their accessibility and the willingness of cattle owners to cooperate. From each herd at least 40 serum samples were collected taking ten animals from each of the following four age groups: 0–1 y, 1–2 y, 2–3 y and more than three years. A total of 7 767 serum samples were collected (**Table 1**).

Data Collection

A detailed questionnaire of the herd history (type of husbandry, disease status and vaccination history) and relevant data on the animals (age and sex) being sampled was completed at the sampling sites. In parallel, data on the number of outbreaks declared and the suspected lung lesions detected in abattoirs over a ten-year period (1997–2006) were also collected respectively from local veterinary services and from abattoir files.

Serum Collection and Serological Testing

Since vaccination against CBPP is practised in Mali, the date of the last registered vaccination against the disease exceeded three months at the time of sampling. Blood samples were collected by jugular vein puncture using sterile disposable needles and syringes. Samples were allowed to clot at room temperature and then centrifuged to harvest the serum that was aliquoted and stored at –20 °C until tested for antibodies (Ab) against *MmmSC*.

The *Mmm*SC-specific antibody (Ab) response was assessed in serum samples using a competitive enzyme linked-immunosorbent test (cELISA) (Institut Pourquier, Montpellier, France), according to the protocol provided with the kit. This test is recommended by the OIE as the standard serological method for CBPP diagnosis (Niang et al., 2006). Moreover, as the antibodies induced by T1/44 vaccines do not persist at a detectable level for more than three months after vaccination, this c-ELISA kit according to its manufacturer can be used for the detection of natural infections, even in areas where vaccination is used. For this reason, assurance was made that in all sites visited, the date of the last registered vaccination against the disease exceeded three months at the time of sampling.

Briefly, 90-well pre-coated microplates with *Mmm*SC antigens were used for the assay. Sera were diluted one in ten and incubated with the monoclonal antibody. The reaction was then revealed by a peroxidase anti-mouse conjugate and a tetramethyl benzidine (TMB) substrate. The reaction was stopped by addition of sulphuric acid solution and optical densities were read at 450 nm with a Titretek Multiscan Plus MKII microplate reader (Flow Laboratories, Finland) and recorded by the ELISA Data interchange (EDI) version 2.2 software connected to the reader.

Data Analysis

National and regional/District prevalences were estimated by calculating the proportion of sera giving a positive result over the total number of tested sera. The herd prevalence was estimated by calculating the proportion of herds with at least one positive animal over the total number of herds visited.

RESULTS

Results on the disease seroprevalence are presented in **Table 2**. Overall, the national prevalence was 16.28% (1 242/7 628), while the herd prevalence was 85.18% (161/189). The prevalence per region ranged from 4.63% in Tombouctou to 26.52% in Mopti, while herd prevalence ranged from 60% in Tombouctou to 96.97% in Mopti. In the Bamako District the prevalence was 28.33%, while the herd prevalence was 100%.

The prevalence of *MmmSC* reactors by age is given in **Table 3**. Analysis of the results reveals that animals aged between 2 and 3 y and above had higher prevalence rates than those of the youngest age groups.

A total of 121 outbreaks of the disease occurred during the period 1997–2006 (**Table 4**). Except in the region of Kidal and the District of Bamako where no cases were reported, all the other regions had reported outbreaks with the highest number being in the regions of Segou (30), Koulkoro (26), Kayes (21) and Sikasso (20)

Table 2. Individual and herd prevalence rates of CBPP antibodies in the different regions of Mali.

Regions	Sampled herds	Sampled sera	Positive sera	Individual antibody prevalence (%)	Positive herds	Herd antibody prevalence (%)
Kayes	28	1 127	125	11.09	21	75.00
Koulikoro	28	1 112	125	11.24	23	82.14
Sikasso	28	1 120	199	17.77	26	92.86
Ségou	28	1 160	245	21.12	25	89.29
Mopti	33	1 346	357	26.52	32	96.97
Tombouctou	20	800	37	4.63	12	60.00
Gao	18	723	86	11.89	15	83.33
Kidal	ND	ND	ND	ND	ND	ND
Bamako	6	240	68	28.33	6	100.00
Total	189	7 628	1242	16. 28	161	85.18

ND — not done.

Table 3. Prevalence of CBPP antibodies according to age groups in different regions.

Regions	Age group (y)											
	0–1 y			1–2 y			2–3 y			> 3 y		
	Sera	Pos.	%	Sera	Pos.	%	Sera	Pos.	%	Sera	Pos.	%
Kayes	280	21	7.50	280	30	10.71	280	41	14.64	287	33	11.50
Koulikoro	280	15	5.36	273	32	11.72	279	40	14.34	280	38	13.57
Sikasso	280	27	9.64	280	59	21.07	280	51	18.21	280	62	22.14
Segou	283	49	17.31	296	62	20.95	294	58	19.73	287	76	26.48
Mopti	355	69	19.44	343	93	27.11	328	103	31.40	320	92	28.75
Tombouctou	200	2	1.00	200	10	5.00	200	11	5.50	200	14	7.00
Gao	190	29	15.26	181	21	11.60	180	19	10.56	172	17	9.88
Kidal	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D. Bamako	60	11	18.33	60	18	30.00	60	20	33.33	60	19	31.67
Total	1928	223	11.56	1913	325	16.98	1901	343	18.04	1886	351	18.61

ND = not done.

Table 4. Summary of annually reported CBPP outbreaks in the different regions of Mali (1997–2006).

Year	Number of reported outbreaks per Region									
	Kayes	Kkro	Sikasso	Segou	Mopti	Tmbtou	Gao	Kidal	Bamako	Total
1997	6	3	3	3	0	0	0	0	0	15
1998	1	2	0	5	1	0	0	0	0	9
1999	2	3	0	5	2	0	0	0	0	12
2000	4	1	2	3	1	1	0	0	0	12
2001	1	7	3	2	0	1	1	0	0	15
2002	1	0	3	1	0	0	0	0	0	5
2003	1	5	4	3	1	0	0	0	0	14
2004	2	0	2	2	0	0	1	0	0	7
2005	2	3	1	4	1	3	8	0	0	22
2006	1	2	2	2	0	2	1	0	0	10
Total	21	26	20	30	6	7	11	0	0	121

Table 5. Summary of the annually reported CBPP lung seizures in the different regions of Mali (1997–2006).

Year	Number of reported lung seizures per region									
	Kayes	Kkro	Sikasso	Segou	Mopti	Tmbtou	Gao	Kidal	Bamako	Total
1997	967	104	0	13	0	0	0	0	0	1084
1998	1 074	132	0	67	0	0	0	0	0	1 273
1999	1 239	140	0	30	0	0	0	0	0	1 409
2000	925	135	0	59	0	0	0	0	0	1 119
2001	1 266	137	4	46	0	0	0	0	0	1 453
2002	1 142	136	0	49	0	0	0	0	0	1 327
2003	970	135	14	20	0	0	0	0	0	1 139
2004	1 039	135	0	17	0	0	0	0	0	1 191
2005	1 029	145	41	42	0	0	0	0	0	1 257
2006	1 049	122	7	36	5	0	0	0	0	1 219
Total	10 700	1 321	66	379	5	0	0	0	0	12 470

and the lowest number registered being in the regions of Mopti (6), Tombouctou (7) and Gao (11).

A total of 12 470 lungs were seized due to CBPP lesions during the 1997–2006 period (**Table 5**). Most seizures occurred in the regions of Kayes (10 700 cases), Koulikoro (1 321 cases) and Segou (379 cases). Conversely, the regions of Kidal and the District of Bamako did not report any cases.

DISCUSSION

This first nation-wide serological survey has provided data for a better assessment of the prevalence of CBPP in Mali. Serum samples collected from 7 628 cattle in different parts of the country were tested for specific antibodies against CBPP by c-ELISA. The test is recognised by the OIE as reference test for the serodiagnosis of CBPP (Niang et al., 2006), but like any serological test, it has its limitations since its does not differentiate between antibodies from vaccinated and non-vaccinated animals. Nevertheless, according to the manufacturer, this kit can detect antibodies after infection even in areas where vaccination is practised because it is accepted that the antibodies induced by T1/44 vaccines do not persist for more than three months at a level detectable by this test in animals vaccinated against CBPP. Therefore, the positive results obtained in this study likely reflect the presence of specific antibodies post-infection since at all sites visited, the last recorded vaccination against CBPP was more than three months before the time of sampling.

The survey showed the prevalence of the disease nation-wide, with higher individual and herd prevalence rates observed in the central and southern regions which include Mopti, Segou, Sikasso and the District of Bamako. This strengthens the view that these regions are endemic areas. This might be due to the fact that these regions happen to include many common dry pastures used by transhumant herds not only from neighbouring regions within Mali but also from neighbouring countries. Therefore the close contacts among healthy and carrier animals within these dry season pastures probably enhanced the transmission of the infection.

Highly noticeable was the high individual and herd seroprevalence rates observed in the capital District of Bamako (28.33% and 100%) where the communal corrals are supposedly well managed and the owners utilise the well-organised State and private veterinary services extensively to assist production. This is probably due to the presence in Bamako of many cattle markets where animals of unknown status converge. These cattle are purchased by cattle owners and then newly introduced into the communal corrals. The status of the region of Koulikoro (11.24% and 82.14%) which is located between the central and the northern part of Mali is somewhat confusing but may be explained by the presence of common pastures in its northern part which are used by transhumant herds coming from Mauritania.

In contrast to the above regions, prevalence rates were relatively lower in the northern and western regions which include Toumbouctou, Gao and Kayes. Although these regions have some common pastures used by transhumants, the concentration of animals coming from neighbouring countries is not as high as that of the southern and central regions. Also, in contrast to the southern and central regions, the climate in these regions is harsh and might not be favourable for the long-term survival of the *Mycoplasma* organisms.

The results of the survey also revealed that older animals had the highest prevalence rates, suggesting that adult cows are more susceptible than young animals which tend to develop joint lesions instead of pulmonary disease (Masiga et al., 1996).

The only statistics available on the epidemiology of CBPP in Mali are those provided by the DNSV which unfortunately include only the number of outbreaks from which morbidity and mortality rates are deduced. These data are clearly very incomplete and do not address the prevalence of the disease, its exact distribution per region and area and per age groups taking into account the structure of the herd.

Similarly few data exist at both regional and continental level. In the West African sub-region, only in Guinea has a limited serological survey been used to evaluate the prevalence of CBPP. This gave a prevalence rate of 6.3% (Diallo et al., 1999). A similar survey conducted in Eritrea showed a seroprevalence rate of 1.43% (T. Tekleghiorghis and U. Ghebremicael, 2004).

Analysing the number of outbreaks declared from the field and the suspected lung lesions detected in abattoirs, it appears that their distribution varied among regions but did not correlate with the observed seroprevalence rates. Indeed, highly noticeable was their low number nationwide, specifically in the region of Mopti which is considered to be a highly endemic area as shown by the results of the present serological survey. This may be due to

inadequate reporting of CBPP cases or to the refusal of herdsmen to report outbreaks because of the fear of quarantine or other sanctions. Also, lung sequestra indicating the chronic stage of CBPP may be mistaken by inexperienced meat inspectors for other lung lesions such as abscesses, tubercular nodules, *Echinococcus* cysts and farcy (nocardiosis) lung lesions.

CONCLUSIONS

The main objective of this study was to evaluate the seroprevalence of CBPP among cattle in Mali and its geographical distribution. Preliminary results indicate the prevalence of the disease nationwide, with higher individual and herd prevalence rates occurring in the central and southern regions of the country than in its northern and western regions. The total number of outbreaks declared from the field and the suspected lung lesions detected in abattoirs varied between regions but did not correlate with the recorded seroprevalence rates.

It is hoped that this study will pave the way for the other West African countries to undertake similar studies for the establishment of a successful sub-regional coordinated CBPP control programme.

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Surveillance of Antibodies to Bluetongue Virus in Livestock in Mongolia using C-ELISA: Preliminary Results

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ABSTRACT

A competitive enzyme-linked immunosorbent assay (C-ELISA) was used to conduct surveillance of bluetongue virus antibodies (BTV) in sheep, goats and cattle in Mongolia. The highest prevalence was recorded in goats (86%) followed by sheep (51%) and cattle (9%). The results are the first confirmation of the presence of such antibodies in Mongolian livestock. Studies are now underway to conduct more detailed investigations concerning bluetongue, including to determine the virus serotypes that are and have been circulating in the country.

Key words: *bluetongue, serosurveillance, C-ELISA, prevalence.*

INTRODUCTION

Bluetongue disease is a non-contagious, arthropod-borne viral disease of ruminants, mainly sheep and less frequently of cattle, goats, deer and antelope. The pathogenic virus belongs to the genus *Orbivirus* and is a member of the Reoviridae family. There are 25 serotypes. A midge, *Culicoides imicola* and other culicoid species are responsible for its transmission.

The disease can cause serious losses to small ruminant production as well as constituting yet another constraint to trade. Not suspected to have occurred in Mongolia, the disease was, until recently, limited in its geographical distribution to areas south of latitude 40°. However, it appears to be moving north as a consequence of climate change resulting from global warming and it is now established in Inner Mongolia of the People's Republic of China and in the north-east of that country. Also, an alarming spread north has occurred from the Mediterranean basin but, as yet, there is little information available about the situation from the eastern side of the Eurasian continental mass although there are anecdotal reports of bluetongue occurring in northern China (Manchuria) and Inner Mongolia close to the Mongolian border which are a cause for concern that a similar situation is developing there.

Our aim therefore was to establish the status of bluetongue in Mongolia as an early warning of possible future disease problems so that the country can prepare to protect its 24 million small ruminants which generate a significant proportion of income for livestock keepers.

MATERIALS AND METHODS

Blood Sampling

The serosurvey was planned between August and September 2007 and data collected over the following eight weeks followed by laboratory testing between November 2007 and August 2008. Results were analysed between November 2008 and April 2009. Blood samples were collected based on epidemiological statistical calculations and random selection (Putt et al., 1993) Administratively, Mongolia divided into 22 aimags (districts) which are subdivided into 332 soums (municipalities) comprising 1 386 bags. Eighty soums were chosen randomly from all aimags and two cities, the number of soums selected being proportional to the number of livestock. For each soum, six herds were selected at random, each defined on the basis that they had different grazing pasture, and from each selected herd, ten cattle, five sheep, and five goats were chosen at random.

A two-stage sampling method was chosen, the primary sampling unit being the soum and the secondary unit, the animal. Sample size was calculated to detect a prevalence of 5% at soum level with a 95% level of confidence. Overall, the survey involved 4 800 cattle, 2 400 sheep and 2 400 goats. Information was collected on the age, status, geographical location, name of owner, bag, soum and aimag/administration unit.

Testing of Sera and Calculation of Prevalences

Screening of sera for bluetongue was through a competitive (ELISA) using a horseradish peroxidase labelled bluetongue virus-specific monoclonal antibody for detecting BTV antibodies (test kit 5010.20, VMRD Inc., Pullman,USA). The kit has a demonstrated sensitivity of 100% and a specificity of 99%. Testing was conducted at the Department of Infectious Diseases and Microbiology, Mongolian State University of Agriculture.

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N.E. Odongo, M. Garcia & G.J. Viljoen (eds), Sustainable Improvement of Animal Production and Health. Food and Agriculture Organization of the United Nations, Rome, 2010: **341–342**

Table 1. Serological prevalence of bluetongue virus in livestock in Mongolia.

Species	Neg	Pos	Total	AP[1]	TP[2]
Cattle	4 277	427	4 704	9.1%	8.2%
Sheep	1 165	1 217	2 382	51.1%	50.6%
Goats	334	2 041	2 375	85.9%	85.8%
Total	5 776	3 685	9 461	38.9%	38.3%

[1] Apparent prevalence
[2] True prevalence

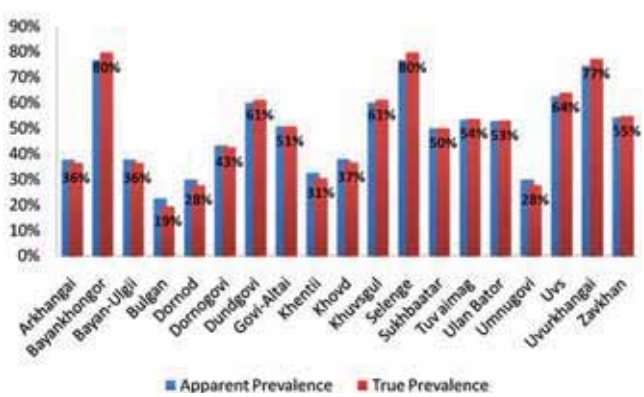

Figure 1. Apparent and true prevalence of bluetongue in sheep by aimags.

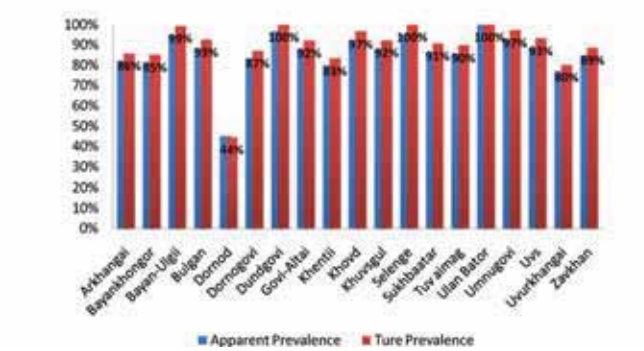

Figure 2. Apparent and true prevalence of bluetongue in goats by aimags.

RESULTS AND DISCUSSION

Results of the serological testing are summarised for each aimag and livestock species in **Table 1** and **Figures 1–2**. They show a wide variation in prevalence among the different aimags and that while bluetongue virus antibody was detected in all aimags and cities, highest prevalence was recorded in goats (86%) followed by sheep (51%) and cattle (9%).

However, because of BTV`s wide pathogenic variability and the fact that cross reactions may occur between other orb viruses, a positive ELISA result with the bluetongue group test does not mean that clinical signs were caused by BTV itself.

CONCLUSIONS

The results presented here record the first confirmation of BTV antibodies in sheep, goats and cattle in Mongolia. Future studies should be undertaken to investigate bluetongue in more detail, and in particular to determine the BTV serotypes that are and have been circulating in Mongolia.

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SESSION 4

ONE HEALTH

One Health: Is there a Need for a Global Research Agenda?

E. Schelling[1]* & J. Zinsstag

ABSTRACT

Zoonotic infections receive internationally more and more attention as neglected diseases impoverished communities. Professional organisations and government institutes have created joint public and animal health working groups and numerous surveillance and research and programmes under the umbrella of 'One Health'. Still, there remains a divide between human, animal and ecosystem health specialists. Because most zoonoses go unrecorded, a rethinking of research and control efforts and their economic consequences is needed. Innovative research approaches promise to better capture the impacts of zoonoses from a societal perspective through more comprehensive frameworks that consider benefits and costs of zoonoses control in different sectors, notably the public health, livestock and private sectors. Such cross-sectoral studies promise to foster communication and exchange of information between sectors. Building on established national and regional technical support agencies for avian influenza will be important, despite the challenges to capture the momentum of partnerships, to establish permanent dialogue between sectors and to create sustainable national and regional bodies. Regional and international research groups applying a 'One Health' approach are well placed to generate the data required on levels of under-reporting, disability adjusted life years (DALY) estimates of zoonoses and the epidemiological and financial information for analyses of costs and benefits and cost-effectiveness and thereby identify the appropriate option for zoonoses prevention and control in particular settings from a range of possible interventions.

Key words: *One Health, neglected zoonoses, DALY, cost-benefit, cost-effectiveness, control.*

BACKGROUND

Most zoonoses occur more frequently among the poor and the poor are more vulnerable to the dual burden of zoonotic infections in people and livestock (WHO, 2006). Poor communities are also characterised by co-infection of several zoonoses and co-morbidity with other severe diseases. Risk factors of cystic echninococcosis in people, for example, are low socio-economic conditions including education levels, poor diagnostic facilities, the lack of dog population control measures and the absence of anthelmintic treatment (Macpherson, 2005). Zoonoses now belong to the category of neglected (tropical) diseases (WHO, 2007). Many countries lack information on the distribution of zoonotic diseases.

Control of zoonoses and improved food safety are public goods and therefore the public sector is seen as an appropriate delivery channel (Ahuja, 2004) although the State may sub-contract tasks such as vaccination and meat inspection to the private sector. However, services in developing countries continue to be poor, particularly in sub-Saharan Africa. As a consequence of the acute human resources crisis affecting the health sector (Wyss et al., 2003) and the structural adjustment programmes followed by the privatisation of health and veterinary services, large areas are not covered by sufficient qualified public and animal health professionals to assure surveillance and reporting of new outbreaks.

Communication between physicians and veterinarians is nearly absent or very weak. Veterinarians and physicians perceive the risks of zoonoses differently in the same context. For example, veterinarians rather than physicians speak with people living with HIV about the zoonotic risks of pet keeping (Grant and Olsen, 1999; Kahn, 2006). One obstacle to improved communication between public health specialists and veterinary professionals — the basis for cooperation between the health and veterinary sectors — has also been the lack of a common measure for the importance of zoonoses. On the human health side, the disability-adjusted life year (DALY) has been developed as a standardised measure of disease burden in people combining the years of life lost (YLL) due to premature death and the years of life lived with a disability (YLD). Since their introduction in the mid 1990's, DALYs have been used to prioritise disease control in the health sector by comparing interventions on the basis of effectiveness to reduce disease burden. Yet, disease burden of zoonoses in terms of DALYs, and particularly global disease burdens, have so far been determined for very few zoonoses e.g. for echinococcosis (Budke, 2006). Huge gaps in reporting of disease outbreaks exist for individual countries (Cowen et al., 2006). Widespread underreporting of zoonotic diseases may explain why they do not score high from a total disease burden perspective and why they often do not figure high on international public health agendas.

ONE HEALTH INITIATIVES

In the past decade, the 'One Medicine' concept by Calvin Schwabe (Schwabe, 1984) has received new attention. By extension of the concept into aspects of health systems it evolved towards 'One Health' (Zinsstag et al., 2005). 'One Health' was adopted by the World Conservation Union in the Manhattan declaration considering that the survival of wildlife requires healthy animals and healthy people. The American Medical Association (AMA) and the American Veterinary Medical Association (AVMA) have created the 'One World One Health' initiative (Enserink, 2007; King et al., 2008). The Consultative

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Group of International Agricultural Research (CGIAR, the alliance of international agricultural research centres) has set up a platform on agriculture and health. The World Health Organization (WHO), the Food and Agriculture Organization (FAO) and the World Organisation for Animal Health (OIE) have cooperated through a global early warning system for animal diseases transmissible to humans (GLEWS) since 2006. The WHO Study Group on Future Trends in Veterinary Public Health, emphasising the need to expand the links between human and animal medicine, recognises that developing countries, especially in Africa, may lack the necessary technology, infrastructure and resources, leading to poor or non-existent surveillance and even inappropriate setting of priorities (WHO, 2002).

Other integrative concepts go beyond the direct animal-human interconnectedness and explore the relationships between various ecosystem components to define and value the priority determinants of health and human well-being (Forget and Lebel, 2001; Waltner-Toews, 2009). The drivers of emergence and re-emergence of diseases are related to disturbances of social and ecological equilibriums. Environmental changes can either not or hardly be reversed, for example pollution with heavy metals (Forget and Lebel, 2001). Ecohealth approaches have been conceptualised and promoted for many years by the International Development Research Centre (IDRC). Ecohealth can be defined as systemic, participatory approaches to understanding and promoting health and well being in the context of social and ecological interactions (Lebel, 2004). Patz et al. (2004) have summarised results that have been achieved with ecohealth approaches. An example is the recognition of fish becoming a human health risk in the Amazonas because with deforestation and soil erosion increased mercury loads were washed into the water.

Shared surveillance databases for disease events in animals and people are being developed such as the GLEWS by WHO, FAO and OIE, the Program for Monitoring Emerging Diseases (ProMED)-mail electronic early warning system (Cowen et al., 2006); other databases are designed to monitor antimicrobials and antimicrobial resistance or environmental toxicology (Zinsstag et al., 2009). In many countries working groups and task forces with members from both the health and livestock sectors were established for preparedness and control of avian influenza. Some countries, for example Ethiopia, started including other zoonoses in their mandate, which makes them permanent partners in a countries' disease control strategy (WHO, 2009). These countries have likely perceived the benefits and added value of coordinated work across sectors, and it is now important to sustain these efforts and the finances invested (US$ 2.7 billion until 2009) in preparedness and control of avian influenza.

The challenge then is to create functional linkages and better cooperation between the human and animal health sectors and aligning these with the needs and potentials of communities and policy makers, while acknowledging that a 'One Health' agenda should embrace ecological thinking. Coordinated working across and between sectors (i.e. 'intersectoral cooperation' for the achievement of a common goal is described below, involving primarily the public health and veterinary sectors, the private sector, and others for example the financial sector.

TAKING A MULTSECTORAL PERSPECTIVE

Communities play an essential role at the first level of any surveillance system (Jost et al., 2007) and are often excellent observers and know the priority diseases of humans and animals in their context. Thanks to livestock holders' reports on perceived poor anthrax vaccine quality, contamination problems in local vaccine production were detected in Chad (Schelling et al., 2008). Interdisciplinary working groups ideally include anthropological and social scientists along with physicians and veterinarians. Ownership by the communities for such endeavours is possible with their participation in knowledge generation as equal partners together with local authorities and scientists. Where an interdisciplinary team involves communities, authorities, lay people and local professionals with knowledge to a given question, then priorities, acceptable institutional and legal arrangements can be identified. Such a process with regular consultation of key partners from academia and society is a transdisciplinary process.

Joint field research studies by mixed public health-veterinary-livestock production-wildlife-plant health teams can serve as the nucleus for enhanced sharing of information. In former Soviet Union countries such as Kyrgyzstan, brucellosis was rather well controlled by veterinarians. However, after the break-up, the veterinary services lacked the needed organisation and human and financial resources. The health sector was then confronted with a sharp increase in human cases and criticised the veterinarians' inability to control the disease. In return, the veterinarians stated that the health sector exaggerated the seriousness of the problem. A representative sampling of people and their livestock then showed the current disease situation. In the aftermath of this survey, the health and veterinary sectors exchanged information more regularly (Zinsstag et al., 2009). Simultaneous surveys in people and their animals allow the recognition of the most important animal species involved in transmission to people (Schelling et al., 2003). In addition, joint studies in the human, animal and wildlife populations can provide the needed evidence for authorities to start joint preparedness and surveillance planning (Kuehn, 2006). However, in reality public health and veterinary governmental authorities often only start cooperating when faced with an outbreak of a disease.

Attempts to assess burdens of diseases in terms of DALYs for neglected zoonoses are hampered by missing knowledge from underreporting, economics and also epidemiology of the diseases. Several research groups work on new diagnostic tools for zoonoses because improved tools are essential for correct diagnosis, to estimate underreporting of zoonoses and to monitor control activities. Efforts go towards developing tests that provide an immediate result, which professionals need to make an appropriate decision, for example on treatment of a patient. These tests are called point of care/bed-side tests for people and pen-side tests for livestock. For example for brucellosis, bed-side and pen-side tests would allow brucellosis to be differentiated from other illnesses causing fever and thus to treat a patient with the recommended antibiotics, as well as to monitor control activities in livestock. Surveys are often needed to obtain an overview on the situation of a zoonosis in a country. Where there is no sampling frame with lists of sampling units, a multi-stage cluster sampling approach can lead to a representative sample. Costs are high for any survey. Therefore, alternative approaches for obtaining accurate estimates are explored such as ecological niche factor analysis and risk mapping.

A good example of an innovative approach is described by Cleaveland et al. (2002). To estimate human rabies deaths (where active detection of human often is too costly because the incidence is very low and passive surveillance of suspect dogs is insufficient [Kitala et al., 2000]), they have surveyed animal bites that are rather frequent. With a probability decision tree using information on bite location and follow-up of bites from suspected rabid animals, they could estimate human deaths. After validation of the approach in field studies, they could show that in rural Tanzania the true incidence of human rabies is ten to 100 times higher than reported.

Costs of uncontrolled zoonoses can be demonstrated in monetary terms for the livestock sector (reduced productivity and market losses), the public health sector (diagnosis, treatment, hospitalisation costs) and the private sector (patient or animal owner out-of-pocket

expenditures, opportunity costs). Where benefits from control within different sectors have been estimated, the cost could be distributed proportionally to the sectors according to their monetary benefit from control. When this comprehensive cross-sectoral analysis was applied, control interventions emerge as highly cost-effective for the public health sector i.e. US$ 25 or less per DALY averted (Roth et al., 2003; Budke et al., 2005; Knobel et al., 2005). Control of cysticercosis is one example of a neglected zoonosis that will likely benefit from a framework that assesses the impact of zoonoses across different sectors (Praet et al., 2009).

Research should also be directed towards demonstrating the additional cost-effectiveness of integrated control of multiple zoonoses compared with efforts focused on individual diseases. This would foster integrated services and control within the human health sector. For example, integrating vaccination of children together with distributing vitamin A, selling of insecticide-treated mosquito nets, de-worming and malaria treatments are encouraged by WHO and UNICEF because serving people with multiple interventions is more equitable and sustainable. Although prior evaluation of the cost-effectiveness of combined approaches provides the needed data for prioritising interventions (Grabowsky et al., 2005), there are so far no good examples of implementing a zoonoses control programme with funding from different sectors.

To achieve such cost-sharing between sectors, decision makers from relevant ministries such as the Ministry of Finances should clearly be involved early in the process of outlining financial flows.

However, health services for humans and animals are provided separately. Joint health service provision and information dissemination should nevertheless be considered since sharing of infrastructure, cold chain and personnel may widen the radius of operation of health and veterinary services in rural zones. McCorkle (1996) argues that an intersectoral approach combined with traditional, non-Western patterns for the joint delivery of basic healthcare services to both humans and animals would be more appropriate and feasible than imposing Western medical approaches alone.

CONCLUSIONS

Zoonoses are among the most important animal and public health problems that affect the well-being of societies worldwide, yet they are too often forgotten or neglected. Since most zoonoses go unrecorded, a rethinking of research and control efforts and the economic consequences is required. Despite the challenges to capture the momentum of partnerships between sectors, building on established national and regional technical support agencies for avian influenza will be important to establish permanent dialogue between sectors and to create sustainable national and regional bodies applying the 'One Heatlh' concept. A more widely, applied 'One Health' research agenda fosters South-South and South-North collaborations, which, in turn, accelerates exchange on lessons learned in different parts of the world. International bodies like the OIE , FAO and the WHO have joined forces to establish global standards for zoonosis surveillance and control, which is very encouraging.

New collaborations, alliances and institutional arrangements can support countries to identify where they can add value to health programmes by strengthened cooperation between different sectors. Public services will certainly continue to play an important role in zoonosis control and service delivery, but conventional strategies are increasingly complemented and, in some cases, replaced by contracting with private operators and by public - private initiatives (also between resource-poor and industrialised countries).

Regional and international research groups with a 'One Health' approach such as the Foodborne Disease Burden Epidemiology Reference Group (FERG), the Cysticercosis Working Group in Eastern and Southern Africa, and the EU-funded research consortium on Integrated Control of Neglected Zoonoses in Africa (ICONZ) are well placed to generate the needed data on levels of underreporting, DALY estimates of zoonoses and the epidemiological and financial information needed for analyses of costs and benefits and cost-effectiveness and thereby identify the most appropriate option for zoonoses prevention and control in particular settings.

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Integrating Results of Laboratory Surveillance of Human Illness and Monitoring of Animals and Foods

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ABSTRACT

Cost-efficient monitoring of food contamination and surveillance of foodborne diseases requires a coordinated multidisciplinary approach with the participation of stakeholders from all sectors of the 'farm-to-consumption' continuum including the public health sector. To facilitate communication and coordination, establishment of a coordinating body with the participation of relevant stakeholders is recommended. Furthermore, relevant surveillance data from all stages in the food production chain and from the surveillance of human disease should be continuously collected and analysed to evaluate trends and sources of foodborne disease. The establishment of a dedicated multidisciplinary surveillance unit involving epidemiological and microbiological expertise from all sectors can facilitate this type of coherent data analysis and feed back. Systems such as these can be operated at the national, regional and global levels.

Key words: *food safety, foodborne diseases, surveillance, communication, coordination, multidisciplinary.*

INTRODUCTION

Food safety is the assurance that food will not cause harm to the consumer when it is prepared and/or eaten. The provision of this assurance covers an incredibly complex area of roles and responsibilities. It crosses multiple sectors of government, including Ministries of Health, Agriculture and Trade, and requires the involvement of multiple professional disciplines and a broad array of stakeholders. An effective food safety system, national and international, requires the sharing of information and expertise in order to face the global nature of modern food safety issues.

The contamination of food is a worldwide public health concern and a leading cause of trade issues internationally. Food contamination by chemical hazards may occur through environmental pollution of the air, water and soil, e.g. with toxic metals, polychlorinated biphenols (PCBs) and dioxins, or through the intentional use of various chemicals, such as pesticides, animal drugs and other agrochemicals. However, this paper will not address specific issues related to trade or chemical food contamination, but focus mainly on the control of foodborne pathogens and disease throughout the food production chain.

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Microbiological food contamination and subsequent transmission can occur at any point of the food production chain, from livestock feed, via the on-farm production site, at the slaughterhouse or packing plant, in manufacturing, processing and retailing of food, through catering and home preparation. Each link in the chain is responsible for the quality and safety of its products. Since there are numerous possible routes for transmission of contamination throughout production, isolated actions (e.g. decontamination of animal feed) will in most cases not ensure lasting consumer protection. Therefore, control measures against the introduction and transmission of foodborne pathogens should be considered at all levels of production.

CONTROL OF FOODBORNE DISEASES

In order to control foodborne disease, it is important to have knowledge of the potential source(s) of food contamination hazards and the most important route(s) of transmission. Once some basic knowledge of the origin and modes of spread of contamination is present, efficient control strategies can be devised and the burden on human and animal health decreased.

Professionals and technical staff working in veterinary, food and public health disciplines have a number of tools available that rely on each other. There are diagnostic tools that can help detect and distinguish foodborne pathogens, chemicals, toxins or other types of residues. There are epidemiological methods to analyse these data and expose association between factors and disease. There are mathematical modelling tools to help understand the complexity of food safety issues and how factors inter-relate. All these technical tools together provide the basis for informed decision support to risk managers and decision makers.

When results obtained from these tools are combined intelligently, food safety and public health systems can be transformed from a reactive to a proactive approach. This means that when signals of contamination early in the chain are communicated timely and accurately, the food safety system can expand from outbreak investigation and trace-back to include predicting forward, and from control to prevention of food safety events. Regular and open communication between all major stakeholders in food safety is imperative for the successful integration and coordination of all food safety efforts.

MAJOR STAKEHOLDERS IN FOOD SAFETY

The food industry is responsible for the quality and the safety of its products and is therefore a major stakeholder in food safety. Production may be monitored through, for example, certification programmes, process control schemes or HACCP (Hazard Analysis

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Critical Control Points) based control programmes. These control activities generate data that can constitute an important contribution to national surveillance programmes. During outbreak investigations, additional sampling may be required to trace-back human illness to the point of contamination in the food-production chain. Close cooperation between the private and public sectors is therefore imperative.

In general, the main stakeholders in food safety representing the government are the Ministries of Health and the Ministries of Agriculture/Food, but may also include Ministries of the Environment, Trade/Commerce/Industry, Consumers and others. Under them, are agencies that are responsible for the legislative, technical and practical implementation of food safety programmes, and each agency often has a dedicated reference laboratory associated with it. The access to surveillance data often goes through these laboratories. These governmental organisation structures often run independent of each other. In order to get a comprehensive view of the national food safety status, the Ministries and their respective agencies and reference laboratories should work closely together.

Finally, other stakeholders of food safety are the non-governmental organisations. They may represent consumer groups, food industry workers or environmentalists. Although these organisations seldom are directly involved in the generation of data, they can influence the launching of food safety initiatives and serve as a driving force behind initiation of surveillance efforts.

The main challenge is to develop structures that ensure the systematic collection, collation, analysis and interpretation of surveillance data and communication to all public and private stakeholders involved (**Figure 1**). Such a structure relies on the acknowledgement of the major stakeholders that they all play an important role in food safety and that they all gain from this collaboration. For this purpose, one or more coordinating bodies or steering committees with representatives of all stakeholders may be formed. The integration of all surveillance data from farm-to-consumption in a coherent analysis and subsequent interpretation may be the task of a specialized multidisciplinary research unit, which reports to the relevant coordinating bodies or steering committees. The evaluation by these committees can then lead to coordinated efforts of prevention and control.

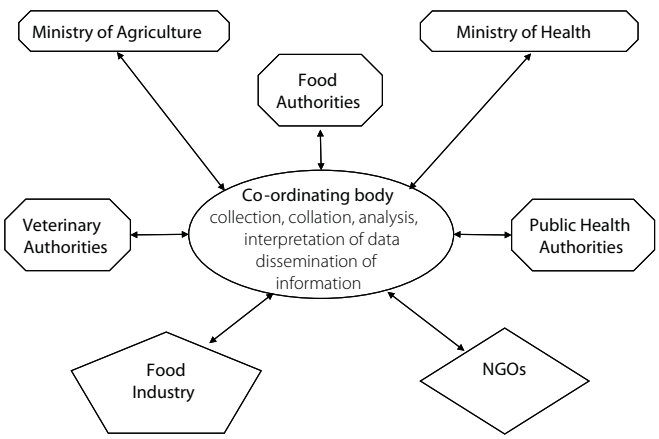

Figure 1. Schematic presentation of the collection, collation, analysis and interpretation of surveillance data and the subsequent dissemination of information to major stakeholders in food safety.

INTEGRATION OF FOODBORNE PATHOGENS AND DISEASE SURVEILLANCE

Integration of surveillance activities at the national level facilitates optimisation and cost efficiency in the generation and utilisation of surveillance data. The challenge is to optimise the sensitivity of the surveillance system while minimising the costs. For example:

- Integration of surveillance components within and between links of a production chain, e.g. to investigate possible associations between the levels of food-borne pathogens in food animals and in food products at retail;
- Integration of different surveillance programmes of the same production animal, e.g. using the same serum samples for the detection of antibodies against both *Salmonella* and porcine reproductive and respiratory syndrome (PRRS);
- Integration of different surveillance programmes for different production animals, e.g. to estimate the relative contribution of the main reservoirs to the total number of human cases of foodborne illness;
- Integration of national surveillance programmes to rapidly recognise and report international outbreaks, a so-called network of networks, e.g. PulseNet International, OzFoodNet, and the Global Foodborne Infections Network (GFN), formerly known as WHO Global Salm-Surv;
- Integration of surveillance programmes run by international organisations, such as the Global Early Warning System for Major Animal Diseases, including Zoonoses (GLEWS), a joint system that builds on alert mechanisms of the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO) and the World Organisation for Animal Health (OIE) and the International Food Safety Authorities Network (INFOSAN) of FAO and WHO.

The integration of foodborne pathogens and disease surveillance activities can be achieved through: i) communication, ii) collaboration, iii) coordination and iv) central collection, collation, analysis and interpretation of data. Communication between major stakeholders can be maintained during regular meetings and direct, informal contact between veterinary and public health workers in key positions. Collaboration consists mainly of the routine exchange of data and participation in outbreak investigation and response. Control activities and the sharing of information need to be coordinated, within and between programmes. Managing a central database containing all surveillance data or linking databases which contain complementary data allows for coherent analyses of the relation between foodborne pathogen reservoirs and disease in time and space. These four components ensure the optimal use of data that are already being generated.

SALMONELLA SURVEILLANCE IN DENMARK: AN EXAMPLE OF AN INTEGRATED APPROACH

In Denmark, the successful implementation of a number of surveillance and control programmes can be accredited to the close cooperation between the public sector and private industry (Wegener et al., 2003). The authorities have delegated the responsibility for technical coordination of the programmes to committees with representatives from the industry, government bodies and science. In the planning and implementation of programmes, there has been a close involvement of microbiologists and epidemiologists. In addition, there is a very close collaboration between medical and veterinary epidemiologists and microbiologists in assessing the effect of the programmes on the incidence of human infection.

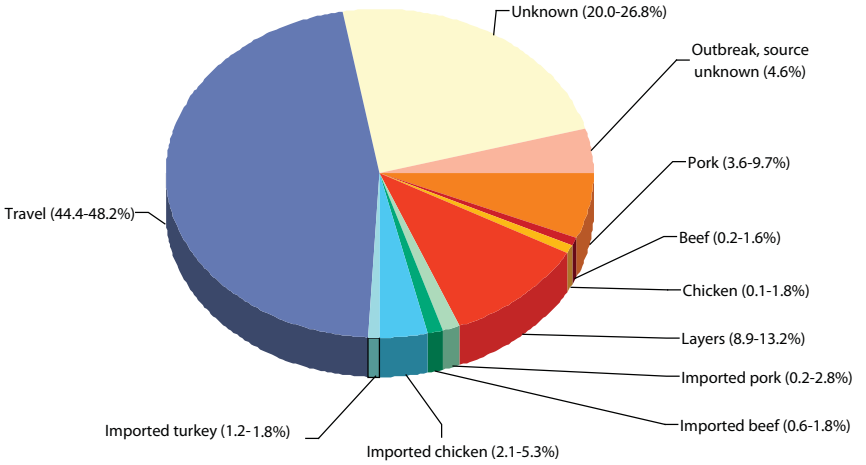

Figure 2. Estimated sources of 1 647 registered cases of human salmonellosis in Denmark, 2007. Source Danish Zoonosis Centre.

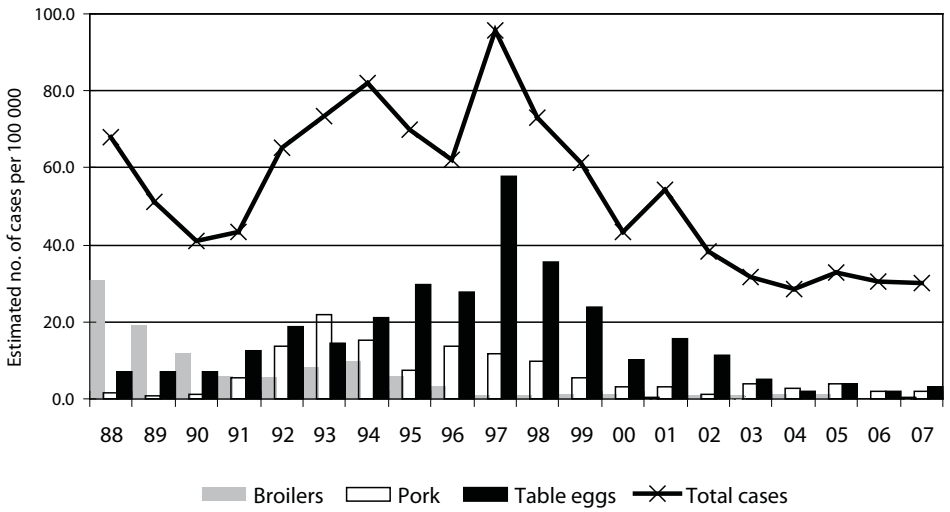

Figure 3. Trends and sources of human salmonellosis in Denmark, 1988 to 2007. Source: Danish Zoonosis Centre.

To initiate and generate the basis for targeted action, The Danish Zoonosis Centre was established in January 1994. The Zoonosis Centre is an epidemiological surveillance and research unit hosted by the National Food Institute, Technical University of Denmark. As the national coordinating body for food safety and zoonosis, the Zoonosis Centre collects all data from all national surveillance and control programmes on zoonoses and conducts an ongoing analysis of the national zoonosis situation from farm-to-consumption, including the identification of outbreaks, the assessment of sources of human foodborne disease as well as basic epidemiological research. An overview of the data is published and commented on in hard copy (Anonymous, 2009) and on the internet (http://www.food.dtu.dk/Default.aspx?ID=9606). The report includes an annual account of major sources of foodborne salmonellosis based on surveillance (**Figure 2**), as well as an overview of the trends in the estimated attribution of these sources to human infection since 1988 (**Figure 3**). The principle of the attribution method is to compare the number of human cases caused by different *Salmonella* subtypes with the distribution of the same subtypes in the various animal and food sources (Hald et al., 2004).

Figure 3 shows that Denmark experienced three major waves of human salmonellosis incidence between 1988 and 2007 that were mainly associated with broilers, pork and eggs, respectively. Following the introduction of a control programme in broiler production in 1988, the broiler-associated salmonellosis incidence (cases/100 000) has been reduced from 30.8 in 1988 to 0.2 in 2007; following the implementation of a control programme in pig herds in 1993, the pork-associated salmonellosis incidence has been reduced from 22.0 in 1993 to 2.0 in 2007; and the egg-associated salmonellosis incidence has been reduced after implementation of a control programme in laying hen production in 1997, from 57.7 in 1997 to 3.3 in 2007. By combining data from animal, food and human sources in a comprehensive analysis, the attribution method has proven to be a useful tool to support the Danish risk managers in their decision to

implement new intervention strategies and evaluate their impact on public health (Anonymous, 2009).

The success of the Danish control programme which has reduced the reported human salmonellosis incidence from 95.6 in 1997 to 31.5 in 2003 has been an inspiration for many countries. However, the incidence has remained at approximately 30/100 000 since then. The risk management options to further reduce the human incidence are less clear. Additional information may be needed to identify sources that are currently not being monitored, including abroad, and implement new prevention measures to decrease the human incidence further.

Notwithstanding this, the recognition of the central role, expertise and leadership displayed by the Danish Zoonosis Centre, has led to its nomination as the Danish reference laboratory for zoonosis epidemiology in EU (NRL–5), WHO Collaborating Centre for Antimicrobial Resistance in Foodborne Pathogens and Zoonosis Collaboration Centre (ZCC) of the European Food Safety Authority (EFSA) with the main task of preparing the Community Summary Report on trends and sources of zoonoses, zoonotic agents and antimicrobial resistance in the European Union (e.g. EFSA, 2009).

An increasing number of other countries have in recent years established similar or related structures to integrate surveillance efforts and facilitate communication and coordination. These countries include Sweden, Norway, Finland, UK, Ireland, Canada and many more. There is also a growing interest of countries to adapt existing or develop new methods to attribute human foodborne illness to specific animal and food sources (Batz et al., 2005).

GLOBAL CAPACITY BUILDING FOR INTEGRATED SURVEILLANCE AND CONTROL

There are various national and regional initiatives that aim to build and improve technical capacity of laboratory diagnostics or foodborne outbreak investigation. However, few initiatives focus specifically on the integration of efforts between sectors and disciplines that are involved in food safety and zoonoses.

The WHO Global Foodborne Infections Network (GFN) (formerly known as WHO Global Salm-Surv) is a capacity-building network to detect, control and prevent foodborne and other enteric infections from farm to table. GFN promotes integrated, laboratory-based surveillance and intersectoral collaboration among human health, veterinary and food-related disciplines (www.who.int/gfn). Created in 2000, the programme now has over 1 500 members from more than 700 institutions in 177 countries. GFN has six main programme components: International Training Courses, Global Salmonella Country Databank, the External Quality Assurance System, the Electronic Discussion Group, Focused Regional and National Projects, and Reference Testing Services.

GFN conducts training courses for microbiologists and epidemiologists from veterinary, food and human health disciplines at 17 training sites around the world. The training modules include benchtop training for microbiologists for laboratory testing of a variety of foodborne pathogens, lectures and case studies for epidemiologists as well as joint activities for all course participants such as integrated surveillance, risk assessment and attribution methodology. To date the programme has held over 65 international training courses in Chinese, English, French, Portuguese, Spanish, and Russian for approximately 1 300 microbiologists and epidemiologists from over 120 countries. Also, more than 80 countries have provided data to the Country Databank on over 1.5 million human isolates and close to 400 000 isolates from non-human sources to help provide a global overview of the epidemiology of *Salmonella*. The External Quality Assurance System of GFN is one of the world's largest annual proficiency tests with more than 150 laboratories participating worldwide (Hendriksen et al., 2009). Though originally focusing on *Salmonella* diagnostics and epidemiology, the training programme has evolved into a capacity-building platform that accommodates a variety of foodborne and other enteric pathogens and diseases of importance in the various regions.

CONCLUDING REMARKS

The detection of changes in patterns of foodborne diseases and variations in the contamination in the food production process are an absolute necessity for the monitoring and continuous improvement of food quality and safety. These programmes need to be sensitive, sensible and cost efficient. Food contamination monitoring and foodborne disease surveillance at national level provide a timely and comprehensive overview of the veterinary and public health status of a nation. The integration of foodborne disease surveillance has the goal to gather all national surveillance activities in a common public service that carries out many functions using similar structures, processes and personnel. The infrastructure of an established surveillance programme in one area may serve as a framework for strengthening other surveillance activities. Though some foodborne diseases may have specific information needs requiring specialised systems, there may be the potential for synergy and the sharing of common resources.

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Molecular Characterisation of Human and Animal Fascioliasis in the Americas

M.D. Bargues & S. Mas-Coma[1]*

ABSTRACT

In Latin America, fascioliasis is an important human and animal health problem in many countries. Molecular studies were performed to determine the genetic characteristics of both liver flukes and lymnaeid vectors by combined haplotyping. Molecular markers obtained were the complete sequences of the nuclear ribosomal DNA ITS–1 and ITS–2 and the mitochondrial DNA genes *cox*1 and *nad*1 and the respective amino acid sequences of the proteins COX1 and NAD1. Fasciolid flukes from Latin America showed a surprising homogeneity both at nuclear rDNA and at mtDNA levels. Differences detected when comparing *cox*1 and *nad*1 were so few that bootstrap values obtained in phylogenetic analyses by using one or the other gene independently proved to be insufficient. Significant values in mtDNA were only obtained by combining both genes within the same analyses and when comparing different countries. These results contrast with those obtained through sequencing studies of lymnaeid snails, in which several different species showing vectorial capacity appeared. Almost all vector species proved to belong to the problematic *Galba/Fossaria* group of small-sized lymnaeids, and, with few exceptions, endemic areas had more than one vector species involved in disease transmission. Thus, owing to the intraspecific genetic homogeneity of the fasciolids, the differences in transmission patterns and epidemiological situations may be related to differences in the lymnaeid vector species present in endemic areas. Results obtained open new doors for future molecular research to establish appropriate control measures for endemic areas in different Latin American countries.

Key words: *fascioliasis, lymnaeid snails, genetic analysis, haplotyping, molecular markers.*

INTRODUCTION

Fascioliasis is a zoonotic disease of domestic ruminants caused by liver fluke parasites and transmitted by freshwater lymnaeid snail vectors. This disease is of well-known veterinary importance because of its great pathogenicity and impact in livestock, especially sheep, goats and cattle, but also pigs, buffaloes and donkeys, as well as horses, camelids and other domestic herbivores. Moreover, this disease is today emerging in humans in Europe, Africa, Asia and the Americas, with 51 countries recording human infection. This parasitic disease is caused by the liver fluke species *Fasciola hepatica* and *F. gigantica*, whose geographical distribution differs. *Fasciola hepatica* is present in Europe, Africa, Asia, the Americas and Oceania, whereas *F. gigantica* is only found in Africa and Asia (Mas-Coma and Bargues, 1997; Mas-Coma, 2004). Although being long recognised for its great veterinary importance, fascioliasis by both *F. hepatica* and *F. gigantica* has only recently been shown to be a widespread human health problem (Mas-Coma et al., 1999a,b; Mas-Coma et al., 2005) with severe symptomatology and pathology in both acute and chronic phases (Chen and Mott, 1990; Mas-Coma et al., 1999b, 2000; Valero et al., 2003). Recent estimates suggest that between 2.4 million (Rim et al., 1994) and 17 million people (Hopkins, 1992) are infected and the figure may be even higher considering the lack of data from many countries, mainly of Asia and Africa (Mas-Coma, 2004).

This old disease has a great powers of geographical expansion due to the large colonisation capacities of its fasciolid causal agents and freshwater lymnaeid snail vector species (Mas-Coma et al., 2001), and is at present emerging or re-emerging in many countries, showing both increases in prevalence and geographical expansion (Mas-Coma, 2004). Throughout its wide geographical distribution range, human fascioliasis shows very unique epidemiological characteristics and human endemic areas that range from hypo- to hyperendemic (Mas-Coma et al., 1999a, b). A recent global analysis of these characteristics concluded that fascioliasis was the vector-borne parasitic disease with the widest latitudinal, longitudinal and altitudinal distribution known (Mas-Coma et al., 2003; Mas-Coma, 2004).

In Latin America, large hot spots of disease have been detected in high altitude areas of Argentina, Chile, Bolivia, Peru, Ecuador and Venezuela, with very high prevalences in livestock and humans in endemic areas where transmission and epidemiology follow whether altiplanic-permanent or valley-seasonal patterns related to vectors of the *Galba/Fossaria* group. Other hot spots include Caribbean Islands like Cuba and Central American countries like Mexico where transmission and epidemiology are determined by lymnaeids such as *Lymnaea cubensis* and *Pseudosuccinea columella*, and where animals have very high prevalences and intensities of infection and a hypoendemic situation with periodic epidemics exists in humans (Mas-Coma, 2005; Mas-Coma et al., 2005).

Molecular studies have been performed during several years to determine the genetic characteristics of liver flukes and to classify lymnaeid snail vectors in the New World. Like other vector-borne infectious diseases, the vectors are crucial for establishing the patterns of transmission and epidemiological features of the disease (Bargues and Mas-Coma, 2005). Lymnaeids are freshwater snails which pose great difficulties in classifying specimens, because of their anatomical uniformity and the intraspecific variability of the shell morphology: sometimes even expert malacologists cannot classify

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specimens correctly (Bargues et al., 2001). In the case of controversial groups, such as *Galba/Fossaria* and *Radix* which include the main vector species of *F. hepatica* and *F. gigantica* respectively, accurate classification of specimens is only possible by DNA marker sequencing (Bargues and Mas-Coma, 2005).

MATERIALS AND METHODS

The specific objectives of the studies reported was to genetically characterise both liver flukes and lymnaeid vectors by combined haplotyping. A total of 141 adult flukes, obtained from animal livers in slaughterhouses of 37 different localities from the endemic countries of Argentina, Bolivia, Chile, Ecuador, Mexico, Peru, Uruguay and Venezuela, were sequenced. Lymnaeid vectors were collected during field activities in human endemic areas of the same countries. For liver flukes, the molecular markers obtained were complete sequences of the nuclear ribosomal DNA (rDNA) internal transcribed spacers ITS–1 and ITS–2, as well as the complete sequences of the mitochondrial DNA (mtDNA) genes of the cytochrome c oxidase subunit I (*cox*1) and NADH dehydrogenase subunit I (*nad*1) and the respective amino acid sequences of the proteins COX1 and NAD1 (Mas-Coma et al., 2009a). Primers used for ITSs were the same as previously used (Mas-Coma et al., 2001 and 2009a); mitochondrial markers were designed on sequences of the complete mitochondrial DNA genome of *F. hepatica* from Australia (Le et al., 2001). For lymnaeid snails, the molecular markers used included the complete sequences rDNA ITS–1 and ITS–2 and mtDNA *cox*1, plus the small subunit or 18S rRNA gene (Bargues et al., 2007).

Sequences were aligned using CLUSTAL-W, homologies assessed using BLAST, genetic distances were measured using PAUP, and pairwise alignments were made with MEGA. Genetic variation was evaluated using DnaSP and a hierarchical analysis of molecular variance (AMOVA) performed using Arlequin. Phylogenies were inferred by maximum-likelihood (ML) using PAUP and PHYML. The evolutionary model was determined using the hierarchical Likelihood Ratio Test (hLRTs) and the Akaike Information Criterion implemented in Modeltest. A median-joining network analysis was performed using Network. Distance-based phylogeny was obtained using the neighbour-joining algorithm with the ML pair-wise distances. Statistical support was evaluated with 1 000 bootstrap replicates. A Bayesian phylogeny was applied to obtain posterior probabilities with MrBayes (Bargues et al., 2007 and 2008).

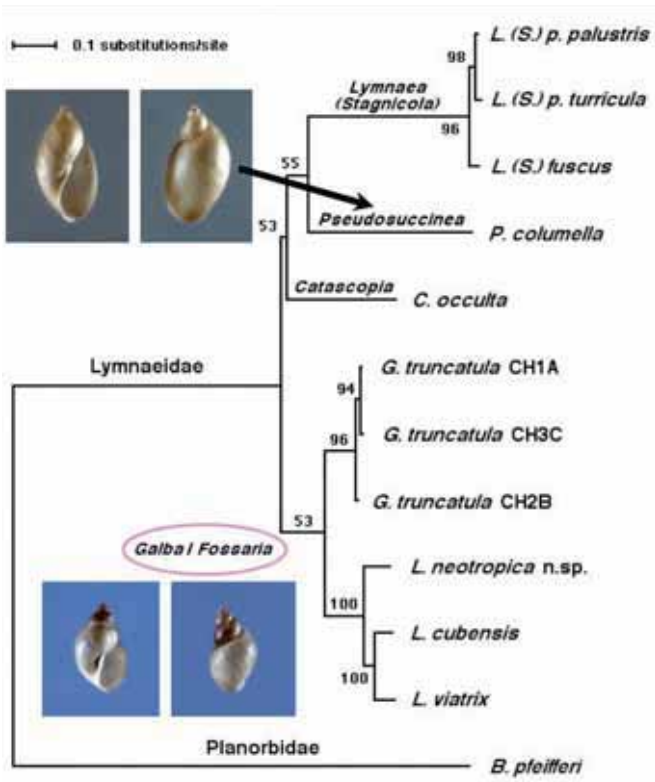

Figure 1. Phylogenetic tree of lymnaeid vector species from Latin America and Europe, based on combined sequences of rDNA ITS–1 and ITS–2, derived from the maximum likelihood (ML HKY85+G) model. Numbers represent the percentage of 1000 puzzling replicates. Scale bar indicates the number of substitutions per sequence position. Modified from Bargues et al. (2007).

RESULTS

DNA Sequencing and Phylogenetic Analyses of Fasciolids

In fasciolids from Latin America, the complete sequence lengths and nucleotide compositions of rDNA ITS–1, ITS–2, and mtDNA *cox*1 and *nad*1 were 432 nucleotides and 51.85% GC content, 364 nucleotides and 48.35% GC content, 1533 nucleotides and 37.24% average GC content, and 903 nucleotides and 34.83% average GC content, respectively. The proteins of the two mtDNA genes were 510 aa long, with start/stop codons of ATG/TAG in all individuals analysed; in the case of COX1, they were 300 aa long, with start/stop codons of GTG/TAG in all individuals analysed, in NAD1.

Fasciolid flukes showed a surprising homogeneity both at nuclear rDNA and at mtDNA levels. No one nucleotide difference appeared in the ITS–1 sequences of the different South American, Central American and Caribbean countries, whereas only one mutation appeared in the ITS–2 sequence in 41.6% of the populations. Similarly, differences detected when comparing *cox*1 and *nad*1 sequences were so few that bootstrap values obtained in phylogenetic analyses by using one or the other gene independently proved to be insufficient. Significant values in mtDNA were only obtained by combining both genes within the same analyses and when comparing different countries.

Interestingly also, when comparing the sequences of both ITS–1 and ITS–2 of all fasciolids of the Americas with corresponding sequences of 'pure' *F. gigantica* from sub-Saharan African countries, the same differences appeared in the polymorphic sites 24, 114, 208, 286 and 306 in ITS–1, and in positions 234, 273, 279, 330 and 337 in ITS–2.

DNA Sequencing and Phylogenetic Analyses of Lymnaeid Vectors

Results obtained in lymnaeid snails contrasted with those obtained in the sequencing studies of fasciolid flukes in that very different sequences were found. These sequences corresponded to several different vector species having the capacity to transmit fascioliasis. Almost all vector species proved to belong to the *Galba/Fossaria* group of small-sized lymnaeids, and, with a few exceptions, endemic areas had more than one vector species involved in disease transmission. The main species found within this problematic group were *Lymnaea viatrix* (= *L. viatrix* var. A ventricosa), *L. cubensis*, *L. neotropica* (= *L. viatrix* var. B elongata) and *Galba truncatula*. The only species

not belonging to the *Galba/Fossaria* group is the widely dispersed, large-sized *Pseudosuccinea columella*.

The phylogenetic analysis with maximum likelihood (HKY85 + G) showed a tree (**Figure 1**) in which the close relationship appears between the American *L. viatrix*, *L. cubensis*, *L. neotropica* and the species *G. truncatula* of European origin. Additionally, *P. columella* , a well-known vector species, appears within the clade of secondary vector species as the stagnicoline lymnaeids, separating the Palaearctic *Catascopia occulta* from the European *L.* (*Stagnicola*) *palustris*, *L.* (*S.*) *turricula* and *L.* (*S.*) *fuscus*.

DISCUSSION AND CONCLUSIONS

Studies on Liver Flukes

The uniformity of American fasciolids with respect to rDNA ITS–1 and ITS–2 and their very low intraspecific variability in nucleotide differences in the two mtDNA genes *cox*1 and *nad*1 are worth emphasising (i) due to its contrast with the high diversity shown by the liver fluke in the Old World linked to its geographical origin and spreading peculiarities in the pre- and posdomestication periods, as verified by the same DNA markers (Mas-Coma et al., 2009a), and also detected in Ireland, The Netherlands and Greece with the mtDNA PCR-RFLP technique (Walker et al., 2007), and (ii) because of the problem they pose for future analyses if only fragments of the two aforementioned mtDNA genes are used (e.g., Hashimoto et al., 1997; Itagaki et al., 1998) (**Figure 2**). Results obtained with liver flukes in American countries using the complete sequences of the *cox*1 and *nad*1 genes show that mutations in the Americas are so few that phylogenetic analyses are not able to furnish significant results when using each mtDNA gene independently. Significant results were only obtained when combining both complete gene sequences and only when comparing flukes from different countries. This means that (i) the uncomplete sequences of the short fragments of these two mtDNA genes that have been always used until now (**Figure 2**) are useless in the Americas, and that (ii) even when combined, the complete sequences of these two mtDNA genes are also useless for analysing disease transmission within a specified endemic area. This suggests that DNA markers evolving much faster than *cox*1 and *nad*1 or other molecular methods involving banding of larger sequences (e.g. RAPDs and microsatellites) will be needed to analyse disease transmission.

A positive conclusion can, however, be deduced from the homogeneity of the liver flukes in the Americas, namely the simplification of both field epidemiological studies and disease control activities (Mas-Coma et al., 2009a). This picture appears to be different from the one known in the Old World, where different epidemiological scenarios and transmission patterns are known (Mas-Coma et al., 1999a, b; Mas-Coma, 2005). In the Americas, nevertheless, the possibility that climate change has different effects on fascioliasis transmission and epidemiology in different areas such as Andean countries and Caribbean Islands cannot be overlooked (Mas-Coma et al., 2008 and 2009b).

Among the several conclusions which can be reached from this analysis of fascioliasis in Latin American endemic countries, the following shall be emphasised because of their applied interest. The homogeneity of liver flukes in the Americas suggests that domestic livestock trade between different countries is important in disease spread. For the same reason, no important clinical and pathogenic differences are to be expected in different endemic areas, except those possibly linked to breed or ethnic group-related differences in susceptibility. Likewise, the susceptibility of flukes to treatments and their capacity to give rise to resistance may be expected to be uniform throughout. The later constitutes a real problem due to the high risk of rapid spread of resistance to triclabendazole if it appears one day in the Americas, the drug most used in animals (Fasinex®) (Fairweather and Boray, 1999; Fairweather, 2005) and the drug of choice of human treatment at present (Egaten®) (Savioli et al., 1999; Mas-Coma et al., 1999b and 2005).

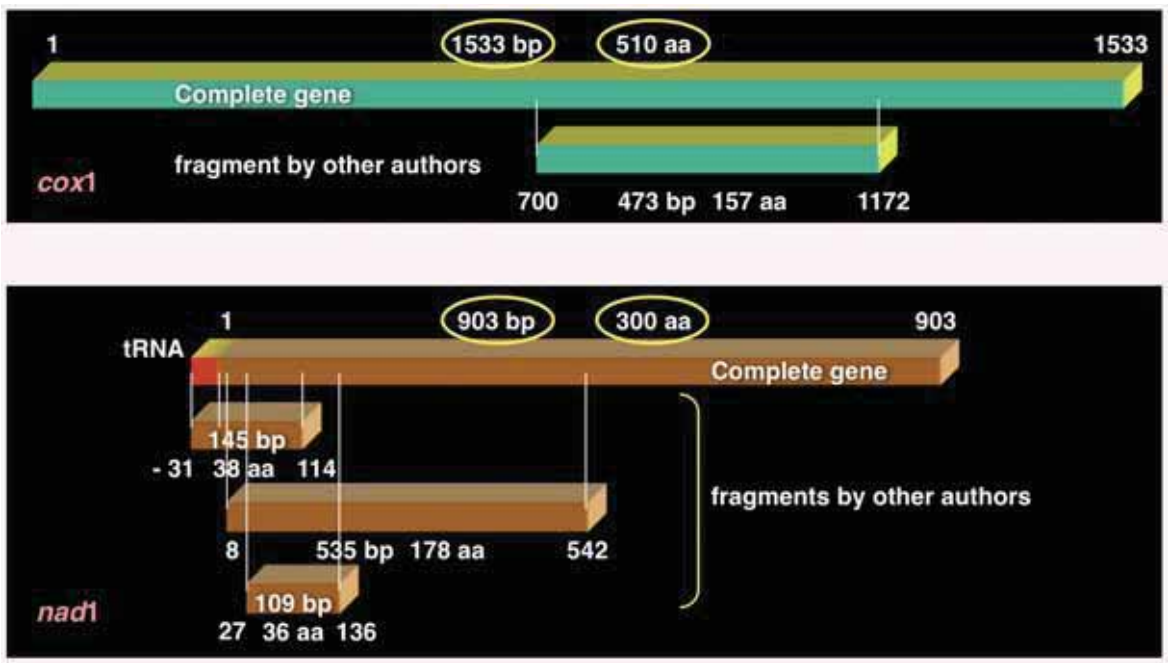

Figure 2. Comparison of the complete sequences of the mtDNA genes *cox*1 and *nad*1 with uncomplete sequences of fragments having been used until now, illustrating nucleotide sequence regions and respective information lost when only fragments are considered.

Studies on Lymnaeid Vectors

The first conclusion from the results of the sequencing studies on lymnaeid snails is that compared with Europe, lymnaeid vector species in the Americas are much more complex genetically. In Europe, there is only one main vector species of human and animal fascioliasis, *G. truncatula*, and with stagnicolines as the only secondary lymnaeids able to transmit the disease under specific circumstances (Bargues et al., 2001, 2003 and 2006a). Contrarily, Latin America lymnaeid vectors linked to endemic areas of zoonotic transmission of fascioliasis appear to involve different species of the *Galba/Fossaria* group, including both autochthonous American species and introduced European ones (Bargues et al., 2006b and 2007). The lymnaeids of this problematic group pose considerable classification problems, because the different species look very much alike in terms of both shell morphology and inner anatomy. Moreover, they appear to show similar ecological characteristics which increases the dificulties in their differentiation. Together, these will represent huge challenges for health workers charged with assessing the characteristics of transmission and epidemiology of the disease in different endemic areas and designing appropriate control measures.

Owing to the intraspecific genetic homogeneity of the causal fasciolids, the differences in transmission patterns and epidemiological situations may be related to differences in the lymnaeid vector species present in endemic areas. Indeed, results described here fully agree with previous assumptions about transmission patterns in human endemic areas and which, in the Americas, can be distinguished as follows according to analyses from field studies (Mas-Coma, 2005):

- a very high altitude pattern related to only *F. hepatica* transmitted by imported *Galba truncatula* in Andean countries following transmission throughout the year; within this category, two subpatterns may be distinguished according to physiographic and seasonal characteristics:
  - — the altiplanic pattern, with transmission throughout the whole year (e.g. in the northern Bolivian Altiplano and the Puno Altiplano);
  - — the valley pattern, with seasonality and with prevalences and intensities related to altitude (e.g. in the Peruvian valleys of Cajamarca and Mantaro);
- a Caribbean insular pattern, with reduced but repeated outbreaks in human hypoendemic areas and lymnaeid species other than the main vector species being involved in transmission (e.g. the Pinar del Rio Province in Cuba).

Finally, in Latin America, knowledge of lymnaeid vectors is required for control initiatives and the results presented here open new doors for future, crucial molecular research as a means to establish the appropriate control measures for endemic areas of the different Latin American countries.

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Climatic Characteristics of Areas with Lymnaeid Snails in Fascioliasis Endemic Areas of Mendoza Province, Argentina

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ABSTRACT

Fascioliasis is a zoonotic trematodiasis which is both emerging and spreading all over the world, with important human endemic areas in South America. Its prevalence in Argentina, and particularly in Mendoza Province, appear to be high. This study was designed to characterise the main climatic conditions of sites in endemic areas of fascioliasis where freshwater snails of the Lymnaeidae family (the intermediate vectors of Fasciola hepatica) are present This was done by analysing the sites by digital climatic analysis using DIVA-GIS 5.2 software, coupled with information gained through earlier research. Temperature showed a small dispersion among sites, possibly indicating that temperature may have a greater influence on the distribution of lymnaeids than precipitation. Also there was convergence in the dispersion graphic between the values for 'minimum temperature of the coldest month' and 'precipitation of the driest month', showing that these aspects could be considered as limitations to the snails' survival. It is concluded that lymnaeid snails have great adaptability and survival capacities, enabling them to colonise and survive in extreme and diverse environments such as the high altitudes of the Andes and the arid plains of central Mendoza Province. The impact of global climate change should not be overlooked as a factor enhancing vector spread.

Key words: *fascioliasis, lymnaeid snails, climatic analysis, adaptability and survival.*

INTRODUCTION

Fascioliasis is a zoonotic trematodiasis caused by the liver flukes *Fasciola hepatica* and *F. gigantica*, which are transmitted by freshwater snails of the family *Lymnaeidae*. Definitive hosts of fasciolid flukes are mainly herbivorous ruminants, especially livestock species such as sheep, cattle and goats. In the New World fascioliasis is only caused by *F. hepatica* (Mas-Coma, these proceedings). Its high pathogenicity causes substantial economic losses in animal production throughout all countries of South America (Torgerson and Claxton, 1999). From a public health standpoint, the disease has become increasingly important over the last two decades, affecting an estimated 17 million people worldwide. In fact, human infections have been noted in most South American countries (Mas-Coma et al., 1999a and 2005), with important human endemic areas being in Andean areas of countries such as Chile, Bolivia and Peru (Mas-Coma et al., 1999b; Apt et al., 1993; Esteban et al., 2002).

As in all other vector-borne diseases, the transmitting freshwater snails (family *Lymnaeidae*) are crucial in determining epidemiological and transmission patterns of the disease, with snail host population dynamics being related to disease intensities, distribution and transmission. Lymnaeid snail population dynamics, like those of other snails are profoundly affected by climatic factors such as air temperature, rainfall and/or potential evapotranspiration.

Rainfall is known to be crucial in fascioliasis transmission, as the appearance of temporary fresh water bodies or the widening of permanent ones offer lymnaeid snails the necessary habitats for their fast evolving populations (Mas-Coma et al., 2009). Thus rainfall is crucial in regions such as the Argentinian latitudes, where the weather characteristics follow a marked seasonality. Snail population dynamics are also highly dependent on temperature. Climate change is therefore likely to affect the distribution and survival of intermediate hosts like lymnaeid snails as well as influence the rates of reproduction and maturation of the parasites carried by them (Mas-Coma et al., 2008).

The prevalence of fascioliasis in the Mendoza Province of Argentina appears to be very high. Most positive animals (76%) from a total of 258 infected cattle came from Andean valleys in the localities of Las Heras, Malargüe, San Carlos, Tunuyán and Tupungato (Mera et al., 2005, 2006; Gonzalez et al., 2006).

Regarding the lymnaeid snails present in the Province, the species of most concern is *Galba truncatula* (the most effective vector known to date); it has recently been characterised by DNA sequence comparisons in affected areas (Bargues et al., 2006 and 2007). Considering the above, the aim of this study was to characterise the climate of sites where snails of the Gastropoda: *Lymnaeidae* family are present in endemic areas of fascioliasis; as well as to identify some probable limiting climatic factors. The information generated thereby could be important for assessing risk areas and control measures, as well as for predicting the future impact of the disease in the event of climate change.

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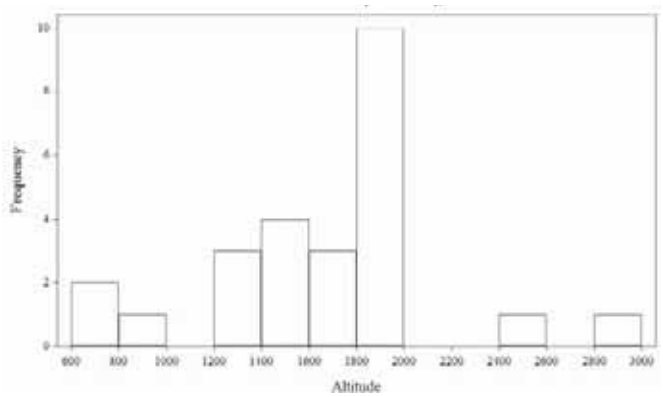

Figure 1. Altitudinal groups.

MATERIALS AND METHODS

A digital climatic analysis of fascioliasis endemic areas with confirmed presence of lymnaeid snails was performed by using DIVA-GIS 5.2 software.

The study performed took into account 25 sites where lymnaeids had been sampled during the last five years by continuous field studies covering the most important river basins of the Province, targeting particularly those areas described in previous research as being endemic for livestock fascioliasis (Mera y Sierra et al., 2005 and 2006; Gonzalez et al., 2006). Coordinates were registered with a standard GPS (Garmin Vista Cx®).

The altitudes of the sampling sites were represented in a histogram (Statistix 7.0), in order to obtain a sampling distribution by altitudinal groups. By using the DIVA-GIS 5.2 software and WorldClim climate data (WorldClim 1.4, 2.5 min resolution climatic layers, Hijmans et al., 2005), digital climatic information was obtained for every site. WorldClim provides monthly maximum and minimum temperatures and monthly precipitation, as well as 19 derived bioclimatic variables. This information was analysed by altitudinal groups with descriptive statistics (Statistix 7.0). A combined dispersion graphic (provided by DIVA-GIS 5.2 — Outliers Graphic, on Ecological Niche Modeling) was developed for the 19 bioclimatic variables, representing every site.

RESULTS

A histogram enabled separating the samplings into three altitudinal groups for the purposes of analysing the information. Group 1: 600 –1000 m above sea level (m.a.s.l.), with three sites (3/25); Group 2: 1200–2000 m.a.s.l., with 20 sites (20/25); Group 3: 2400–3000 m.a.s.l., with two sites (2/25) (**Figure 1**).

The main climatic characteristics of each group were as follows:

- Group 1 (**Table 1** and **Figure 2**): annual mean temperature (15.6 °C), maximun temperature in the warmest month (31.5 °C); minimun temperature in the coldest month (0.03 °C), mean temperature of wettest and driest quarters (22.1°C and 8.2 °C), precipitation in wettest and driest quarters (105.3 and 20 mm), and precipitation in warmest and coldest quarters (102.7 and 20 mm).
- Group 2: annual mean temperature (7.41 °C), maximun temperature in the warmest month (22.46 °C); minimun temperature in the coldest month (–5.36 °C), mean temperature of wettest and driest quarters (3.54 and 11.03 °C), precipitation in wettest and driest quarters (153.90 and 51.95 mm), and precipitation in warmest and coldest quarters (58.65 and 144.85 mm) (**Table 1** and **Figure 2**).

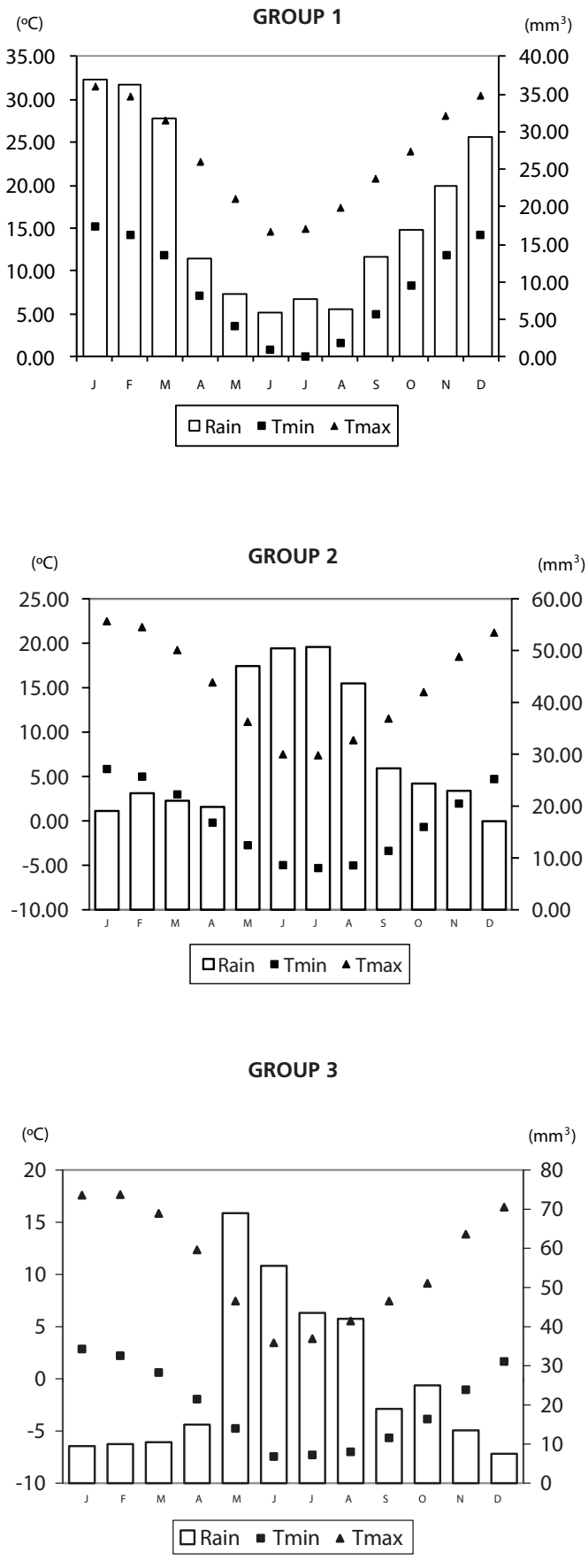

Figure 2. Mean temperatures and monthly precipitation.

Table 1. Climate conditions during the period of the study.

Bioclimatic Variable	Group 1		Group 2		Group 3	
	Mean	s.d.	Mean	s.d.	Mean	s.d.
Annual mean temperature	15.60	0.721	7.41	4.417	4.15	0.78
Mean monthly temperature range	15.60	0.265	15.13	0.615	13.50	0.57
Isothermality (*100)	49.50	0.755	54.45	1.504	53.80	0.57
Temperature seasonality (STD *100)	589.30	16.076	489.27	36.016	461.25	11.38
Max temperature of warmest month	31.53	0.709	22.46	5.010	17.70	1.13
Min temperature of coldest month	0.03	0.416	–5.36	3.810	–7.45	0.35
Temperature annual range	31.50	0.436	27.82	1.635	25.15	0.78
Mean temperature of wettest quarter	22.07	1.361	3.54	6.529	–0.80	0.57
Mean temperature of driest quarter	8.20	0.529	11.03	4.575	9.70	0.99
Mean temperature of warmest quarter	22.63	0.874	13.49	4.803	9.70	0.99
Mean temperature of coldest quarter	8.20	0.529	1.44	4.100	–1.50	0.57
Annual precipitation	228.67	20.984	365.95	122.392	320.00	26.87
Precipitation of wettest month	37.33	0.577	57.40	26.428	69.00	18.38
Precipitation of driest month	5.67	1.528	15.05	4.097	7.50	4.95
Precipitation seasonality	63.10	9.968	45.82	18.333	76.65	27.08
Precipitation of wettest quarter	105.33	4.041	153.90	72.424	168.00	45.25
Precipitation of driest quarter	20.00	5.196	51.95	13.256	27.00	16.97
Precipitation of warmest quarter	102.67	4.509	58.65	10.956	27.00	16.97
Precipitation of coldest quarter	20.00	5.196	144.85	76.024	141.00	36.77

- Group 3 (**Table 1** and **Figure 2**): annual mean temperature (4.2 °C), maximun temperature in the warmest month (17.7 °C); minimun temperature in the coldest month (–7.5 °C), mean temperature of wettest and driest quarters (–0.80 °C and 9.70 °C), precipitation in wettest and driest quarters (168 and 27 mm), and precipitation in warmest and coldest quarters (27 and 141 mm).

The combined dispersion graphic showed a small dispersion between temperature-related variables (1–11), but the greatest divergence between precipitation-related ones (12–19). However, convergence between values is specially noticeable for variables 6 (minimun temperature of coldest month) and 14 (precipitation of driest month) (**Figure 5**).

DISCUSSION AND CONCLUSIONS

The small dispersion seen on temperature variables may indicate that at Andean sites temperature may have a more important influence on lymnaeid distribution than precipitation. Also, the convergence noted between minimun temperatures of the coldest month and levels of precipitation in the driest month, suggests that these aspects could be considered as limitations to the survival of snails. In this regard, the minimun temperature of the coldest month may reflect the lowest temperature at which these snails are capable of surviving (with an extreme minimum temperature of –7.7 °C, at 2 971 m.a.s.l.), which is well below water freezing point (the 'ice point'). Also, precipitation during the driest month (5.67 mm^3, in Group 1 sites) reflects the extreme conditions of some of the sites, with great risks of drought. In both situations it is known that these snails may survive by burrowing themselves into soil, while waiting for better external conditions.

It was also noted that at Group 1 sites at lower heights the driest quarter is also the coldest, as rains mainly occur in summer, while in Groups 2 and 3 the situation is quite different, as the main precipitations occur during the coldest quarter in the form of snow. In these places during summer, snails are present on the streams fed by snowmelt.

In light of these results, it is apparent that lymnaeid snails have great adaptibility and survival capacities, enabling them to colonise and survive in extreme and diverse environments such as the high altitudes of the Andes (with a mean minimun temperature in the coldest month of –7.45 °C, in Group 3) or on the arid plains of central Mendoza Province (with precipitation in the driest month of just 5.67 mm^3, in Group 1). Clearly, the impact of global climate change should not be overlooked, as changing climatic conditions may enhance the spread of lymnaeid vectors and the colonisation of new sites, thereby increasing the risk of fascioliasis.

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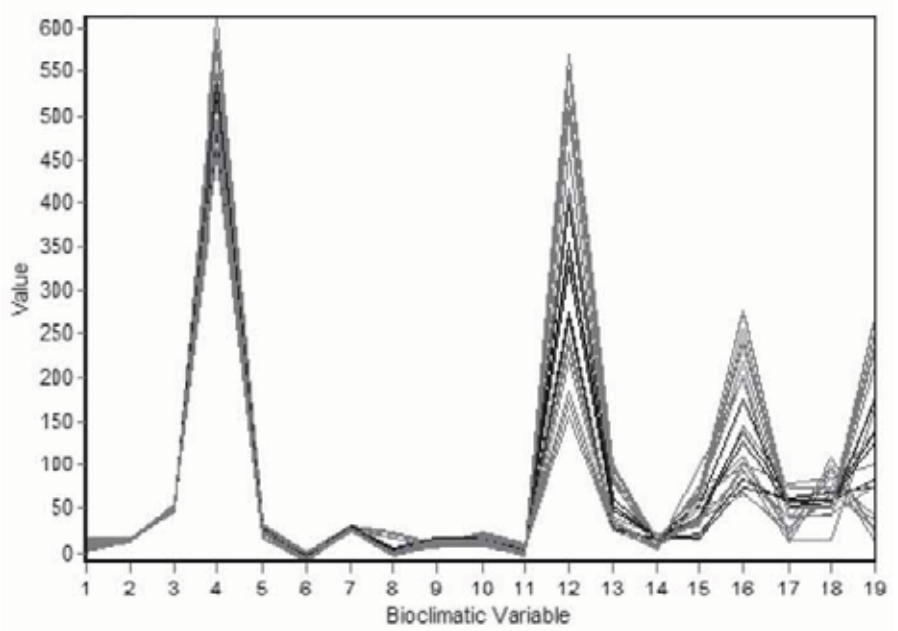

Figure 5. Dispersion graphic showing the 19 bioclimatic variables.

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Prevalence of Rift Valley Fever IgG Antibody in Various Occupational Groups before the 2007 Outbreak in Tanzania

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ABSTRACT

Rift Valley fever (RVF), caused by RVF virus is a mosquito-borne viral disease that is a significant global threat to humans and livestock. In Tanzania, RVF virus infection in human is not well studied. In this study we aimed to determine the seroprevalence of RVF, assess its zoonotic importance and identify factors associated with seroprevalence. A cross-sectional serological survey of 199 apparently healthy persons from various occupations was carried out in Tanga, Tanzania in November 2004 to determine exposure to RVF virus. Sera were tested for the presence of antibodies to RVF virus by an inhibition enzyme linked immunosorbent assay (ELISA) for detecting immunoglobulin G (IgG). All reactive sera were further tested using an immunoglobulin M (IgM) capture ELISA test for detection of specific RVF virus to determine if there were any instances of recent infection. Eight samples (4%) tested positive for IgG but none of them tested positive for IgM antibodies. Among the occupational groups examined, the seroprevalence was 7.3, 1.5 and 9.5% respectively among 'abattoir workers', 'livestock keepers' and 'other' categories. Seropositivity was higher in males (5.3%) than females (1.5%), with no significant differences among the age groups and sexes. The results indicate that a small proportion of people in Tanga municipality were exposed to RVF virus infection prior to the 2007 disease outbreak in Tanzania. Public health actions for RVF control will need to target not only the occupational groups at risk of infection with severe forms of this disease, but also the general population at large.

Key words: *Epidemiology, sero-prevalence, zoonoses, vector-borne, Tanzania.*

INTRODUCTION

Rift Valley fever (RVF), caused by RVF virus is a mosquito-borne viral disease that is a significant global threat to humans and livestock. Transmission in humans is via direct contact through infected animal products or contaminated foods or aborted foetuses and from the bites of infected mosquitoes, most commonly the *Aedes* species (Corso et al., 2007). Humans infected with RVF virus typically develop a mild self-limited febrile illness, but retinal degeneration, severe encephalitis, fatal hepatitis and haemorrhagic fever may also occur (Swanepoel and Coetzer, 2004). Although few studies have been conducted to asses the economic impact attributable to human RVF, it is thought to be substantial (Meegan, 1981; Davies and Martin, 2003; Davies 2006).

Previous RVF outbreaks in Tanzania, were confined mainly to livestock and mostly affecting northern parts of the country and recorded in 1956, 1978/79 and 1997/98 (Kondela et al., 1985; Woods et al., 2002). The recent re-emergence (in early 2007) of the disease among humans and livestock covering nine different geographical regions, and the fact that RVF virus replicates in a wide range of competent mosquito vectors (Turell et al., 2008) have raised concern that the virus might spread further into non-endemic regions of Tanzania (e.g. Tanga, Coast, Mtwara, Ruvuma, Rukwa, Kigoma and Shinyanga). These threats emphasise the need to have capable surveillance tools and a sound disease control strategy in place. Unpublished, hospital-based reports from the recent outbreak in Tanzania indicate that RVF claimed 144 lives with a corresponding case fatality rate of 46.6% (WHO, 2007).

RVF is known to be endemic in most sub-Saharan countries and in some regions in Tanzania (FAO/UNDP/OAU/IBAR, 2001; FAO, 2002). Between epidemic waves, RVF virus circulates at very low prevalence and without noticeable clinical manifestations in both humans and animals (Davies and Martin, 2003). Much less is known of the prevalence in man and of the effect on human health in this region of the world. This information is important when designing appropriate strategies that would help reduce its prevalence and effects. This inadequacy of data, and the availability of a serum bank comprising samples collected in 2004 prompted the initiation of this study with the aim of establishing past exposure to RVF virus before the 2007 outbreak. The objective of the present study was to estimate the RVF antibody prevalence in various, apparently healthy, occupational risk groups of inhabitants in the Tanga Municipality of Tanzania. The overall purpose was to collect baseline data to enhance understanding of the epidemiology of the disease.

MATERIALS AND METHODS

Study Area

This cross-sectional field study was conducted in Tanga Municipality, Tanga Region, one of eight regional districts in the country. The area is located between latitude 4° 21′ and 6° 14′ S, and longitude 36° 11′ and 38° 26′ E, in northeast Tanzania. It has a human population of around 242 640 growing at 2.9% per annum (URT, 2002). Most rural inhabitants are subsistence farmers, whilst Tanga town, the regional centre, provides many jobs in industry and trade. Tanga Municipality has a hot and humid tropical climate with two rainy

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seasons: an intense one observed during the months of March, April and May, and a mild one occurring in November and December. The mean annual rainfall varies from 500 to 1 400 mm/y. The relative humidity of the day ranges from 60–90% for most of the year. Monthly mean temperatures range typically from 15 °C between June and August and 35 °C between December and March, and the area receives between 2 300 to 3 100 h of sunshine /y. Subjects for the study were selected according to the willingness of individuals to be included in the study frame for testing.

Data Collection

A pre-tested individual questionnaire comprising closed ended questions was used to obtain demographic (age, gender, location) and occupational data (categorised as 'abattoir workers'; 'livestock keepers'; 'non-livestock keepers'; 'veterinary/livestock farmers' and 'other'). The 'other' category comprised people from the general community outside the traditional occupational risk groups i.e. business people, housewives, students, soldiers etc. Other information collected included contact with livestock at home, type of activities in which the individual was engaged, as well as awareness regarding zoonoses including RVF.

Ethical Consideration

During survey visits, interviewers introduced themselves and explained the objectives and all procedures to all potential interviewees participating in the study. A written consent form was obtained from adults or guardians of those individuals aged less than 18 y prior to inclusion. Ethical review and approval were granted by the Human Ethics Committee of the National Institute for Medical Research (NIMR), Dar-es-Salaam, Tanzania. Research clearance was obtained from the Tanzania Commission of Science and Technology.

Laboratory Analysis of Sera

During 2004, a total of 199 human (aged from 14 to 84 y.) serum samples were collected from urban and peri-urban areas surrounding Tanga town. The radius was about 30 km of the town centre. Serum samples from each individual were then stored at -20 ^{0}C until further use. Serodiagnosis of RVF was performed using a Rift Valley fever Inhibition ELISA kit (batch no. 2007/10) obtained from the National Institute for Communicable Disease, Sandringham, South Africa. Results or cut-off values were expressed as percent inhibition (PI) values using the equation: [(100 – (mean net optical density (OD) of test sample/mean net OD of negative control) × 100]. For ease of interpretation, and comparison with other studies, test sera were classified as seropositive if the PI was ≥ 38.6%. RVF IgG positive (past exposure to virus/infection before or during November 2004) samples were tested using a capture ELISA to evaluate the level of anti-RVF IgM antibodies (which reflect recent infection at least one month prior to November 2004) using test kit batch no. 2007/06. Results were expressed as percent positivity (PP) values of optical densities (Paweska et al., 2005), relative to those of a strong positive control serum. Threshold PP values ≥ 7.1% were considered to be positive and values less than this as negative.

Statistical Analysis

Questionnaire and laboratory data were handled and analysed using Epi-info (version 6.04, CDC, Atlanta, USA). The differences in RVF antibody prevalences were compared across the investigated variables using the Mantel-Haenszel chi-square. A value of $P < 0.05$ was considered significant. Biostatistical analysis was performed using Epi-info (CDC, 1996).

Table 1. The proportion of individuals in each category of each variable investigated during the study (n = 199)

Variable	Category	No examined	%	No positive	Prevalence (%)
Occupation group					
	Abattoir	41	20.6	3	7.32
	Livestock farmer	67	33.6	1	1.50
	Non-livestock keepers	38	19.1	0	0
	Veterinary/meat inspectors	11	5.52	0	0
	Other	42	21.1	4	9.5
Sex					
	Female	67	33.7	1	1.50
	Male	132	66.3	7	5.30
Age (y)					
	≤ 20	18	9.04	0	0
	20-30	48	24.12	3	6.25
	30-40	67	33.6	4	5.97
	40-50	41	20.6	0	0
	50-60	14	7.03	0	0
	≥ 60	11	5.52	1	9.09
Total		199	100	8	4.02

RESULTS

Descriptive Statistics

Most of the sampled subjects (n = 156; 78%) were between 20 and 50 y of age, and two-thirds were male and one-third female. The abattoir group consisted of only males. All of the non-livestock farmers group and almost all persons of the 'other' group did not have cattle at home. Non-livestock farmers were those involved in other agricultural activities like growing crops. Some of the abattoir workers (n = 9; 23%) and most of the veterinary staff (n = 1 0; 90%) reported keeping livestock at home and being involved activities related to cattle keeping (**Table 1**).

Presence of Antibodies to RVF Virus Infection

The overall prevalence of anti RVF virus immunoglobulin (IgG) antibody was 4.02% and none had IgM antibodies. The sero-prevalence of anti-RVF virus immunoglobulin (IgG) antibody was higher in males than in females but the difference was not statistically significant (P=0.34). Seroprevalence increased markedly in males aged between 20 and 40 y (data not shown), while antibodies were not detected in young people under 20 y. Among the occupational groups examined, the seroprevalence was 7.3%, 1.5% and 9.5% in the 'abattoir workers', 'livestock keepers' and 'other' categories, respectively (**Table 1**) although no statistical difference was found between any of these occupational groups (P = 0.086). None of the study participants considered RVF to be a zoonotic disease.

DISCUSSION

There is a paucity of virological and epidemiological information concerning the seroprevalence of RVF infections in the general population and various occupational groups in Tanzania. Overall, 60% of the sera investigated were from people who had contact with cattle, either through cattle keeping at home, or through occupations like working in abattoirs and as veterinary staff, and 40% were from people who had no close contact with cattle, like crop farmers and others. This last group might still have contact with cattle products, like raw meat and milk when preparing or consuming food. Interestingly, the detected seropositive status of the general population before the 2007 outbreaks support the endemicity of this disease in Tanzania (Kondela et al., 1985; FAO/UNDP/OAU/IBAR, 2001; Woods et al., 2002).

The low reactor rate for females in this study could probably be due to the small number of females (n = 67) studied compared with males (n = 132). Recent RVF outbreaks in Tanzania showed that the ratio of male to female mortalities was 1.6:1 and more patients aged 16–60 yrs died than in any other age group (MoHSS, 2007). The higher proportionate infection rate in males than females agrees with the findings of other workers (Nabeth et al., 2001). Traditionally, the majority of abattoir and cattle keeping activities in Tanzania are carried out by men. This may, in part, explain the high prevalence rate detected in males and in abattoir workers.

The overall prevalence of infection in the present study was lower than that reported from other parts of Africa such as a prevalence of 14.8% (Olaleye et al., 1996) in Nigeria, 24.4% (Nabeth et al., 2001) in Mauritania, and 22.3% in Senegal (Wilson et al., 1994). On the other hand, the prevalence in Tanga was higher than found from studies in pregnant women in Mozambique (Niklasson et al., 1987), where a prevalence of 2% was reported. This variability could be attributed to differences in sampling techniques, investigator technical know-how, climate-agro-ecological factors, as well as the diagnostic methods used. The IgG-sandwich ELISA and the IgM-capture ELISA used here, when compared with the virus neutralisation test (sensitivity 96.2% and specificity 95 2%), have sensitivities of 100% and 96.47% respectively and specificities of 99.95% and 99.44% respectively on field sera (Paweska et al., 2005).

Tanga Municipality is characterised by hot, humid and wet climate variables. This is thought to allow a build-up of high densities of mosquitoes of veterinary and medical importance (James, 1979) and may therefore increase the abundance of mosquito species that are potential vectors of RVF virus i.e. *Aedes* and *Culex* (Turell et al., 2007 and 2008). Laboratory-based vector competence and field isolation studies carried out with selected African mosquitoes suggest *Culex zombaensis*, *Culex poicilipes*, *Culex pipiens* and *Aedes caspius* to be potential vectors for RVF virus transmission (Meegan et al., 1980; Diallo et al., 2005; Turell et al., 2007 and 2008). Although *Aedes* and *Culex* species are known to be prevalent in Tanga, their role in transmission of RVF warrants further investigation (Mboera et al., 2000; Magesa et al., 2006).

A number of studies, (Wilson et al., 1994; Elfadil et al., 2006; Turell et al., 2008), have shown that the presence of, and exposure to bites from a wide range of mosquito vectors e.g. *Aedes* spp. was associated with an increased likelihood of the presence of antibodies to RVF virus in the human population. This would be consistent with RVF virus being transmitted by mosquito vectors in addition to any contact that individuals might have with infected animal parts (tissue, blood, urine, uterine fluid etc) during slaughtering (Chevalier et al., 2004; Gerdes, 2004) when assisting with the delivery of newborn animals, or iatrogenically by the use of contaminated needles during mass vaccination (FAO, 2002).

The finding that none of the participants was aware of RVF as a zoonotic disease is striking, and this is a reflection of the poor knowledge of zoonoses by livestock keepers, veterinary field staff and staff in the heath facilities coupled perhaps with the inter-epizootic nature of disease occurrence. The limited knowledge level recorded in this study may also be due to the general lack of data on RVF and inadequate communication between veterinary and human healthcare professionals. This shows also that emergency preparedness for RVF epidemics is low. Furthermore, low awareness is likely to expose people to increased risk of contracting RVF since they might not take proper precautions i.e. use protective clothing when dealing with abortion cases and during on-farm activities like slaughtering cattle. For instance, during the current study period most abattoir staff had no protective clothing. Also, the consumption of raw milk, raw blood and raw or undercooked meat is still common practice in some communities in Tanzania (Shirima et al., 2003).

CONCLUSIONS

This study highlights the importance of RVF as a public health risk and occupational disease in the area under study and supports the need for effective surveillance and future prospective studies to assess specific risk factors. Such studies will be crucial for the design of prevention strategies, which are likely to include instituting vector (i.e. mosquito) control measures and avoiding infected animal parts (urine, blood, uterine fluid). The link between relevant professionals needs strengthening on a broader scale (i.e. through combined veterinary/medical student training and continuing education) to embody the principle of collaborative approaches for epidemiological studies and control of zoonotic diseases.

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SESSION 5

ACHIEVING FOOD SAFETY AND SECURITY IN THE 21ST CENTURY

Biosecurity in a Global Market Place

M. Jeggo[1]*

ABSTRACT

International travel and free trade are modern bywords and the international movement of people, animals and livestock products seen as essential for the global market place to function. Yet is this compatible with a national bio-secure environment? Governments around the world seek to manage the risks posed by infectious disease to livestock, man, the environment and related ecosystems whilst at the same time permitting free trade. Ample examples exist of these competing elements as illustrated by recent outbreaks of avian influenza, bluetongue, severe acute respiratory syndrome (SARS) and most recently in Australia, equine influenza. Whilst the recognition that some 70% of new infectious diseases in man come from animals, even those diseases that affect only animals such as foot and mouth disease, can have devastating effects on trade and economies. The word 'biosecurity' now encompasses most of these elements with processes being developed to identify, mitigate or eliminate these biosecurity risks, and ultimately to prevent adverse events. An added dimension to be considered recently is that of bio-terrorism. So is it time for a new global co-ordinated and collaborative approach to managing biosecurity that recognises the need to encourage not restrict, the global market place? Are there newer approaches that could encourage global trade in livestock and livestock products? One such strategy could be to consider the biosecurity risks of the commodity as opposed to the disease status of the country of origin as a more effective approach for the future.

Key words: *trade, infectious diseases, biosecurity, risk assessment, health status, commodity-based, international standards.*

THE GLOBAL MARKET PLACE FOR LIVESTOCK AND LIVESTOCK PRODUCTS

In 1999 it was recognised that a global livestock revolution was underway characterised by a doubling in demand for livestock products over the next 15 years (Delgado et al., 1999). Driven by urbanisation, an increase in available incomes and a move away from a cereal- to a livestock-based diet, it was foreseen that this demand-driven process would provide a major opportunity for those in the livestock sector. Whilst not a global trend, it was clear that in both Asia and Latin America this revolution had the potential to seriously revitalise rural communities and for many provide a way out of subsistence farming. Now ten years on, every indication continues to support this basic premise (Dijkman et al., 2008). However, more recently there has been a shift of production away from the temperate and dryer areas, to the warmer and more humid areas but with an associated increase in livestock disease risks for producers in such regions. In response to these and other pressures, there has been a move from local multi-purpose activities to a more market orientated integrated process-driven production approach. Significantly, this has increased pressure on communal resources such as grazing areas and water.

As the livestock revolution has unfolded, in some regions there has been a focus on large-scale industrial type production systems with a major concentration on monogastric species (pigs and poultry) (Naylor et al., 2005). There are, however, large regional differences e.g. Brazil has emerged as the major global poultry and pig producer with China and Japan as major importers whilst sadly, the situation in Africa has remained static. Critically these changes have been associated with significant threats to both the environment and human health. The overall trend has continued to be associated with significant shifts of production from developed to developing countries (**Figure 1**).

FOOD SECURITY

There has been a growing focus on global food security since the Millennium Development Goals were set, and whilst still targeting developing countries the global nature of this challenge has become increasingly clear. The matter has now become significantly more acute with the new awareness of the impact of climate change on primary food production and the vital role played by water availability in the process. For livestock, the negative impact of methane production and the associated carbon trading issues have somewhat tempered producer opportunities. A major factor leading to volatility

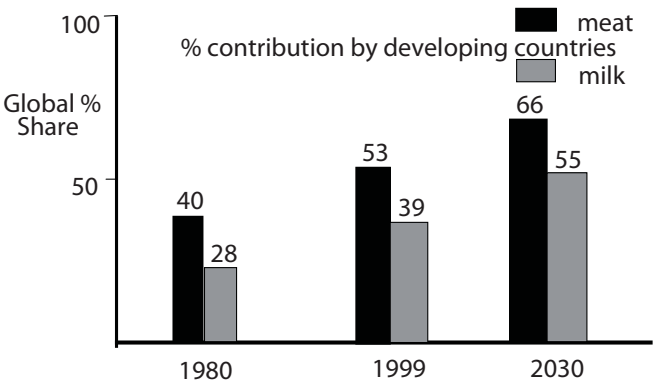

Figure 1. Shifting production of livestock products from developed to developing countrie.

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N.E. Odongo, M. Garcia & G.J. Viljoen (eds), Sustainable Improvement of Animal Production and Health. Food and Agriculture Organization of the United Nations, Rome, 2010: **369–372**

in the financial viability of supply chains has been the availability and price of grain. Considered within the concept of the three 'Fs' (feed, fuel and food), the influence of grain has been significant in the past ten years. Effects such as the availability and price of oil, and the use of grain as an alternative fuel source have had profound effects (Steinfeld, 2003). Yet the financial viability of livestock as a key food source depends intrinsically on the price of grain. The longer term implications of these fluctuations on livestock production profitability remain unclear.

DO INFECTIOUS DISEASES MATTER?

In the late 1980's infectious disease both of man and animals were considered threats of the past. The range of effective vaccines and therapeutics had persuaded many that health risks of the future would be focused on nutritional diseases and those associated with longevity. This paradigm, however, changed somewhat dramatically with the advent of mad cow disease and then subsequently, to name but a few, SARS, avian influenza, foot and mouth disease and most profoundly, AIDS (Woolhouse et al., 2005). By the turn of the century it was clear that not only were new and emerging diseases having a profound impact e.g. SARS, henipavirus infections, but that many diseases thought to be under control were re-gaining importance e.g. tuberculosis, malaria.

An example of the speed of spread can be seen with the introduction of bluetongue into Europe. The disease had been confined to one or two islands in the Mediterranean area but this changed dramatically in 2002 with an introduction into Holland and the subsequent spread throughout Europe.

Importantly, the cost of disease relates not only to the effect on the individual animal and producer, but the knock-on effects to other industries and related areas. The 2001 foot and mouth disease outbreak in Europe, whilst serious enough for livestock producers, had a huge impact on the tourist industry in the UK, whilst for the global SARS pandemic, the biggest economic impact was on the airline industry (**Figure 2**).

Overall, the incidence of infectious disease continues to grow globally, with known infectious diseases gaining further ground e.g. rabies, tuberculosis, salmonella, Rift Valley fever; and with new diseases continuing to arise e.g. acquired immunodeficiency syndrome (AIDS), bovine spongiform encephalopathy (BSE), Hendra virus infections, Nipah virus infections, SARS, and Ebola Reston virus. Significantly 75% of new diseases affecting man now originate in animals (Jones et al., 2008). Gradually the new concept of 'one health' has emerged within the framework built around the 'biosecurity' approach. Biosecurity is essentially about the risks associated with infectious disease and the related causative pathogens and has now been extended by some to include all invasive species and their impact on animals, man and the environment (**Figure 3**).

THE CONCEPT OF RISK

Managing the threats from infectious disease requires an understanding of the risks involved and an underlying perception that there are no certainties (Wooldridge et al., 2006). The threats are unpredictable and the associated risks need to be managed within this framework. Risk is determined through an analysis of likelihood and consequence with a crucial underpinning i.e. understanding that zero — risk does not exist. In risk management it is necessary at the outset to undertake a hazard identification followed by a detailed risk assessment before considering risk mitigation strategies and importantly a process of risk communication. A useful approach is to consider the twin elements of likelihood and consequence within the framework of a 'Bow Tie' structure (**Figure 4**).

A careful review would indicate that the biggest biosecurity risk posed by invasive species comes from those infectious agents that can evolve and adapt both to their current hosts and through host switching. In this context, the virus is ideally suited and therefore

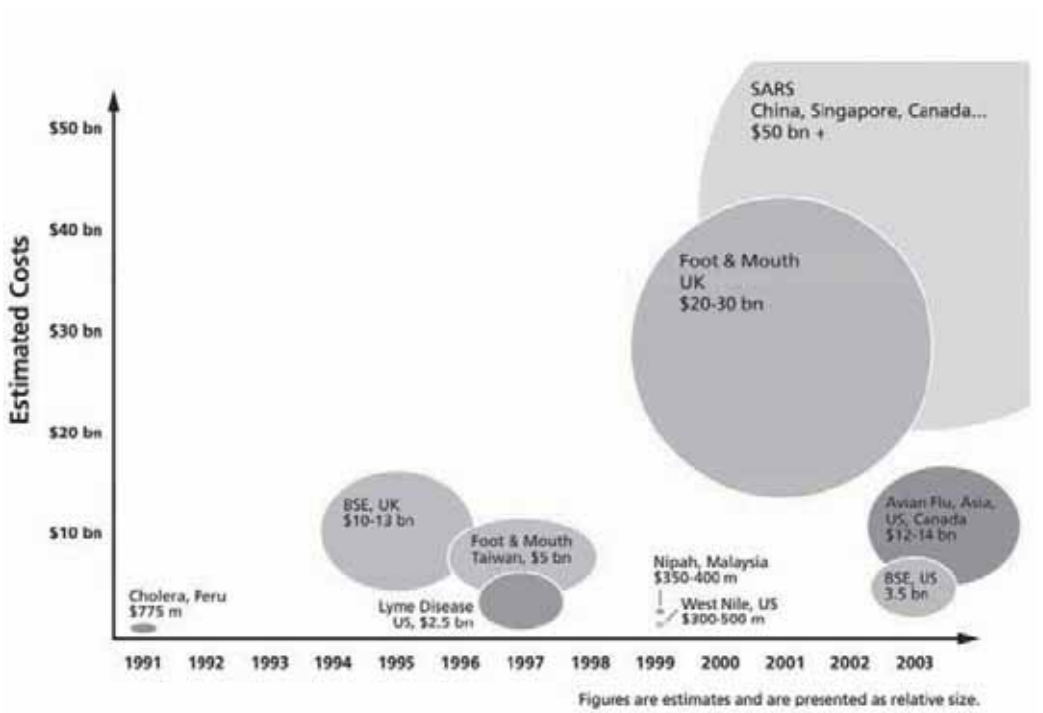

Figure 2. Economic impacts of selected emerging and re-emerging infectious diseases.

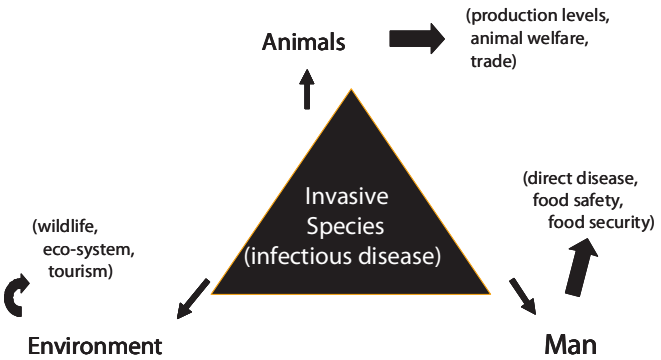

Figure 4. The 'Bow Tie' approach to risk assessment.

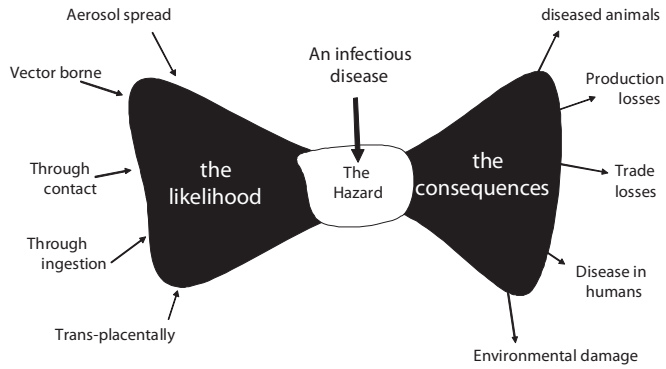

Figure 3. The current biosecurity framework.

not surprisingly seen as the biggest threat. However, other factors serve to enhance the ability of infectious agents to pose new threats including climate change, the global movement of people, animals and their products, the urbanisation and centralisation of people and livestock and the overall trend towards intensification of animal production systems.

World Organisation for Animal Health (OIE)

The OIE was established in 1924 to manage the risks of animal disease brought about by trade in livestock (e.g. rinderpest and foot and mouth disease). At this time it was recognised that the main risks were associated with the movement of live animals and therefore there was a clear focus on establishing the disease status of the country of origin of the animals being imported. This resulted in trade essentially taking place with countries of similar disease status or with those of a better status. Although increasingly this provided a 'global' framework, bilateral processes between countries proved to be the main way of operating for many years.

The OIE approach is not the same today (OIE, 2008) and the OIE's Terrestrial Animal Health Code (TAHC) now permits the use of zonation and compartalisation to assist trade. There is now a more sophisticated risk framework, which clarifies the processes for defining the disease status of a country, detailed guidelines on the laboratory tests to be used, and processes for assessing the quality of national veterinary services and their ability to correctly determine a national disease status. Although not directly a requirement, animal welfare issues are now being addressed by OIE and it works in close co-operation with the World Health Organization in addressing issues around zoonotic diseases. However, despite all these changes, for most developing countries, the fundamental basis for trade is still as it was in 1924 i.e. the health status of the 'national herd'.

Does the market really care that things have remained fundamentally the same over so many years? Certainly there remains real concern of the risks to the national 'herd' through the importation of infected stock or stock of unknown disease status. Many processes are in place to manage this risk today. Moreover, there was, and is today, considerable trade advantage for countries free of many of the OIE diseases. Additionally and increasingly Governments are concerned not only about the trading issues but the risks to man and the environment posed by such trade. Finally, issues surrounding the management of animal welfare in exporting countries are now having serious trade implications.

But viewed from some perspectives, this is not a 'level' playing field. Developing countries are placed at a serious disadvantage and unfortunately many of these countries which have agriculturally-based economies find themselves unable to trade effectively in their primary commodity. The starkest examples exist in Africa, where resources for establishing and maintaining veterinary services remain limited, international trade in livestock and livestock products is minimal and these countries remain within the 'poverty trap'. Is there an alternative?

Commodity Versus Product

In the past few years, the OIE has begun to accept the principle of commodity-based trading (CBT) and uses this as a guide for bilateral trade agreements (see www.oie.int). It is important to distinguish this from the extensive work undertaken by the FAO/WHO Codex Alimentarius Commission which deals with setting standards for products, albeit confined exclusively to issues of human food safety. There are regrettable overlaps and gaps in the standards established for commodities (OIE) and products (Codex Alimentarius). Harmonising these standards could do a great deal but would still require drastic modification of current certification and auditing procedures to have a real impact.

Building on this Paradigm

Can we therefore build on this alternative approach and trade in the livestock commodity or processed product and not in the live animal — and base the biosecurity risk assessments on the commodity or product rather than on the disease status of the country (Perry et. al. 2005)? For subsistence farmers in developing countries participation in the 'livestock revolution' is essential to create a pathway out of poverty.

Support for driving this change would need to focus on establishing processing plants and operations in developing countries and to undertake production in ways that address any biosecurity risks in the final exported product. The national disease status thereby becomes irrelevant and risk is assessed from the perspective of the commodity and not the animal. This approach would involve further investment in infrastructure to create the necessary post-farm gate processing capability but could have real advantages for the many developing countries currently excluded from the international livestock market place because of their current disease status. In creating this processing infrastructure, ways would need to be found to fully engage livestock producers in the process, through, for example, whole of chain co-operatives. In this way the benefits would feed all the way down the production chain and not just accrue post-farm gate.

Successes in this area have already been achieved, as for example in Kenya and Ethiopia (Perry et al., 2005) but international bodies such as the World Bank need to drive the process further to ensure the necessary investments are available to establish the needed infrastructures including processing plants. Of course, none of this precludes the need to continually improve the disease status of these countries, to improve their veterinary services and reduce the risks from disease on their livestock, people and ecosystems. Indeed the more these issues are addressed at the level of the farm the less will be the need to manage the risks at the processing end.

CONCLUSIONS

Global trade in livestock and livestock products continues to increase and is now clearly demand- rather than supply-driven. Ensuring a food secure environment will therefore in part only be achieved through meeting this demand. Biosecurity and particularly the risks from infectious diseases in live animals limit our ability to meet this demand at the global level. Investments in processing capacity at or near livestock production areas and importantly, prior to export from a country, could significantly reduce biosecurity risks. This has the potential to provide substantial benefits to those countries currently excluded from the opportunities provided by the livestock revolution because of their poor national disease status. Is this the way for livestock producers in developing countries to trade their way out of the poverty trap?

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Quality Comparison between Gamma-Irradiated and E-beam Irradiated Pork Patties

B.S. Song, W.G. Kim , J.G. Park, J.H. Kim, Y. Yoon, J.I. Choi , M.W. Byun & J.W. Lee[1]*

ABSTRACT

This study compared the effects of gamma and electron beam (e-beam) irradiation on the quality of pork patties. Pork patties (diameter: 100 mm, thickness: 10 mm) were vacuum-packaged and irradiated by gamma ray (^{60}Co with a 490 kCi source) and e-beam (2.5 MeV) at five, ten, 15, and 20 kGy at room temperature. During accelerated storage at 30 °C for 10 d, determination of total bacterial populations, hardness, and sensory evaluation was conducted at appropriate sampling intervals. The results of total bacterial populations showed that the gamma-irradiated (GR) samples had lower ($P < 0.05$) total bacterial counts than e-beam-irradiated (EB) samples during sotrage at 30 °C for 10 d, regardless of irradiation dose. The hardness and sensory properties such as colour, chewiness, taste, and overall acceptability of pork patties were decreased depending upon irradiation dose. GR samples had lower hardness and sensory scores than those of EB samples. In conclusion, gamma irradiation on pork patties should be useful in decreasing bacterial populations when compared with e-beam irradiation. However, further studies should be conducted to reduce the quality deterioration of GR pork patties.

Key words: *pork patties, gamma irradiation, E-beam irradiation, bacterial populations, hardness, sensory properties.*

INTRODUCTION

The meat processing industry has grown substantially in recent years, and the development of new processed meat products has increased because of the demand for ready-to-eat meat products and the excellent nutritional properties of the foods. However, slaughter, cutting, and processing procedures may increase the possibility of microbial contamination of foods. The studies by Taha (1999), and Woodburn and Raob (1997) showed that fresh meat and processed meat have been implicated in the transmission of foodborne pathogens such as *Escherichia coli* O157:H7, *Salmonella*, *Pseudomonas* spp., *Listeria monosytogenes*, *Staphylococcus aureus*, and *Bacillus cereus*.

One of the decontamination technologies for ensuring the microbiological safety of meat is radiation processing. In addition to spoilage bacteria, meat products may contain parasites and pathogenic bacteria, which can be inactivated by irradiation (Olson, 1998). Many researchers have also reported that gamma or electron beam (e-beam) irradiation in low doses (< 10 kGy) kills most microorganisms with no deterioration of food quality (Mohamed, 1999; Thayer et al., 1995; Youssef, 1994). Indeed, several reports have demonstrated the antimicrobial effects of radiation in meat products such as bacon, ham (Weirbicki and Heilgman, 1980), hamburgers (Dempster et al., 1985) and sausage (Kiss et al., 1990).

Meanwhile, Mitchell (1994) suggested that e-beam processing is regarded more favourably than gamma irradiation by consumers who may associate gamma processing with the nuclear industry. Thus, comparison was needed of the effects of gamma and e-beam on microbial, physicochemical and sensory properties of different foods. However, few studies have been conducted to compare the effects of gamma and e-beam irradiation on quality and reduction of bacterial populations in meat and meat products (Mitchell, 1994; Song et al., 2009; Park et al., 2010). Therefore, the objective of this study was to compare the effects of gamma irradiation and e-beam irradiation on the qualities of the pork patties as well as reduction of microbial population.

MATERIALS AND METHODS

Pork Patty Preparation and Packaging

Pork loins were purchased from three local grocery stores. Ground pork (53 g) was then mixed with various ingredients: (pork back fat: 15 g, ice water: 6 g, ginger: 1 g, onion: 8.5 g, egg white: 4.3 g, tomato ketchup: 1.6 g, isolated soy protein: 4.1 g, dried bread powder: 4.1 g, nutmeg powder: 0.05 g, NaCl: 0.65 g, flavour enhancing wine: 0.41 g, black pepper powder: 0.21 g, red colour reagent: 0.01 g, trisodium phosphate: 0.22 g, sugar: 0.85 g) as described by Lee et al. (2005), and 100 g of the meat batter was used to prepare patties (diameter: 100 mm, thickness: 10 mm) using a patty maker (Large Hamburger press, Tupperware, Inc., Orlando, FL, USA). The patties were then heated in a cooker (NUVUES–3 cooker, Menominee, MI, USA) up to 70 °C of internal temperature, removed from the cooker and cooled down at room temperature (25 °C). Each patty was placed in a retort pouch laminated with polyester, aluminum and polypropylene (MULTIVAC, Wolfertxchwenden, Germany), followed by vacuum-packaging. The internal temperature was monitored with a thermocouple (TES–1300 thermometer, TES, TAIWAN).

Irradiation and Storage Conditions

The vacuum-packaged samples were irradiated at 0 (control), 5, 10, 15, and 20 kGy of gamma rays, while the e-beam irradiated both sides of the patties at same dose as the gamma irradiation. Gamma irradiation was conducted using a ^{60}Co irradiator (point source AECL, IR–79, MDS Nordion International Co. Ltd., Ottawa, Ontario, Canada) in the Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute (Jeoung-Eup, Korea). The source strength was

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N.E. Odongo, M. Garcia & G.J. Viljoen (eds), Sustainable Improvement of Animal Production and Health. Food and Agriculture Organization of the United Nations, Rome, 2010: **373–375**

approximately 300 kCi with a dose rate of 10 kGy/h. Dosimetry was applied using 5 mm diameter alanine dosimeters (Bruker Instruments, Rheinstetten, Germany). E-beam irradiation was performed with an ELV–4 Electron-Beam-Accelerator (2.5 MeV) at the EB-Tech (EB-Tech Co., Daejeon, Korea). The beam currents were 2.5, 5, 7.6, and 10.5 mA for 5, 10, 15, and 20 kGy, respectively.

After irradiation, the patties were stored in an incubator (Mir 552, Sanyo Co., Tokyo, Japan) at 30°C for 10 d.

Total Aerobic Bacteria

Total aerobic bacterial populations in patties were determined on days 0, 2, 5, and 10. The 10 g portions of patties were placed aseptically in sterile nylon bag (10 × 15 cm; Sunkyung Co., Ltd., Seoul, Korea) containing 90 mL of 0.1% sterile peptone water (Difco Laboratores, Detroit, MI, USA) and blended for 2 min using a Lab-blender 400 stomacher (Seward medical, London, UK). The blended sample was used to test the growth of the total aerobic bacterial populations in a plate count agar (Difco Lab., St. Louis, USA). Plates were prepared in triplicate and incubated at 37°C for 48 h, and aerobic bacterial populations on a plate were determined as colony forming units (log CFU)/g.

Hardness and Sensory Evaluation

The hardness of the patties was also determined on day 0 using a penetrating test by a texture analyser system (TA-XT2i, Stable Micro System, England) equipped with a probe (1.0 cm thickness). Sensory evaluation of the patties was conducted by 21 panelists who were trained according to the method described by Civille and Szczesniak (1973). Colour, chewiness, taste, off-flavour, and overall acceptance of non-irradiated, gamma and e-beam irradiated samples were evaluated using a seven-point descriptive scale where 1 = extremely disliked or extremely weak to 7 = extremely liked or extremely strong. After irradiation, patties were removed from pouches and reheated in a cooker (NUVUES–3 cooker, Menominee, MI, USA) at 130 °C for 10 min for sensory evaluation.

Statistical Analysis

One-way analyses of variance (ANOVA) were used to determine the effect of a combined treatment of *Kimchi* on the growth of the microorganisms and the quality properties of four groups by a Statistical Package for Social Sciences (SPSS, 10.0). Duncan's multiple range test was used to compare the differences among the means at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of Irradiation on Bacterial Growth

After irradiation (day zero), gamma irradiation decreased bacterial populations more than e-beam irradiation ($P < 0.05$), and GR samples at more than 5 kGy and EB samples at more than 10 kGy had levels

Table 1. Effect on growth of total aerobic bacteria of pork patties with vacuum packaging and gamma ray or electron beam irradiation during storage at 30 °C (log CFU/g).

Days	Gamma ray (kGy)					Electron beam (kGy)				
	0	5	10	15	20	0	5	10	15	20
0	3.65	ND[2)]	ND	ND	ND	3.56	3.32	ND	ND	ND
2	–[1)]	6.89	5.76	3.32	ND	-	7.91	6.83	6.61	5.51
5	-	-	6.49	5.72	3.75	-	-	-	-	-
10	-	-	-	7.26	5.46	-	-	-	-	-

1 indicates no determination of cells because of spoilage.

2 not detectable.

Table 2. Evaluation of hardness and sensory qualities of gamma ray or electron beam irradiated pork patties after vacuum packaging.

	Dose (kGy)	Hardness (g)	Colour	Chewiness	Taste	Off-flavour	Overall acceptance
Gamma ray	0	431.76±45.35^{a}	6.8±0.8^{a}	6.7±0.7^{a}	6.8±0.6^{a}	1.2±0.1^{c}	6.8±0.8^{a}
	5	395.67±50.32^{a}	6.1±0.6^{a}	6.1±0.5^{a}	6.2±0.4^{a}	2.2±0.2^{b}	5.7±0.4^{a}
	10	385.06±27.59^{a}	5.6±0.5^{ab}	5.7±0.4^{ab}	5.6±0.6^{a}	2.7±0.3^{ab}	5.3±0.5^{ab}
	15	381.43±20.32^{a}	5.1±0.4^{b}	5.2±0.4^{b}	5.3±0.4^{ab}	3.1±0.3^{a}	4.6±0.2^{b}
	20	375.69±28.35^{a}	4.7±0.5^{b}	4.4±0.3^{b}	4.1±0.3^{b}	3.3±0.2^{a}	4.2±0.4^{b}
Electron beam	0	431.76±45.35^{a}	6.7±0.4^{a}	6.9±0.6^{a}	6.7±0.7^{a}	2.1±0.2^{b}	6.9±0.5^{a}
	5	424.38±36.22^{a}	5.9±0.6^{a}	5.8±0.6^{ab}	6.5±0.3^{a}	2.3±0.2^{ab}	5.6±0.4^{b}
	10	423.21±61.62^{a}	5.8±0.5^{ab}	5.5±0.3^{b}	5.8±0.5^{ab}	2.7±0.1^{a}	5.4±0.4^{bc}
	15	419.93±83.64^{a}	5.4±0.3^{b}	4.6±0.4^{c}	5.4±0.6^{b}	2.9±0.2^{a}	4.9±0.2^{c}
	20	407.34±69.88^{a}	5.1±0.4^{b}	4.3±0.3^{c}	4.3±0.4^{c}	2.7±0.2^{a}	4.4±0.4^{c}

$^{a-c}$ Means within the same column different letters differ significantly ($P < 0.05$).

below detection limit (2 log CFU/g) (**Table 1**). This was also found in studies by Chung et al. (2000), Song et al. (2009) and Park et al. (2010) indicating that gamma irradiation was more effective than e-beam irradiation for the destruction of *P. fluorescens* or total aerobic bacteria in refrigerated beef or beef patties. However, bacteria in the samples below the detection limit were recovered during accelerated storage at 30 °C.

Hardness and Sensory Evaluation

The results of the hardness and sensory evaluation of patties are shown in **Table 2**. The hardness of the GR and EB samples significantly decreased ($P < 0.05$) depending on the irradiation doses. The hardness of the GR patties was lower than that of the EB patties. Yook et al. (2001) studied the effect of gamma irradiation on morphological properties and post-mortem metabolism in bovine *M. sternomandibularis* with special references to ultrastructure, shear force, pH, and ATP breakdown. This observation suggests that the bonds between myosin and actin are disrupted by irradiation and is supported by the report of Lee et al. (2000), that myosin was denatured by gamma irradiation.

Sensory properties such as colour, chewiness, taste, and overall acceptability of the GR and EB samples were decreased depending on the irradiation doses (Luchsinger et al., 1996; Park et al., 2010). The GR samples had lower sensory scores than the EB samples. These adverse changes (off-flavour) may be caused by free radicals generated from irradiation (Smith et al., 1960). However, the generation of off-flavour in irradiated meat and meat products can be reduced by various methods such as modified atmosphere packaging, reducing the temperature (freezing) prior to irradiation and addition of antioxidants (Brewer, 2009).

CONCLUSIONS

Gamma irradiation is more effective for inactivating microorganisms in patties than e-beam irradiation. However, gamma irradiation decreased the hardness and sensory scores of patties to a greater extent than e-beam irradiation. Therefore, combination treatments such as modified atmosphere packaging, reducing the temperature (freezing) prior to irradiation and addition of antioxidants will be necessary for quality improvement of irradiated patties.

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Current Status, Surveillance and Control of Avian Influenza in Domestic and Wild Bird Populations in Bulgaria

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ABSTRACT

This report describes the history and current status of avian influenza (AI) infection and control in Bulgaria. The country has a unique geographic position in Europe with regard to wild bird populations and their migration routes which pass through its territory. In recent years, Bulgaria did not remain free from AI. The region with the highest rate of isolation of H5N1 virus strains were the Black Sea coast and wet territories connected with the Via Pontica migration pathway in the administrative districts of Dobrich, Varna and Bourgas. Low pathogenic (LP) AI strains isolated from ducks were subtypes H3, H4 and H6 from the areas of Plovdiv, Pazardjik, St. Zagora, Yambol, Sliven and Haskovo. Raising ducks for liver production is a popular practice in south and southeast Bulgaria. From an epidemiological standpoint, controlling circulation of AI viruses among duck flocks, especially before their gathering in larger farms for fattening is a mandatory requirement of official authorities. To prevent the spread of highly pathogenic (HP) AI, surveillance of domestic poultry as well as wild birds should be strengthened in countries at risk, especially along bird migration routes. Monitoring, sampling and analysis of the viral subtypes of AI found in wild birds needs to be carried out to fully understand their role in the propagation and spread of HPAI viruses.

Key words: *Avian influenza, Bulgaria, migratory routes, National Veterinary Service, wetlands, wild birds.*

INTRODUCTION

Avian influenza (AI) is a highly contagious viral disease affecting domestic poultry (chickens, turkeys, quails, guinea fowl, etc.), as well as pet birds and wild birds. It is a disease of varying severity but may be of great importance for animal health, with serious implications for the poultry industry and, in some cases, for human health. Influenza A viruses infecting poultry can be divided into two distinct groups on the basis of their ability to cause disease in susceptible birds: low-pathogenic AI (LPAI) and highly pathogenic AI (HPAI) (Lamb and Krug, 2001; Fouchier et al., 2005). Highly pathogenic avian influenza (HPAI) virus spreads rapidly, and may cause serious disease and high mortality in affected birds (up to 100% within 48 h). The low pathogenic avian influenza (LPAI) causes mild disease that may be undetected as some species of birds show no symptoms. Subtyping of influenza A viruses is based on antigenic differences between the two surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). To date, 16 HA and 9 NA subtypes of influenza A viruses have been identified.

Wild birds play a role on the circulation of influenza A virus. They often carry LPAI viruses (Alexander, 2000; OIE, 2004; Mekushinov, 2006), and infected individuals can spread these over a wide area during their migration between breeding and wintering grounds. The mallard is one of the most abundant waterfowl in the world, and Munster et al. (2005) detected numerous influenza A virus subtypes, including the strains H5 and H7, which may sometimes be highly pathogenic in this species (Mekushinov, 2006).

Once domestic birds are infected, outbreaks caused by HPAI can be difficult to control and often have major economic impacts for poultry farmers in affected countries, since mortality rates are high and infected birds must be destroyed in order to prevent the spread of the disease. Indeed, since 1997 millions of domestic poultry died or had to be destroyed due to outbreaks of HPAI H5N1 in the countries of Southeast Asia.

AVIAN INFLUENZA IN BULGARIA

The Danube Delta forms the most extensive wetland in Europe after the Volga delta. This is one of Europe's most important sites for breeding, passage and wintering of water birds, particularly wintering; it regularly holds more than 20 000 water birds. In winter 2005–2006, the Danube Delta area faced several outbreaks of HPAI H5N1 in both domestic and wild birds. H5N1 avian influenza was first reported in Romania in October 2005. In February 2006, the OIE confirmed a further outbreak in poultry in the Jurilovca district of Tulcea County. Several outbreaks had previously been reported in this County, although this was the first in the Jurilovca district. Birds on the infected farm and neighbouring premises were culled and movement controls on people and poultry applied.

The poultry livestock industry in Bulgaria is still not affected by the AI subtype H5N1 virus. However, in 2006 a pathogenic strain was isolated from five sick and dead swans and in the course of surveillance for AI viruses (AIVs) low pathogenic strains of types H4, H5, H6, H7 and H10 were also isolated. These findings show that the country is threatened by potential AI infection connected with wild migratory water fowl.

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Bulgaria possesses a unique flora and fauna with some 394 wild bird species recorded in the country. Bulgaria has a special geographic position in Europe with regards to wild bird populations (Nankinov et al.1997; Kostadinova and Gramatikov, 2000) and is the second country in Europe after Spain in terms of the diversity of its bird fauna. Although the territory represents only 1% of the European land mass, it contains 76% of the European ornitho-fauna (Nankinov et al.1997). Many of them are migratory birds and some are included in the Red Book of endangered species. There are two major migratory routes that wild birds use to cross the territory of Bulgaria - the Via Pontica and Via Aristotle. These are used by migratory birds from Northern, Central and Eastern Europe and western Siberia.

Anatidae (ducks, geese and swans) are a group of water birds that are ecologically dependent on wetlands for at least some aspects of their annual cycle. These species use a wide range of wetlands, from the high arctic tundra, rivers and estuaries, freshwater or saline lakes, and ponds or swamps, to coastal lagoons and inter-tidal coastal areas such as mud-flats, bays and the open sea. They also utilise man-made wetlands such as rice fields and other agricultural areas. Many Anatidae populations migrate between wetlands in the northern breeding areas and southern non-breeding areas and in doing so, regularly cross the borders of two or more countries.

These migrations create possibilities for multiple contacts with other bird and animal species and enhanced circulation and spread of AIVs. It is well known that most of the influenza A subtype viruses circulate in their principal host, the wild duck. The main migratory species in Bulgaria belong to two orders, Anseriformes (ducks, gooses, swans, etc.) and Charadriformes (*Arenaria inteprea, Vanellus spinosus, Scolopax rusticola, Sterna fuscata, Larus crassirostria,* etc).

One of the widespread migrating species in the country is the mallard (Anas *platyrhynchos*), which has a breeding population of up to 6 000 birds and a wintering population of up to 148 600 birds (BirdLife International, 2004). The greatest numbers are observed in October, with numbers falling until the end of December, and increasing again in January (Munster et al., 2005; Olsen et al., 2006). This is because the autumn migration has two stages. The first one is the movement of mallards to the Mediterranean Sea and western Asia, mainly during October and November, while the second comprises mallards that come for wintering in Bulgaria at the end of December and beginning of January. During the spring migration, which takes place in February until mid- March, mallards go back to northeastern Russia and Siberia (Munster et al., 2005; Olsen et al., 2006). Wintering of birds from Central Europe in Bulgaria is considered an exception (Munster et al., 2005).

High and Low Pathogenic Strains Isolated

The prevalence of infection with AI has a seasonal character, being greatest in late autumn and winter in the Northern hemisphere, when the birds come back from the regions where they lay their eggs and care for the young, namely the Arctic zone where there is a very dense presence of different bird species and nests and oral/faecal contamination is massive. Clinical symptoms of the disease appear in some of the resting stops during the migration e.g. an H5N1 epizootic in Romania in 2005 and cases of infected swans and wild ducks in the period after January 25, 2006 in Bulgaria. The severe cold of –25 °C was thought to be the cause of dissemination in all directions and to neighbouring Romania of subtype H5N1by sick and infected swans.

The regions in Bulgaria within the last three years where isolates of highly pathogenic viruses of the H5N1 subtype and the low pathogenic viruses of H4, H6, H7 and H10 have been found have been clearly defined (**Table 1**). The region with the highest rate of isolation of H5N1 virus strains completely overlapped the Black Sea coast and wet territories connected with Via Pontica in the administrative districts of Dobrich, Varna and Bourgas. Only one H5N1 strain was isolated in 2006 in the Vidin region as a result of the migratory movement of swans infected with this viral type originating from the Danube river delta. The preserved territory of Poda covers a surface of 2 270 hectares. It is located on the south of Bourgas, close to and connected with the Black Sea. Two hundred and forty five bird species occur in the region, some of them being included in the Red Book of Bulgaria and some of them of European importance. Many migrating and wintering species are found. The territory is on the migration route Via Pontica.

The first case of HPAI in wild birds was found in a dead mute swan found on the river Danube near the town of Vidin. It was confirmed as H5N1. This case was likely the result of spread by H5N1-infected migrating swans and wild ducks from the Danube delta. Mute swans were also found dead around Lake Durankulak at the coast in Kraimorie, Burgas as a consequence of AI-H5.

The Bulgarian National Reference Laboratory on Avian Influenza and Newcastle disease in birds also holds several LPAI isolates:

- influenza A/H10N7/Mallard/ Montana/08., isolated from the fresh faeces of live mallards, with no clinical signs inhabiting the river Ogosta in the district of Montana;
- influenza A/H7N7/Mallard/ Han Krum/08, isolated from the internal organs of a shot mallard, inhabiting the river Kamchia, near Han Krum village in the district of Shumen.

Areas and Species at Risk

Based on AI cases in Bulgaria over the last three years, several regions can be identified: highly pathogenic viruses of the subtype H5N1 and low pathogenic subtypes H4, H6, H7 and H10.

The risk zones of AI penetration are connected with the wet areas and territories via the main migratory path of wild birds and the Black Sea coast (**Figure 1**). The national early warning and surveillance system was adapted to and covered these risk areas on the basis of periodic risk assessment.

Two well equipped laboratories in Sofia and Varna cover the needs for sample investigation and research activities connected with AI in the country. The national surveillance plan includes domestic and wild bird populations and domestic small and large scale poultry production in the country.

Based on risk assessment, we believe that future research should focus on the populations of several species of wild migratory ducks wintering at Shabla Lake (district of Dobrich), Varna-Beloslav Lake (district of Varna) and the wetlands of Poda connected to the Mandra Lake (district of Burgas). Surveillance will include marking of caught birds to monitor their AI status in case they are caught again. These three lakes have been selected based on the advice of the Bulgarian Society for the Protection of Birds (BSPB) Varna, and on the basis of their hydrologic features which make placing of ornithological nets for catching ducks possible. Other lakes do not allow access by boat or they freeze over in the winter.

Geographic coverage

Shabla Lake Complex

Area: 3 195 ha; altitude: 0–40 m.

Shabla lake (Dobruch district) one of the favorite places where wild birds stop when they migrate. The lake complex includes the lakes of Shabla, Ezerets and Shabla Tuzla, located over Sarmatian limestone in northeastern Bulgaria, 5 km northeast of the town of

Table 1. AIVs isolated in Bulgaria during 2005–2008.

Isolate	Date	District	Location	Sample	Bird species	Virus isolates
A/malard duck/05	11.11.2005	Bourgas	Poda dam	Faecal sample	Malard duck	H6N2
A/swan/Vidin/06	31.01.2006	Vidin	Danube river	Internal organs	Swan	H5N1
A/swan/Varna/06	06.02.2006	Varna	Tzonevo dam	Internal organs	Swan	H5N1
A/swam/Kraimorie/06	07.02.2006	Bourgas	Kraimorie	Internal organs	Swan	H5N1
A/swan/Dobrich/06	06.2.2006	Dobritch	Duranculak Lake	Internal organs	Swan	H5N1
A/swan/Bourgas/06	17.02.2006	Bourgas	Chengenez Skale village	Internal organs	Swan	H5N1
A/mule duck/Parvomay/06	25.04.2006	Plovdiv	Parvomay town	Cloacal swab	Mule duck	H6N5
A/mallard/Pazardjik/06	21.03.2006	Pazardjik	Kovatchevo village	Faecal samples	Malard duck	H4N6
A/mule duck/Rajevo konare//07	14.05.2007	Plovdiv	Rajevo konare village	Cloacal sample	Mule duck	H4N6
A/malard/Krepost/07	18.04.2007	Haskovo	Krepost village	Cloacal sample	Mule duck	H4N2
A/mule duck/Rajevo konare/07	22.11.2007	Plovdiv	Rajevo konare	Cloacal swab	Mule duck	H6N5
A/malard/Chan Krum/08	31.01.2008	Shoumen	Chan Krum village	Internal organs	Malard duck	H7N7
A/malard/Montana/07	31.01.2008	Montana	Ogosta river	Faecal sample	Malard duck	H10N7

Figure 1. Areas in which there is a high risk of HPAI in Bulgaria.

Shabla. Shabla Lake unites two closely located coastal firth lakes — Shabla and Ezerets — connected through an artificial canal. On the east, the lake is separated from the sea by a 30–50 m sand strip. Shabla Tuzla is a semi-saline lagoon, located 1.5 km southeast of Shabla Lake and separated from the sea by high dunes. The territory of Shabla Lake complex supports 259 bird species and is of strategic importance for the globally threatened red-breasted goose in winter, as, together with Durankulak Lake, it holds almost the entire global population of this species. The lake is one of the sites with considerable concentrations of whooper swan and mallard.

Varna- Beloslav Lakes Complex

Area: 4 681.8 ha; altitude: 0–101 m.

The complex includes two lakes, Varna and Beloslav, connected by an artificial canal and located to the west of the city of Varna. Varna Lake is a coastal firth lake of natural origin, although Beloslav Lake was a closed freshwater firth until 1923. Due to the digging of artificial canals connecting Varna Lake with the Black Sea and another one between the two lakes, the water salinity increased. Since lakes do not freeze in winter, they are preferred as a wintering site by different ducks, cormorans and other waterfowls.

Mandra-Poda Complex, District of Burgas

Area: 5 988 ha; altitude: 0–101 m.

The complex includes Mandra Lake with its adjacent wetlands. Mandra Lake is located at the Black Sea coast and is the southernmost of the Burgas lakes. Its northeastern part touches on the city of Burgas. This former semi-saline lake has been turned into a freshwater reservoir. A lagoon covering the areas of Poda and Uzungeren has been preserved between the reservoir wall and the Black Sea (Roberts, 1978). The complex has international importance for the regular wintering of up to 69 000 waterfowl belonging to 82 species.

Bourgas lake system (Mandra Dam, area Poda and Vaya Lake

The areas with the highest number of isolated subtypes H5N1 virus of AI cover almost entirely a strip along the Black Sea coast and wet territories under Via Pontica in the areas of Dobrich, Varna and Bourgas.

Lake system and wet territories in Varna and Dobrich districts

Two findings were identified as important for AIV infections based on the epidemiological data. First, the availability of water reservoirs, some of which are warm waters being located near to electric power stations; and second, the availability of farms with ducks which are stocked with one-day chicks imported from France or produced from imported breeding eggs delivered by the same suppliers. Ducks are raised extensively in the first three months and afterwards are transferred to fattening farms with relatively good biosecurity standards.

Epidemiology and Surveillance

As shown in **Table 1**, isolated low pathogenic viruses from ducks were of subtypes H4 and H6 in the areas of Plovdiv, Pazardjik and Haskovo. Raising ducks for liver production on such farms is a popular practice in south and southeast Bulgaria (regions of Yambol, Sliven, Stara Zagora, Bourgas, Haskovo).

Table 2. Species and numbers of birds of interest for surveillance in the lakes.

Species	Size of wintering population on Shabla Lake	Size of wintering population on Varna-Beloslav Lake	Size of wintering population on Poda Lake
Ruddy Shelduck	1–27	0–8	1–6
Common Shelduck	1–20	1–43	8–641
Eurasian Wigeon	3–550	3–112	20–3 530
Gadwall	8–15	2–15	19–102
Common Teal	4–408	15–336	175–3 700
Mallard	80–62 210	97–4 004	268–11 883
Northern Pintail	3–19	1–8	3–57
Garganey	3–14	18–87 at the time of migration	Up to 1 112 at the time of migration
Northern Shoveler	6–42	2–35	30–1 109
Red-crested Pochard	4–32	1–28	1–54
Common Pochard	36–3 520	1 125 – 10 240	371–1 3170
Ferruginous Duck	Up to 88 at the time of migration	0–2	4–45
Tufted Duck	16–735	75–2408	486 – 12 800
Greater Scaup	2–26	-	5–100
Longtailed Duck	3–6	-	0–3
White –winged Scoter	-	-	2–12
White-headed Duck	-	3–5	24–202
Black Scoter	-	-	0–3

From an epidemiological standpoint, control over the circulation of AIVs among duck flocks, especially before their gathering in bigger farms for fattening, is mandatory but often overlooked by official authorities due to lack of sufficient resources. During the first three months of rearing the ducks are exposed to high risks of contact with other domestic and wild birds, including migrating waterfowl. If researched, the study will ensure monitoring of a very important factor for circulation and ecological migration of AIVs in the country. Understandably, it will help the local duck industry (a major export earner for the country) to improve its biosecurity standards.

In the past few years the surveillance programme of the Bulgarian National Veterinary Service has included both passive and active surveillance of wild birds. In fact, mainly dead birds were submitted to laboratories, and very rarely faecal samples from places the wild birds inhabit. Also, attempts had not been made either to catch live wild waterfowl, or to monitor the status of marked birds and assess their potential for carrying of AIVs during different life stages. For that reason active surveillance and using microsatellite markers for bird migration tracing will improve the early warning and preventative AI control system.

The currently ongoing outbreaks caused by HPAIV of the subtype H5N1 are of concern not only to the poultry industry but also to public health. This virus, which causes a high fatality rate among infected patients, may adapt to efficient human-to-human transmission and thus initiate a new human influenza pandemic (Alexander, 2000; BirdLife International, 2004; Wallenstein et al., 2007). Since 1996, when the ancestor virus was identified in domestic geese from China, outbreaks have spread and now encompass countries in Asia, the Middle East, Europe, and Africa. This spread of HPAIV among poultry flocks is traditionally thought to occur by transport of infected poultry, contaminated equipment, and persons associated with the poultry industry. HPAIV has occasionally been detected in wild birds near affected poultry flocks, but these birds have had limited or no role in virus dissemination. In the current outbreaks, however, wild birds are suspected of playing a major role as long distance virus vectors.

During the expansion of HPAI (H5N l) outbreaks from Asia to Europe, two events implicated wild birds, particularly water birds, as long distance virus vectors. First, virus outbreaks in 2005 spread rapidly westward from Russia and Kazakhstan in July and August to Turkey, Romania, and Ukraine in October. Wild water birds were suggested as vectors because the virus spread through areas that had no previous record of any virus presence and coincided with the fall migration of wild water birds between these areas. Second, at the beginning of 2006, HPAIV (H5N1) was detected in many wild water birds in western Europe, often in areas where no outbreaks had been detected among intensively surveyed poultry; this event overlapped with unusual water bird movements associated with cold weather in the Black Sea.

To prevent further spreading of H5N1, surveillance in domestic poultry as well as in wild birds should be strengthened in countries at immediate risk, especially along migrating bird routes. Resources should be focused on the reduction of close contacts between humans, domestic poultry and wildlife through better management practices and improved biosecurity in poultry production enterprises, especially those that are small and 'open-air' - where domestic poultry and waterfowl are allowed to mingle with wild birds.

Official competent authorities such as the Chief Veterinary Officer would also need to monitor 'wet' and wildlife markets, where wild and domesticated species are kept in close proximity and are at risk of exposure to a wide range of pathogens. Limiting contact with wild birds should therefore be part of any AI control strategy. The control

of AI infection in wild bird populations at this stage is not feasible from logistical, environmental and biodiversity points of view. Indiscriminate culling of wild migratory bird populations would be ineffective in preventing further spread of AI and their hunting would likely cause dispersion of the birds.

PRESENT AND FUTURE NEEDS

Monitoring, sampling and analysis of the viral subtypes of AI found in wild birds needs to be done in order to fully understand their role in the propagation and spread of HPAI viruses. Multidisciplinary research is required that brings in the competencies of veterinarians, wildlife specialists, ornithologists, virologists, molecular biologists and other specialities. Besides the current regional and country specific AI projects being implemented by FAO, Mongolia has been assisted through a regional technical co-operation to review emergency preparedness and surveillance activities for HPAI since the outbreak in wild birds was reported.

A Global Strategy for the prevention and control of HPAI has been prepared by FAO and OIE under the umbrella of the Global Framework for the Control of Transboundary Diseases (GF - TADs). This Global Strategy addresses country level activities as well as the indispensable regional and international coordination. Within the epidemiological context of the current HPAI outbreaks, there is an urgent need to strengthen the joint FAO/OIE/WHO Global Early Warning System (GLEWS) so as to improve the regional capacity for early detection and response to AI incursions. Immediate support to national Animal Health Services will be required in Eastern Europe for emergency preparedness, surveillance and early response activities Diagnostic capability in the region for avian influenza have been substantially enhanced by national efforts, with coordination and support provided by international organization. However, surveillance methods are still seriously inadequate to allow confident national and regional decisions to be made.

Specifically, surveillance efforts should be shifted from simple case-finding to identification of risk factors influencing maintenance of infection, and integrate surveillance procedures into control strategies. Also, for each country, the transmission pathways which are considered to have been responsible for infection dissemination in the current epidemic should be identified, and a surveillance strategy developed which will allow each of these pathways to be monitored, and changes in prevalence to be assessed. For example, market surveillance and interviews should be used to identify high-risk markets, bird types and bird sources, and control strategies built around this information. Further, for each country which no longer has active infection (or cases), surveillance measures that would minimise the time to detect a new incursion should be determined within the limits of available resources.

Other steps would include applying nuclear molecular epidemiological investigation methods more comprehensively, to clarify epidemiological processes which are influencing the evolution of the epidemic: assessing the level of human exposure under various circumstances, in order to evaluate the risks of emergence of a virus capable of human to human transmission, and use this information to help guide the allocation of resources to different elements of the control strategy. Further needs include developing rapid and standardised methods for the routine analysis of surveillance data which would identify important changes in the H5N1 situation, and enable notification of this information to the competent authorities; and harmonised collection and presentation of surveillance and control data across countries in the region, so that information can be interpreted in a compatible way across the region.

CONCLUSIONS

The region with the highest rate of isolation of H5N1 virus strains completely overlapped the Black Sea coast and wet territories connected with the Via Pontica migration pathway in the administrative districts of Dobrich, Varna and Bourgas. Isolated LPAI strains from ducks were of subtypes H3, H4 and H6 in the areas of Plovdiv, Pazardjik, St. Zagora, Yambol, Sliven and Haskovo.

Raising ducks for liver production is a popular practice in south and southeast Bulgaria. From an epidemiological standpoint, control over circulation of AIVs among duck flocks, especially before their gathering in larger farms for fattening is a mandatory requirement of official authorities. To prevent the spread of HPAI, surveillance of domestic poultry as well as wild birds should be strengthened in countries at risk, especially along bird migration routes. Monitoring, sampling and analysis of the viral subtypes of avian influenza found in wild birds needs to be done in order to fully understand their role in the propagation and spread of HPAI viruses.

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Vitamin D Metabolism in Experimental Animals: Kinetics of *Solanum glaucophyllum* Active Principle in Cows and Assessment of Calcium, Phosphorus and Vitamin D3 Requirements in Broilers

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ABSTRACT

In 1990 our group began working on the development of a sensitive method to measure the active principle (1,25 dihydroxy-vitamin D_3-glycoside) of *Solanum glaucophyllum*, a plant which grows wild in Argentina and causes calcinosis in breeding cattle. A radioreceptor assay (RRA) was applied to measure the free vitamin D metabolite in the plasma of experimental cows that were fed the plant in order to study the kinetics of the active principle. The 1,25 dihydroxyvitamin D concentration in plasma showed a 33-fold increase four h post treatment. Peak levels were recorded 12 h after dosing, decreased by half between 24–36 h and continued declining until 48 h. More recently, this plant has been proposed as a source of vitamin D activity (VDA) and thereby may contribute to improving Ca and P utilisation by animals and environmental care. The effects of different dietary levels of calcium (Ca) and phosphorus (P) over the range between commercial recommendations (control) and two thirds of NRC requirements (basal) as well as different sources of those minerals were therefore studied in experiments covering either a part or the entire breeding cycle of broilers through measurements of productive, nutritional, skeletal and biochemical parameters. Results indicated that birds fed diets deficient in these minerals exhibited skeletal responses but nevertheless showed better productive responses than those fed control diets. The high levels of vitamin D_3 employed in commercial farms (25 times NRC recommendations) could enable birds fed on deficient diets to increase synthesis of the active metabolite of the vitamin in order to partially overcome deficiencies in these minerals. On the other hand, such high levels of vitamin D_3 might have been unbalanced for optimal efficiency, at least under the experimental farm conditions of the present work.

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INTRODUCTION

Enteque Seco (ES), which affects grazing cattle in Argentina and other countries in South America, is caused by ingesting fallen leaves of the toxic weed *Solanum glaucophyllum (SG)* (Okada et al., 1977). It is characterised by loss of body weight, kyphosis, stiffness of the forelimbs, soft tissue calcification and modifications in Ca and P plasma concentrations (Worker and Carrillo, 1967). The active principle in *SG* is 1,25$(OH)_2$vitamin D_3-glycoside (Haussler et al., 1976). The economic relevance of *SG* could be seen on the one hand as being the causal factor of toxicosis of grazing cattle (negative value), and on the other, as a valuable source of vitamin D_3 active metabolites (positive value). Until now, most scientific work has dealt with the pathology and biochemistry of this calcinotic disease in experimental animals. Conversely, only a few publications have described quantitative studies about the vitamin D synthetic capability of the plant (Weissenberg, 1989), and only one report could be found about the environmental conditions that might influence vitamin D_3 production by the plant (Puche et al., 1980) in addition of the recent report of Dallorso et al. (2008).

Pharmacological applications of *SG* have been reported in human and veterinary medicine as well as in animal production. Potential veterinary uses include the prevention of milk fever, pseudo-vitamin D deficiency of pigs and acidosis in chicks (Weissenberg, 1989). The first assay applied in animal husbandry was about elucidating the effect of *SG* powder from leaves on egg shell quality in laying hens (Gallego et al., 1978/9). Recently, *SG* has been used as an additive in the feed of chicks to improve phosphorus utilisation (Cheng et al., 2004), and a further promising application would be the supplementation of finishing cattle with *SG* to improve meat tenderness (Paaren, 1999).

The present report summarises some unpublished results of research to improve the diagnosis of ES using a radioreceptor assay (RRA) to determine the concentration of 1,25$(OH)_2$vitamin D in plasma (1,25D) based on the knowledge that 1,25vitamin D_3-glycoside is cleaved by intestinal bacterial enzymes to 1,25D (Boland et al.,1987). This enabled us to apply our experience and expertise to the area of Ca and P nutrition in poultry.

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MATERIALS AND METHODS

Studies on ES Diagnosis

The experiment was performed to describe the fate of the active principle in *SG* on experimental cows.

Animals

Two non-pregnant, non-lactating adult Jersey cows without any clinical and pathological signs were used. Animals were housed indoors in individual pens and fed diets supplying 75 g Ca and 35 g P/d. Cows ('A' and 'B') were fed a diet composed of 65% corn silage, the remainder being a mixture of beet pulp, soybean meal, distillers grain and alfalfa hay with a commercial supplement. Each animal was fed 9 kg dry matter/d.

Vitamin D Compounds

The [^{3}H]–1,25D_3 was generously supplied by the National Animal Disease Center, (NADC), Iowa, USA. The 1,25D_3; 1,24,25$(OH)_3D_3$; 1,25,26,$(OH)_3D_3$ and 1a, 25$(OH)_2D_3$–26,23 lactone were kindly provided kindly by Dr. Milan Uskokovic (Chemical Research Department, Hoffmann-La Roche, Inc., N.J., USA.

Solanum glaucophyllum (SG)

The SG was provided by Dr. C. Corbellini (INTA, Argentina). The leaves were collected in Mercedes, Bs. As., Argentina, in May 1995.

Experiment

After 2 weeks on the experimental diet a single dose of 100 g of powdered dried leaves of *SG* was mixed with the silage and given to each of the two cows. Two blood samples were taken 24 h before feeding and just before the single dose of *SG* to obtain baseline physiological data. Sampling was then continued every four h during the first d, at 36 h, 48 h, 72 h, 96 h., and on d seven after dosing. Blood samples were taken from the jugular vein with heparinised syringes, centrifuged to obtain plasma, and kept frozen at –90 °C until analysed.

Determination of 1,25D_3

A one-step extraction procedure was used (Dallorso et al., 2001) followed by analysis using a non-HPLC charcoal binding assay using thymus gland receptor (Reinhardt et al., 1984). Radioactivity was quantified using a scintillation counter (Beckman L.S. 8 000).

Determination of 1,24,25$(OH)_3D_3$, 1,25,26,$(OH)_3D_3$ and 1,25$(OH)_2D_3$–26,23 Lactone

To elute the three more polar vitamin D_3 metabolites, prewashed SPE C18 cartridges were sequentially washed after sample application with double distilled water, methanol:water (50:50), hexane:methylene chloride (90:10), and hexane:isopropanol (99:1). Finally, these three metabolites were eluted with hexane:isopropanol (85:15). To obtain the three metabolites separately, the eluates were evaporated in a vacuum centrifuge, hexane/methylene chloride (2/1): isopropanol:methanol (46:50:4) added, injected onto a HPLC unit (Waters Corp.) and run through a silica column, with monitoring using an absorbance detector (Model 440) at 254 nm. The mobile phase used was hexane/methylene chloride (2/1): isopropanol:methanol (46:50:4). Each metabolite was collected in a different fraction by an ISCO Collector (Model 568) and all were analysed by radioimmunoassay (RIA, Hollis et al., 1996). The antibodies were raised in rabbits in the NADC. The [^{3}H]–1,25D_3 fraction was used to determine the recovery of all the vitamin D metabolites.

Experimental Studies on Calcium and Phosphorus Nutrition in Poultry

Intensive poultry production produces an excess of P in manure. When this is applied on fields as fertiliser, the surplus can cause eutrophication of water bodies (Sharpley et al., 1994). In order to contribute to environmental care through nutritional management the effects of different dietary levels and sources of Ca and P covering the range between commercial recommendations and near two-thirds of NRC (1994) requirements on productive, nutritional, skeletal and biochemical responses were studied in a series of experiments covering part or the entire breeding cycle of broilers. Two of these experiments are presented.

Animals

These were males Cobb–500 from Granja Tres Arroyos, Capilla del Señor, Buenos Aires, Argentina.

Trial 1

This covered the period from 7–20 d of age (initial weight [d 7] was 151 ± 17 g). The feed was mash composed of corn, soy meal (40% crude protein), soybean, soy oil, limestone, calcium phosphate, salt, methionine and a premix containing 5 000 IU vitamin D_3/ kg of diet and covering requirements for this strain of broilers except those of Ca and P in the basal diets. Animals were divided into two groups each of 25 broilers i.e. a control group that received Ca = 0.92% and P_a= 0.41%; and a group receiving a basal diet with 0.56% Ca and O.28% P_a.

Trial 2

This was carried out when animals were between 1 and 49 d of age. The feed was mash composed of corn, soy meal (45% crude protein, soybean, meat meal (41% crude protein), soy oil, limestone, salt, methionine, lysine, threonine and premix containing 5 000 IU of vitamin D_3/ kg diet and covering requirements for this strain of broilers except for Ca and P levels in basal diets. The control animals (five pens of 15 animals each) were on a starter ration of 0.9% Ca and 0.45% P_a, a grower diet with 0.88% Ca and 0.42% P_a, and a finisher of 0.84% Ca and 0.40% P_a; during the last week the diet had 0.78% Ca and 0.35% P_a.. Animals on the basal diet (five pens of 15 animals each) were on a starter ration containing 0.56% Ca and 0.28% P_a; a grower diet with 0.55% Ca and 0.26% P_a and finished on a diet with 0.52% Ca and 0.25% P_a. During the last week, the diet contained 0.49% Ca and 0.22% P_a.

Measurements

Animals were weighed and their feed consumption measured weekly and data were expressed as the average of each pen. At the end of each trial broilers from each pen (n = 5) were taken at random and maintained in cages until euthanised by complete bleeding to be used for some of the following determinations:

- Ca, P_i and 1,25D levels in plasma obtained from heparinised blood samples that were immediately centrifuged at 3 500 rpm for 10 min;
- Ca, P, and ash levels, morphology, weight, density and volume of right tibiotarsi; and
- Ca, P and ash in feed and manure.

Feed samples of each treatment were taken from the appropriate bags after preparation. Manure samples were collected in cages during a period of 8 h prior to euthanasia. Ca was determined by atomic absorption spectroscopy (AAS) on plasma diluted in a solution containing La_3O_2 0.5% and Pi by colorimetry (Fiske and Subbarow, 1925). Bone volume was determined by complete immersion of tibiotarsi in a fixed volume of water in a graduated tube (bone volume (mL) = water volume with bone inside the tube - water volume without bone inside the tube). Bone density was calculated as bone weight in air/bone volume. Tibiotarsi, feed and manure were dried in an electric stove at 80°C (DM), incinerated at 580°C and ash dissolved in HCl: H_2O (1:1) and filtered, and washed with water to a final volume of 50 mL.

[1,25D] was analysed by RIA (Gil and Dallorso, 2002) using plasma samples prepared as described previously (Dallorso et al., 2001). A radioreceptor assay (RRA) was replaced advantageously by RIA to determine the active vitamin D metabolite due to the fact that the binding capacity of the antibody raised against $1,25D_3$ remains constant when stored in solution at –20 °C while the intracellular vitamin D receptor (VDR) employed in RRA, although freeze-dried and stored at –80 °C needs to be prepared frequently, among other disadvantages. Although the antibody binds to 25(OH)vitamin D (25D) with five percent cross-reactivity (100% 1,25D), since 25D was undetectable in the analyte fraction (F2) and conversely, the analyte was not present in the 25D fraction (F1), this method was considered adequate for measurement of [1,25D].

Analysis of data

In trial 1, animals were considered experimental units (n = 25 animals in each group) for the analysis of individual variables under study. Values of body weight were studied in all 25 animals. The other determinations were made on a randomised sample of five animals. In trial 2, pens were considered experimental units (n = 5 pens in each group of 15 animals each). Values of body weight and feed consumption were obtained for all 15 animals in each pen and expressed as an average of each pen. The other determinations were made using a randomised sample of six animals from each pen.

Differences between control and basal groups were detected by Two-Sample t test (P-value ≤ 0.05) using the software Statistix SXW-Version 8.0.

RESULTS AND DISCUSION

Studies on ES Diagnosis

The evolution of the levels of vitamin D metabolites in cows 'A' and 'B' is shown in **Figures 1** and **2**. The 1,25D concentration showed a 45-fold increase to 3 345 pg/mL by 4 h post treatment in cow 'B' and a 20-fold increase to 5 200 pg/mL in cow 'A'. The maximum was observed 12 h after feeding in both cows, decreased to half its maximum between 24 h and 36 h and continued declining until 48 h. The 1α-lactone; $1,24,25(OH)_3D_3$ and $1,25,26(OH)_3D_3$ concentrations were quantified before and at 24, 48, 72, and 96 h and on day 7 post feeding. The $1,24,25(OH)_3D_3$ and **1α**-lactone levels reached a maximum at 24 h with 9.7 and 14-fold increases (1 930 pg/mL and 1 037 pg/mL) respectively in cow 'B' (see **Figure 2**). The $1,25,26(OH)_3D_3$ concentration was only slightly elevated compared with the other three vitamin D metabolites. All three metabolites returned to near baseline levels by 7 d. As expected, there was an increase in 1,25D and $1,24,25D_3$ levels after feeding cows with *SG* which is consistent with our previous findings (Dallorso et al., 1994). It was also observed that in addition to $1,24,25D_3$, the metabolite **1α**-lactone was also a major circulating $1,25D_3$ form. This is in agreement with reports of pharmacological doses of $1,25D_3$ to adult cows (Horst et al., 1983) and with experiments with radio-inert compounds in rats that showed injected $1,25D_3$ to be an efficient precursor of 1α-lactone (Horst et al., 1984). No significant elevation was recorded in plasma $1,25,26D_3$ levels. This situation is in accordance with previous work (Reinhardt et al., 1982). It seems reasonable to conclude that $1,24,25D_3$ and 1 α-lactone arose from further metabolism of the increased $1,25D_3$ levels emanating from the treatment with *SG* since these compounds circulate in low concentrations in normal cows (Reinhardt et al., 1981) and are found to be elevated in different species like the rat (Ohnuma and Norman, 1982), dog (Ishizuka et al., 1984) and cow

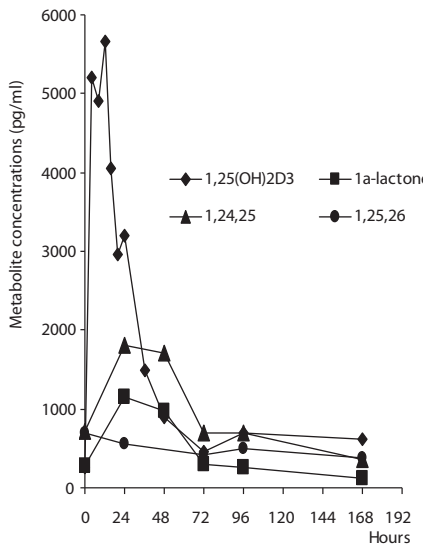

Figure 1. Plasma $1,25(OH)_2$ D_3; 1 alpha lactone: $1,24,25(OH)_3$ D_3 and $1,25,26(OH)_3$ D_3 concentrations after a single oral dose of 100 g *Solanum glaucophyllum* to cow 'A'.

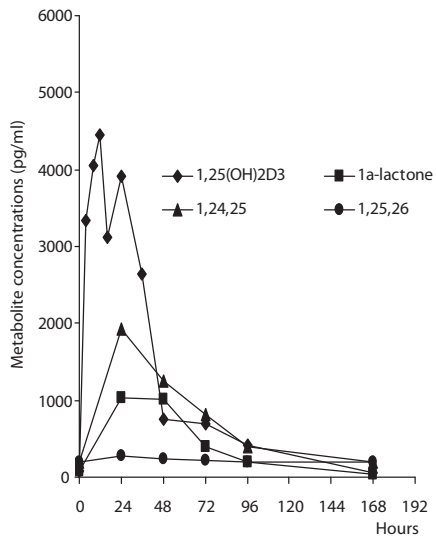

Figure 2. Plasma $1,25(OH)_2$ D_3, 1 alfa lactone; $1,24,25(OH)_2$ D_3 and $1,25,26(OH)_3$ D_3 concentrations after a single oral dose of 100 g *Solanum glaucophyllum* to cow 'B'.

(Horst et al., 1983) treated with vitamin D derivatives. The appearance of each metabolite paralleled the appearance of 1,25D.

The significantly augmented 1 α-lactone or 1,24,25D_3 values caused by the 1,25D_3 degradation recorded after the feeding period cannot be used to diagnose *SG* toxicity. In previous work with rabbits and ewes dosed orally with *SG* (Dallorso et al., 2000a), high plasma levels of the 1,25D metabolite occurred 1 h after administration and remained higher than control animals for up to 24 h after administration. Also, while rabbits dosed orally and subcutaneously showed increments in the plasma metabolite, highest values were recorded in those dosed orally. The calcinotic effects seen in rabbits dosed subcutaneously could have been caused by the 1,25D_3-glycoside without the intervention of enteric microbial enzymes (Dallorso et al., 2000b). This was suspected earlier (Barros et al 1981) who showed specific effects of this active principle in the aorta of rabbits 6 h after intravenous administration of aqueous extracts of *SG* leaves.

Experimental Studies on Calcium and Phosphorus Nutrition in Poultry

There were no significant differences (P = 0.6819) in body weight at 20 d of age between control (746 ± 62.47 g) and basal (736 ± 100.86 g)

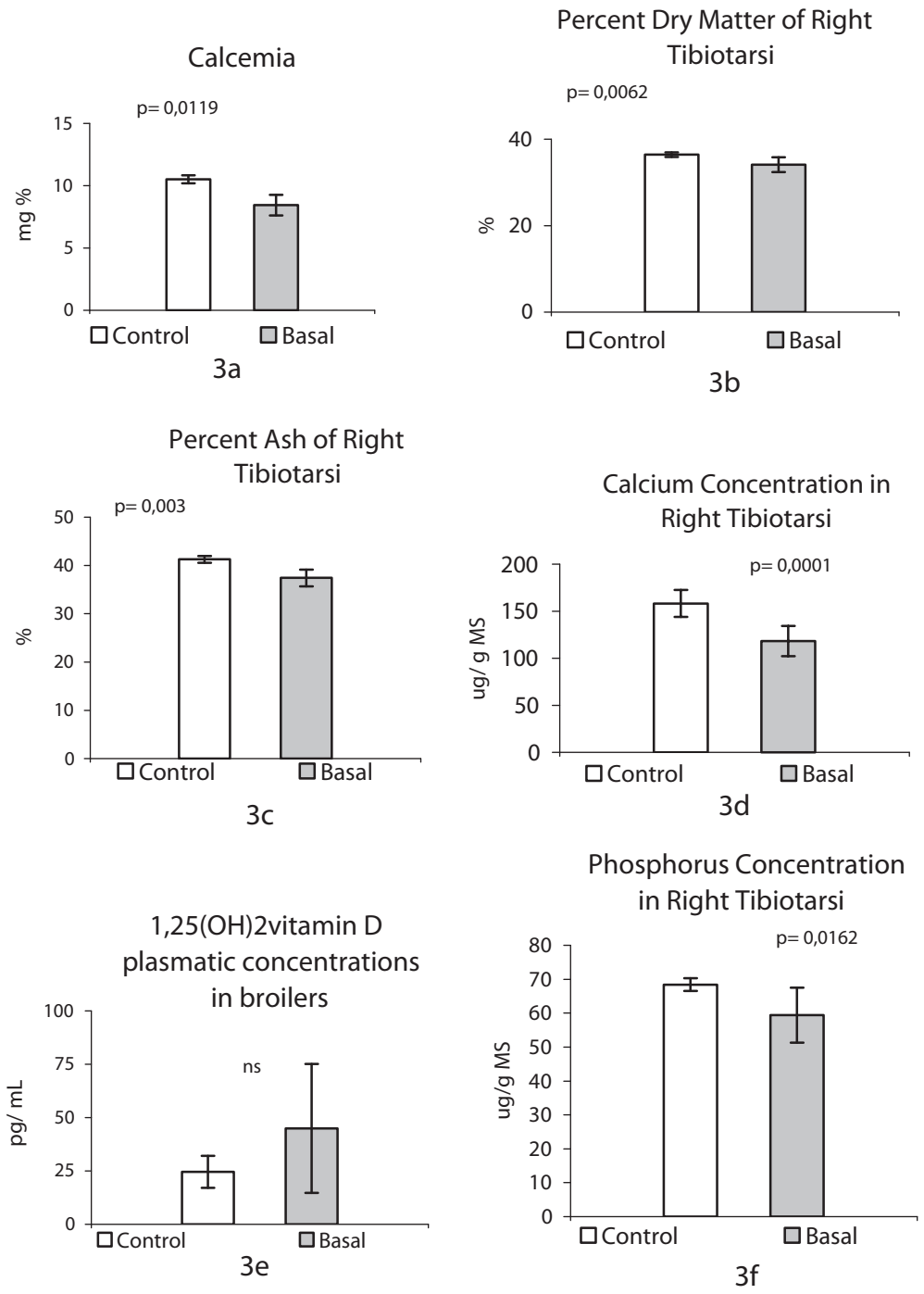

Figure 3. Biochemical and skeletal variables measured at 20 d of age in broilers (Trial 1).

Table 1. Body weight and feed conversion in broilers (Trial 2).

Treatment	Body weight (g)			Feed Conversion (g feed/g weight gain)		
Age(d)	21*	28*	35	21***	28***	35***
Control	750±13	1 276±29	1 901±50	1 426±11	1 516±16	1 633±9
Basal	824±49	1 336±52	1 966±80	1 286±24	1 423±15	1 558±14

Body weight (g) and feed consumption (g) were evaluated weekly and values in table are the mean of five pens (15 animals each).

Differences between control and basal groups within days of age: $^{*}P \leq 0.05$; $^{***}P \leq 0.001$.

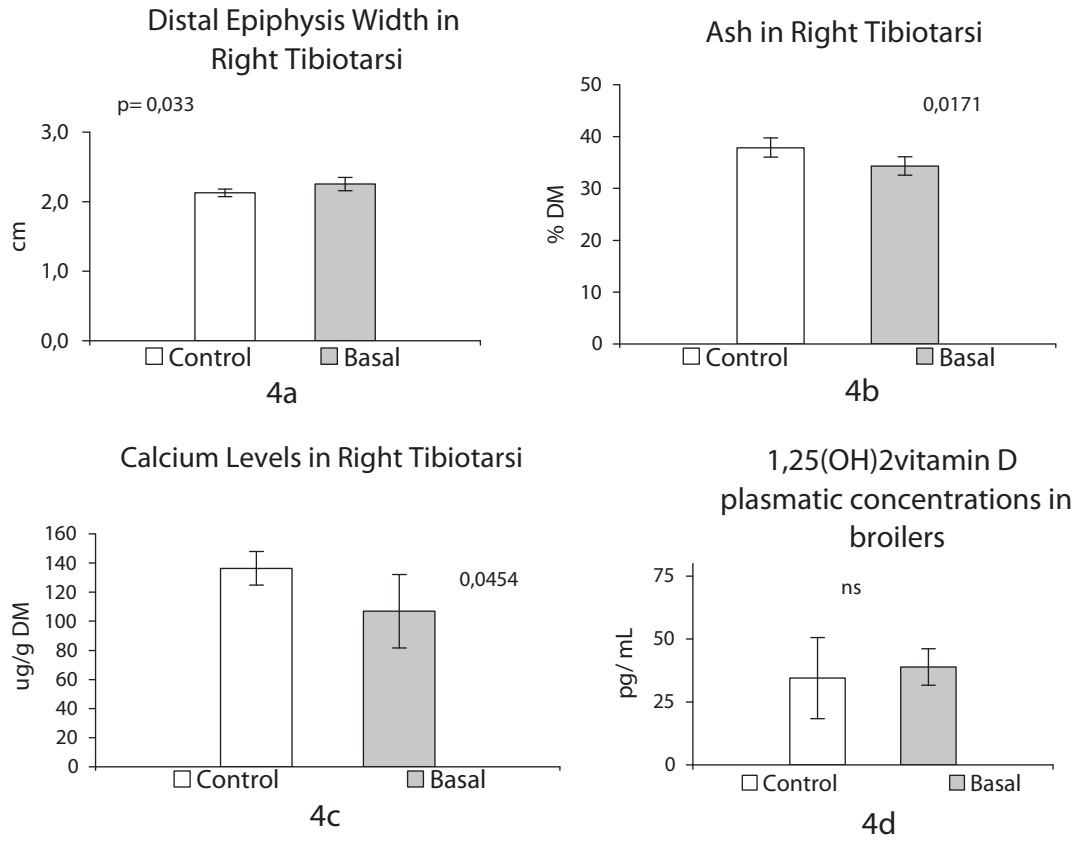

Figure 4. Biochemical and skeletal variables measured at 49 d of age in broilers (Trial 2).

groups in Trial 1 although the controls had higher body weights. Additionally, feed conversion values (g feed consumed / g weight gain) were lower in the controls (1 998 vs 2 099). At 20 d of age lower values ($P \leq 0.05$) were recorded in the basal groups for plasma Ca levels, percentage dry matter and ash, and for Ca and P levels of the right tibiotarsi (**Figures 3a-f**). Levels of vitamin D active metabolite, were twice as high in the basal than in the control group, but the difference was not significant ($P = 0.273$) (see **Figure 3e**). Neither were significant differences detected between the groups in plasma Pi concentrations, fresh and dry weight, and the volume and density of right tibiotarsi. Ca and P levels in manure paralleled those in feed (data not shown).

In Trial 2, although animals of the basal group were fed only two thirds of the recommended levels of Ca and P during their entire breeding cycle they showed significant and favourable differences in body weight at 21 d and 28 d of age, and in feed conversion at 21, 28 and 35 d of life (**Table 1**). Also, significantly lower values ($P \leq 0.05$) for ash and Ca levels accompanied by higher widths of the distal epiphysis and the right tibiotarsi were recorded at 49 d of age in these animals (**Figures 4a–c**). However, vitamin D active metabolite concentrations in plasma did not show significant differences ($P = 0.594$; $n = 5$) between the basal and control groups, but since the mean basal group value was slightly higher a greater number of experimental units should be used in future studies to ensure rigorous statistical analysis (**Figure 4d**). Other nutritional, biochemical and skeletal variables were similar in both groups.

The high levels of vitamin D_3 employed here (25 times the 1994 NRC recommendations) and in commercial farms (Barroeta et al., 2002) could enable birds fed on basal diets to increase synthesis of the active metabolite of vitamin D in order to ameliorate partially the effects of Ca and P deficiencies. Conversely, the diet with high levels of vitamin D_3 together with the recommended levels of Ca and P for lower levels of vitamin D_3 (NRC, 1994) might have been

unbalanced for optimal efficiency, at least in the experimental farm conditions of the present work.

CONCLUSIONS

The determination of plasma 1,25D levels in experimental cows contributed to knowledge about calcinosis of cattle in Argentina, named ES. The results obtained demonstrated the rapidity with which the active principle of *SG* is hydrolysed by digestive enzymes in ruminants, evidenced by the augmented levels of the free vitamin D_3 metabolite circulating in blood four h after oral administration.

The preliminary results obtained with feeding broilers suggest that it will be necessary to investigate the performance of commercial broiler chickens during the entire cycle with different combinations of vitamin D_3, calcium and phosphorus in order to determine the appropriate levels for optimum and deficient diets.

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Animal Health in Albania

A. Lika[1]*

ABSTRACT

The animal health service policy in Albania represents an integral component of overall governmental, social and economic policy in the field of agricultural and rural development, public health, food processing and import/export of animal products. In order to obtain the necessary political, economic and public support, the animal health service attempts to contribute effectively to the overall development of the country which aims at improving the standards of living of its inhabitants. Practical means of contributing to national development include reducing food loses due to animal morbidity and mortality, increasing the productivity of the livestock population, protecting human health against zoonotic diseases and ensuring humane treatment of animals. An animal health strategy contributes to the creation of conditions necessary for uninterrupted animal disease surveillance and control in the country. The main animal health problem in Albania is brucellosis in ruminants, caused by *B. melitensis*. This infection currently affects the entire country, reaching a prevalence of 10% in several districts. The latest and most severe outbreaks of classical swine fever were identified on 1996 when 5 515 animals were infected and 3 683 animals died. The circulation of bluetongue virus (BTV) was detected for the first time in Albania in 2002 with a seroprevalence of 15%. The evidence of BTV circulation in Albania and the absence of the main vector *C. imicola* suggest that other *Culicoides* species could be implicated in virus transmission. H5N1 avian influenza in Albania was confirmed in March 2006 in backyard flocks in the villages of Cuke and Peze-Helmes. In both villages there were no human cases. Rabies was of concern in Albania from 1928 until 1976. The disease re-emerged in March 2001 in the village of Morine in Kukes district affecting a domestic dog and three persons were bitten. Other cases have been reported in northern Albania.

INTRODUCTION

Agriculture plays an important role in Albania, although currently it is practised largely at a subsistence level. Albania's farming sector has been dominated by small private holdings since the collapse of the communist State in 1991, when peasant farmers disbanded the quasi-state collective farms. Agriculture subsequently became an important source of income support in rural areas, and is now undergoing a transition from a largely subsistence sector to a commercial one. Currently, the sector contributes 25% of GDP, which is high compared with neighbouring countries, while average gross income per farm is estimated at about US$ 1 800.

Around 40% of Albania's 28 748 km^2 land area is classified as agricultural land (24% arable and 15% pasture). The rest is divided between forest (36%) and other uses. Over 75% of Albania is hilly and mountainous, and much agricultural land is hilly. Albania is predominantly mountainous in the north and east, with agricultural land concentrated in the more densely populated coastal plains of the west (43% of arable land). A further 34% of agricultural land lies in river valleys; 23% is upland. Albania is located in the Mediterranean climatic zone and has short winters and hot, dry summers. It has abundant precipitation (1 430 mm annual rainfall) concentrated in autumn and winter, with frequent droughts in summer. It also has extensive underground water resources (World Bank, 2007).

The total number of farms in Albania is approximately 370 000, mostly dominated by small farms (average size 1.14 ha according to official statistics, or 0.8 according to HBS data (Albanian Agriculture, 2007). This is a much smaller average size compared with an average of 5 ha for Central and Eastern European countries and 27 ha for Western Europe. This is an important handicap to improving agricultural productivity and encouraging sustainable development of the agricultural sector.

MATERIALS AND METHODS

Identification of Gaps

The vision of the National Animal Health Programme (NAHP) is to improve the health and welfare of animals for meeting the needs of stakeholders, enable safe production of food, improve health of the public, sustain the rural society, and support the rural economy. Effective national food control systems are also essential to protect the health and safety of consumers. Food-borne diseases caused by microbiological contamination remain a major public health problem in Albania. The country is registering an increase in brucellosis, particularly in humans, transmitted either through contact with animal tissue or through the ingestion of contaminated milk and milk products (**Figure 1**).

The concept and requirements for working towards this vision are:

- the current veterinary services in the country, including the veterinary diagnostic institution, are very weak. The few resources in place are fragmented and reflect the historic paradigm of the previous regime with incomplete transition towards a market economy. The veterinary services in the 12 districts/regions appear to have been connected administratively but their field operations are neither connected with the national interest nor geared to the above vision;

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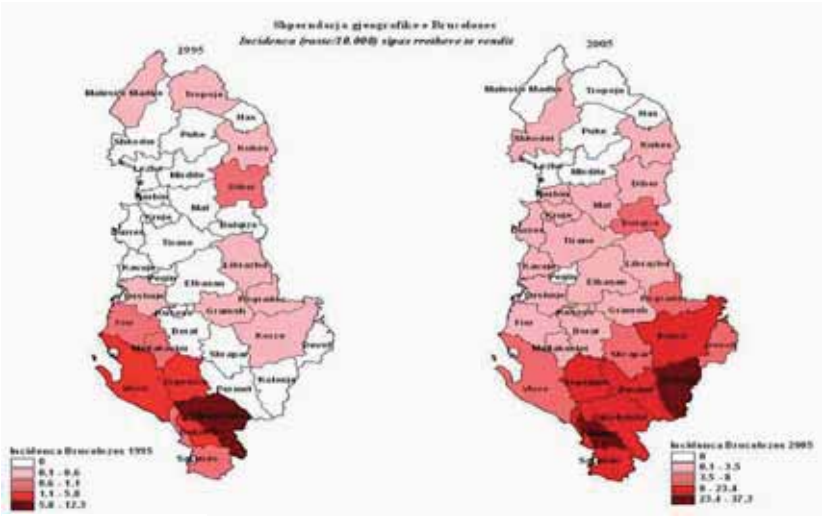

Incidence 1995 Incidence 2005

Figure 1. Geographic distribution of brucellosis. Incidence (cases/10 000 persons) according to district.

- there is a need to link food safety and zoonotic aspects to the public health sectors so that public interest and funding resources can be increased;
- the livestock sector is undeveloped and consumers do not have much influence or organisation. The veterinary service should therefore take this opportunity to present a comprehensive plan for national animal health with benefits for both consumers through safety food and the livestock sector itself through better production and trade;
- budgets need to be in place to meet all needs including coordination with many of the international organisations to secure funding for specific activities within the comprehensive NAHP;
- the foundation of a reliable NAHP is a scientifically based surveillance system in which contingency planning is incorporated for specific health events.

Data Collection

Our data originated from the Department of Animal Health in the Food Safety and Veterinary Institute and direct contact with District Veterinary Services throughout the country.

RESULTS AND DISCUSSION

The Main Animal Diseases

Brucellosis

The main problem is brucellosis in ruminants, caused by *B. melitensis*, which is widespread in several districts of the country. The infection reached its highest levels between 1960 and 1965, and subsequently decreased through the implementation of different control measures. In 1989, the country was proclaimed free of bovine brucellosis, with a low prevalence (0.002%) in small ruminants. During this period, compulsory herd testing and removal of reactors was successfully implemented and helped to eliminate positive animals. Moreover, application of the B–19 strain vaccine in cattle in combination with animal tracing yielded significant results. After the political and economic changes in the 1990s, the infection spread in animals throughout the country and reached its climax in 2000. The uncontrolled movement of animals, the failure to apply sanitary and quarantine measures as well as the low level of cultural and technical education of farmers, together with a limited budget for implementing an eradication strategy (total screening, total elimination of positive heads), led to this expansion across the country. This infection currently affects the entire territory of the country, reaching a prevalence of 10% in several districts such as Saranda and Gjirokaster. The number of persons affected by brucellosis is increasing, particularly in rural areas (Kakariqi, 2006).

A new strategy for the control of brucellosis in Albania using *B. melitensis* rev 1 strain vaccine was introduced in 2003, and is starting to have positive results.

Table 1. Number of pigs vaccinated against classical swine fever.

Year	Number of vaccinated pigs
1996	11 504
1997	13 702
1998	51 775
1999	37 927
2000	29 611
2001	7 115
2002	6 332
2003	51 524
2004	42 045
2005	20 055
2006	10 187
2007	17 679

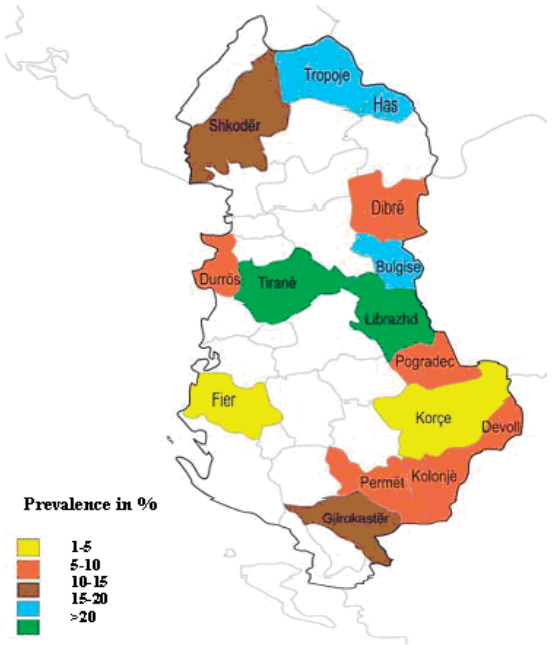

Figure 2. Seroprevalence of BTV in Albania in 2002.

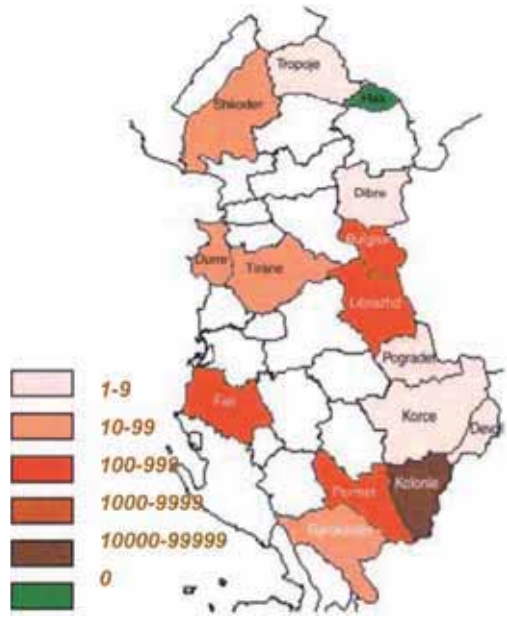

Figure 3. Abundance of *Culicoides* spp. in Albania (October-November 2002).

Classical Swine Fever

This was first diagnosed in 1966 in southeast Albania and outbreaks of the disease were later identified elsewhere based on clinical signs and postmortem lesions (it originated from swine imported from China). The disease was eradicated subsequently following drastic measures which included massive culling, quarantine, etc. The infection reappeared in 1975 in a reserve of wild boars in the south of Albania (Karaburun).

The latest and most severe outbreaks of the disease were identified in 1996 (using direct immunofluorescence test in the Food Safety and Veterinary Institute) when 5 515 animals out of 35 235 swine were infected and 3 683 animals died. The rest of the swine population was subjected to compulsory slaughtering and despite the very restricted financial possibilities, the State reimbursed the cost of the operation. The disease was kept under control by applying surveillance, quarantine, stamping out and vaccination

In general there are now only sporadic outbreaks of infection in the north of the country, although vaccination is still applied in old outbreak areas (**Table 1**).

Bluetongue Virus (BTV)

This was detected for the first time in Albania in 2002 (Di Ventura et al., 2004) with a seroprevalence of 15% (**Figure 2**) determined by competitive ELISA and virus neutralisation assays. During that year a survey for *Culicoides*, was also made. Twenty species were identified in the collections (**Figure 3**). The finding that serotype 9 of the virus was the only one involved suggested that BTV infection came from neighboring countries. However, evidence of BTV circulation in Albania and the absence of the main vector *C. imicola* suggest that other *Culicoides* species could be implicated in virus transmission. The high abundance of the Obsoletus complex, from which BTV was recently isolated in outbreaks where no specimens of *C. imicola* were captured, also suggest that probably the vector belong to this complex (Goffredo et al., 2003)

H5N1 Avian Influenza

The first case in Albania was confirmed on 7th March 2006 in a dead chicken from a backyard flock in the village of Cuke in the Sarande district of southern Albania, and a further case was confirmed on 21 March 2006 in the village of Peze-Helmes near Tirana. Dead chicks were sent to the Animal Health Laboratory in Tirana where avian influenza virus was isolated on SPAF eggs and detected using haemagglutination inhibition (HI) and agar immunodiffusion (AGID) tests. The isolate was sent to the OIE Reference Laboratory in Weybridge, UK which confirmed the presence of H5 with a final typing of H5N1.

In both villages outbreaks were confined to backyerd poultry and there were no cases in humans. Also, infections occurred on holdings with direct access to an adjacent floodplain and appear to have resulted from contact with infected migrating waterfowl. The level of contact may have been no more than some hens foraging on a pasture where waterfowl had grazed some time previously. The severity of the outbreak was limited by the fact that the majority of flocks in the village were either housed or confined within a walled garden. The response measures against AI during outbreaks in Cuke and Peze were well planned.

Following confirmation of the outbreak, the authorities put into operation the contingency plan which had been drawn up for such an eventuality. One of the first tasks was to conduct an awareness campaign among the villagers outlining the measures which would be implemented and the precautions that they should take. At this stage a preliminary appraisal was made of the number of poultry to be slaughtered and the manpower and resources required. A landfill site for the disposal of culled birds and contaminated material was identified and prepared. A number of meetings were held with per-

sonnel engaged in the operation where areas of responsibility were identified and delegated. Training was provided to hired workers on technical and safety aspects of culling and disinfection to ensure a professional and effective performance of these tasks.

Quarantine

A quarantine zone was established around the village with disinfection points for people and vehicles at the main access points. This action appeared to be very successful with a high level of compliance by all inhabitants. Within a three km zone, biosecurity measures were implemented including a requirement that all poultry must be housed and all illness and mortality in birds be reported to the veterinary service of the communes.

Depopulation and Disinfection

Culling and disinfection operations were carried out by three teams each under the supervision of a veterinarian. The teams consisted of a pickup truck and driver, a record keeper, two workers responsible for culling operations and one for disinfection. The teams had police support if required but most flock owners were very cooperative. Birds were killed by dislocation of the neck before being put in black plastic sacks. Initially it was debated whether it was necessary to kill the birds or let them die by suffocation. In practice killing proved both more humane and more efficient as it precluded any escape of birds from a torn bag in transit or at the landfill.

Disposal of Dead and Diseased Birds

The landfill was located about 2 km from the village. The site was well located being easily accessible from the village but isolated from any habitation. It was under constant supervision during the culling operation and was closed with topsoil at the end of each day to prevent scavenging by foxes or dogs.

Rabies

This was a disease of concern from 1928 until 1976. Cases were reported sporadically and most of them in wild animals (wolves, foxes, jackals, etc) and in stray cats and dogs. From 1976 up to 2000, rabies cases were minimal and the country was classified as free from disease, even if in neighbouring counties the disease was increasing. This status of 'freedom from disease' for that period can be explained by the fact that at the time the veterinary service was well organised and the border was protected by a permanent barrier called Clone[1].

The disease reoccurred in Albania in March 2001 in Morine village in the Kukes district affecting a domestic dog and three persons were bitten who forunately received the appropriate treartment quickly. Since then, further cases have been reported in northern Albania. Specifically:

- in November 2002 a fox was diagnosed positive in Qereti village in Puka district;
- in March 2003 a further two foxes were found positive in Gjorica village in Bulqiza district;
- in May 2004 a rabid wolf wandered around the villages of Perollaj, Helshan, and Zahrishte in the Has district. As a consequence of this incident, 21 animals of different species were bitten and about seven of them showed clinical symptoms; (**Figure 4**)
- since then, no cases of rabies have been reported;
- the rabies virus was identified by brain histo-pathological findings and immuno-fluorescence microscopy on fresh brain tissues.

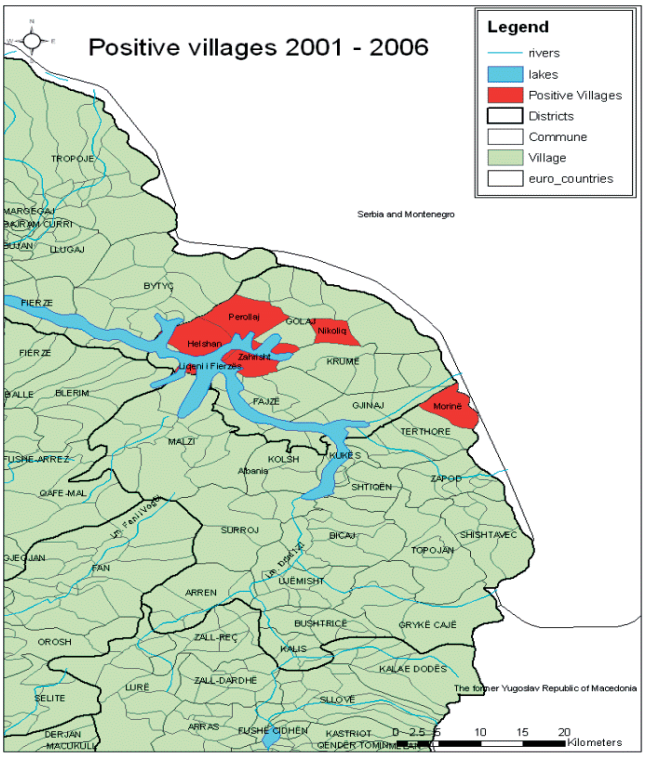

Figure 4. Villages with cases of rabies (in red) from 2001–2006.

Control measures comprise:

- vaccination of remaining dogs in the village;
- disinfection of likely contaminated areas;
- enforcement of quarantine measures;
- suspension of trade in live animals and by-products;
- strict surveillance of remaining dogs in the village;

Rabies monitoring in animals is performed by the Food Safety and Veterinary Institute. Laboratory confirmation of rabies virus is based on positive results obtained by the direct fluorescent antibody test (IFAT) using antirabies test serum from 'SIFIN'-Gmbh, histopathological examination of 'Negri' corpuscles and the mouse inoculation test (MIT). A large-scale rabies survey started in 1997 involving different geographical regions and focussing mainly on red foxes and other wild terrestrial carnivores; dogs, cats and bats are included as well. During the period 1997–2009, 1 220 animals, comprising 681 wild carnivores, 409 bats and 130 domestic animals were destroyed.

Unfortunately, Albania has not yet been able to introduce mass wildlife immunisation with oral vaccines due to lack of well-developed vaccination strategies based on prior ecological studies of target animals, appropriate planning, trained personnel and of course, adequate funding.

CONCLUSIONS

Government expenditures on agriculture have decreased in recent years, with the bulk of Ministry of Agriculture, Food and Consumer

1 Clone is a permanent barrier covering the entire Albanian national boundary and consisting of by a large densely fenced spine wire. This fence was constructed to prevent the illegal crossing of the border, but it also prevents the movement of wild fauna across the border.

Protection (MAFCP) expenditures (48% of the budget in 2005) going to investments in irrigation and drainage infrastructure. However, the 'Agricultural and Food Safety Inspections and Services and Consumer Protection' Programme (the third largest in MAFCP with 15% o f the MAFCP budget), is expected to see significant increases in the coming years to strengthen inspection services.

Given the limited absorptive capacity of relevant institutions, additional funding should be selectively applied and well prioritised. Establishing a national food safety system consistent with the EU Acquis will require increased levels of public expenditures on food safety, veterinary and phytosanitary activities. Establishing a national food safety system consistent with the Acquis is a priority and adequate financial resources should be allocated to support the creation of institutions and systems and to upgrade skills.

The challenge faced by a reliable National Animal Health Programme, is to sustain livestock production, including social needs, in the agricultural community, through modern economic approaches. The NAHP can be the core for this type of sustainability due to the trust of the agricultural community in the veterinary input. However, the NAHP should use police authority to implement necessary quarantine measures in case of tranbaundary diseases.

The overall capacity of the country's laboratories is deficient and should be further developed in order to provide food testing and analytical services that meet international standards and requirements. Although there are 36 laboratories throughout the country, most have inadequate or outdated equipment and infrastructure, a shortage of competent analytical and managerial staff, no official working methods or procedures or business plans, and poorly developed systems for recording test results, reporting, and information management. In some cases, laboratories have obtained sophisticated equipment under international projects, but analysts and technicians lack the necessary skills to operate and maintain them. Other challenges to be overcome include shortages in the power supply and lack of funds to meet operating costs. Many laboratories face difficulties in obtaining essential supplies of materials, reagents and services (disposable materials, reagents, gases, calibration and maintenance services), and lack access to technical support for calibration and reference testing. A further problem is the loss of qualified staff, including individuals trained by donor projects, either through dismissal or transitioning to other jobs.

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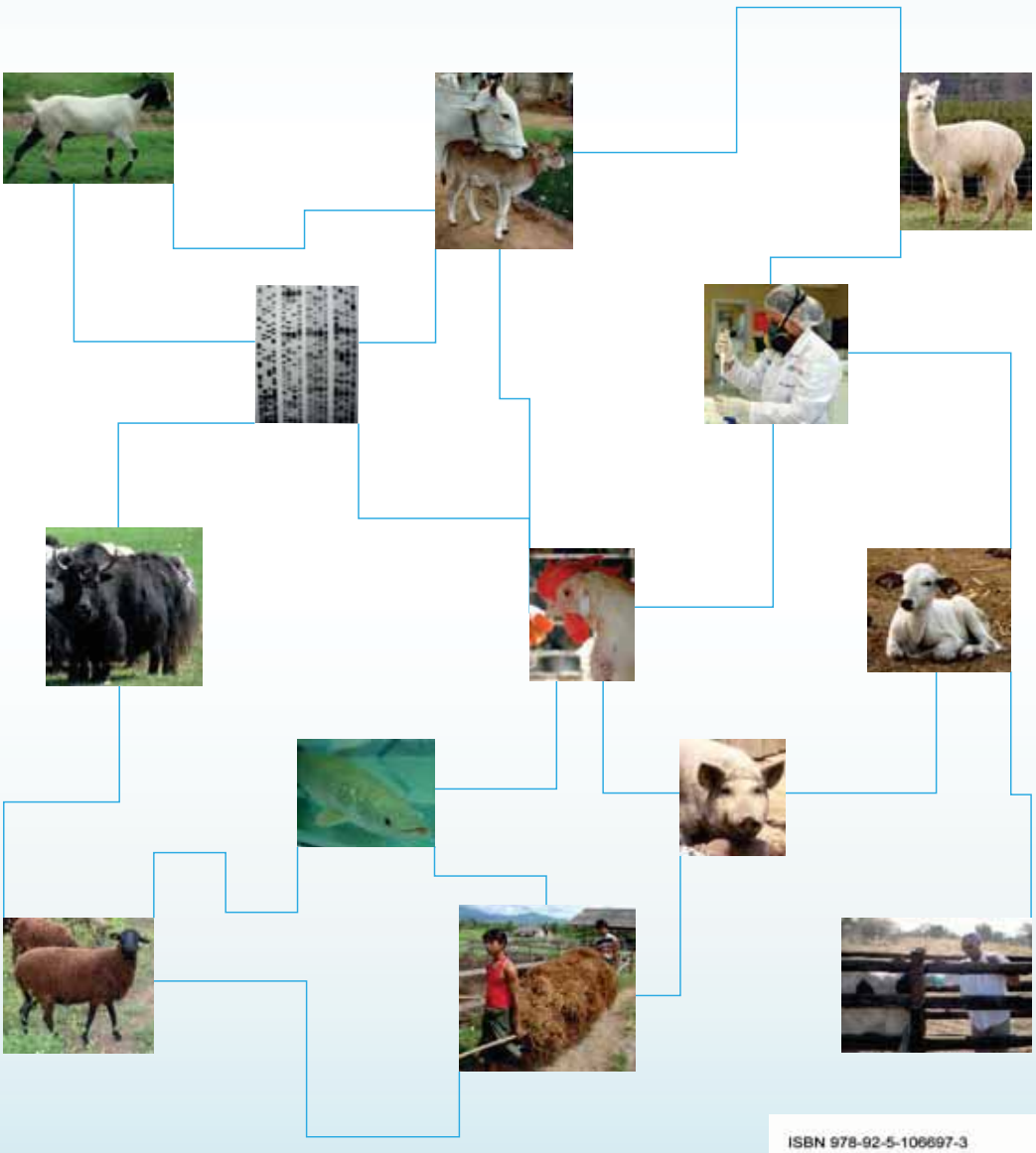
A symposium on 'Sustainable Improvement of Animal Production and Health' that was organised by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture in cooperation with the Animal Production and Health Division of the Food and Agriculture Organization of the United Nations addressed the animal husbandry and public health issues that threaten global food security and safety. The growing world population is vulnerable to limitations in the production of agricultural products and any change, be it climatic realities and/or variations or civil strive upset the delicate balance of providing affordable food for all. It is alarming that the world's poorest people, some one billion living mostly in Africa and Asia, depend on livestock for their day-to-day livelihood. To reduce poverty, fight hunger and ensure global food security, there is an urgent need to increase livestock production in sustainable ways.

This publication provides invaluable information not only on how nuclear and related techniques can be used to support sustainable livestock production systems, but also about the constraints and opportunities for using these techniques in developing countries; it attempts to identify specific research needs and gaps and new options for using these techniques for solving established and emerging problems. As such, it is hoped that the information presented and suggestions made will provide valuable guidance to scientists in both the public and private sectors as well as to government and institutional policy and decision makers.



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