THE POTENTIAL OF CRYOPRESERVATION AND REPRODUCTIVE TECHNOLOGIES FOR ANIMAL GENETIC RESOURCES CONSERVATION STRATEGIES

S.J. Hiemstra[1], T. van der Lende[2] and H. Woelders[1,2]

[1]Centre for Genetic Resources, the Netherlands (CGN), Wageningen University and Research Centre, P.O. Box 65, 8200 AB Lelystad, the Netherlands. sipkejoost.hiemstra@wur.nl; henri.woelders@wur.nl. [2]Animal Sciences Group of Wageningen University and Research Centre, P.O. Box 65, 8200 AB Lelystad, the Netherlands. tette.vanderlende@wur.nl

Summary
Ex situ conservation of genetic material from livestock and fish through cryopreservation is an important strategy to conserve genetic diversity in these species. Conservation strategies benefit from advances in cryopreservation and reproductive technologies. Choice of type of genetic material to be preserved for different species highly depends on objectives, technical feasibility (e.g., collection, cryoconservation), costs, and practical circumstances.

Keywords
Livestock, fish, reproduction, cryopreservation, conservation strategies

Introduction

Global diversity in domestic animals is considered to be under threat. Worldwide, a large number of domestic animal breeds is endangered, in a critical status or extinct already. Of the 6379 domestic animal breed populations, 9% is in critical condition and 39% is endangered [15]. There is worldwide consensus about the global decline in domestic animal diversity and the need to conserve genetic diversity. The vast majority of aquatic genetic resources are found in wild populations of fish, invertebrates and aquatic plants. Domestication of aquatic species has not proceeded to the same level as it has in crop and livestock sectors. According to FAO, there are more than 1000 common aquatic species that are harvested by humans in major fisheries and thousands of additional species are harvested in small-scale fisheries. The number of species in aquaculture is growing and several important species rely on the collection of brood stock or seed from natural populations.

In farm animals, trends in within-breed diversity are as important as between-breed diversity in order to be able to cope with changing requirements and future demands in breeding and selection. A small effective population size in rare or endangered breeds requires monitoring of within-breed diversity and conservation programs to maintain within breed diversity. Several authors also emphasized the reduction in effective population sizes of widely used domestic animal breeds [e.g., 55]. Although introgression of genes for specific traits or characteristics from local breeds to commercial breeds has been very limited so far, Notter [37] suggested that – similar to plants – useful genes may exist in lowly productive types and recommended systematic programs for genetic resources conservation, evaluation and use.

There are several options to conserve genetic diversity. In general, *in situ* conservation or *conservation by utilization* is preferred as a mechanism to conserve breeds. A breed has to evolve and adapt to changing environments and efforts to create a need for products or functions of the breed should be promoted. Conservation without further development of the breed or without expected future use is not a desirable strategy. However, in addition to *in situ*

conservation, methods or techniques to maintain live animals outside their production or natural environment (*ex situ live)* or through cryopreservation of germplasm (*ex situ*) are set up to preserve (germplasm of) rare breeds as well as the more widely used commercial breeds. Moreover, cryopreservation of germplasm is a very good *ex situ* strategy to conserve existing allelic diversity for future use.
There is a growing interest in *ex situ* conservation strategies, serving a variety of objectives [14]. In many countries *ex situ* conservation represents an integral component of conservation strategies [2]. Some strategies focus primarily on preservation of germplasm of rare breeds, but in general there is consensus that *ex situ* collections should be established for all breeds with the aim to capture as much allelic or genetic diversity in conservation programs as possible. Where *in situ* conservation or use of animal genetic resources is not necessarily dependent on high-tech approaches or facilities, the efficiency and efficacy of *ex situ* conservation strategies will certainly benefit from advances in cryopreservation and reproductive technology. Since *ex situ* conservation activities are in general rather costly, debate is going on about priorities, different methodologies and future use and benefits of cryopreservation and reproductive technology.
In this paper focus is on *ex situ* conservation. An overview of the state of the art in cryopreservation and reproductive technology for farm animal and fish is followed by discussion of implications for *ex situ* conservation strategies. This paper is restricted to the main agricultural species. Regarding aquatic species, this paper deals with fish only and focus on aquaculture rather than fisheries.

State of the art in cryopreservation technology

Cryobiologic principles

Cryopreservation allows virtually indefinite storage of biological material without deterioration over a time scale of at least several thousands of years [31], but probably much longer. Important progress in cryobiology was achieved in the second half of the previous century. Much progress resulted from empirical studies. In later years, progress was also strongly stimulated by the development of fundamental theoretical cryobiology.
In so-called ‘slow cooling’ methods, the biological material is cooled at a range of cooling rates that are fast enough to prevent ‘slow cooling damage’ but are slow enough to allow sufficient dehydration of the cells to prevent intracellular ice formation (IIF) [32]. The dehydrated cells in the ‘unfrozen fraction’ that remains between the masses of ice will ultimately reach a stable glassy state, or ‘vitrify’. In so-called vitrification methods, the water content is lowered before cooling by adding high concentrations of cryoprotective agents (CPA). Thus, no ice is formed at all, and the entire sample will vitrify. This allows fast cooling rates without risk of IIF. The CPA concentration of vitrification solutions can be minimised by using very high cooling and thawing rates. By using extremely high cooling rates, vitrification is possible even in complete absence of CPAs [24].

Semen

Semen of most livestock species can be frozen adequately. Also, for a large number of bird and mammal livestock species, dedicated freezing media and equipment for collecting, packing, freezing and inseminating semen have been developed and are available commercially. In the cattle AI industry, in which bulls are selected for ‘freezability’ of their semen, the post-thaw semen quality is quite good, featuring 50-70% motile spermatozoa. Pregnancy or calving rate is the same as that of fresh semen, provided that higher sperm dosages are used for frozen sperm. For other mammalian species the percentage post-thaw motile sperm or membrane-intact sperm is generally somewhat lower, but a fair post-thaw

viability can be expected for most species. For many species the fertility of frozen semen is found to be lower than that of fresh semen. This may depend on the site of semen deposition, the morphology of the female genital tract, and the ability to detect heat or ovulation. For instance in sheep, very poor results are obtained with cervical semen deposition when using frozen ram semen, compared to fresh [33]. There may be considerable differences between breeds and between males, in the 'freezability' of the semen. As a consequence, frozen semen of some genetically interesting breeds or males may not be suitable as a gene bank resource, or can be used only with a poor efficiency.

As to avian livestock species, semen-freezing techniques for fowl, turkey, goose, and duck, render a fair post-thaw sperm survival of up to 60% live spermatozoa. Reasonable insemination results with frozen-thawed semen have been reported for the major avian livestock species [3, see for more references 21]. However, there is a striking variation between studies in the reported percentages of fertilized eggs, as listed in Hammerstedt and Graham [21] ranging from 9-91%. Moreover, the number of spermatozoa that gives maximal fertilisation levels in chickens is much higher for frozen-thawed semen compared with fresh semen [56].

More than 200 fish species with external fertilisation have been tested for sperm cryopreservation [3]. The present state of the art for many species of fish seems to be adequate for the purpose of gene banking. The insemination ratios used may vary according to species and procedure between 10^4 - 10^7 spermatozoa per egg. Even in fish species like the African catfish, in which semen can only be obtained by testis destruction or death of the male, enough semen can be obtained from one single male to produce close to 10^6 larvae [52]. Thus, for gene-bank purposes, storage of only one single vial or straw would be sufficient to generate plenty of progeny of that male.

Freezing media widely vary between the classes (mammals, birds, fish) but also between species within a classis. Most media feature a saline or saccharide bulk osmotic support, a suitable CPA at concentrations varying from 0.2 – 1.5 M, and various protective macromolecular additives, mostly milk and egg yolk components, or lipid components from vegetal origin [4]. Milk or egg yolk is often used in media for mammalian semen. In mammalian semen the egg yolk and milk components protect the spermatozoa during cooling and during freezing and thawing [54]. These additives are generally not used in freezing media for avian and fish species, although in a few studies with fish semen, egg yolk was found to confer protection against cryodamage (e.g., [6]).

Glycerol is widely used as a suitable CPA in mammalian, bird, and fish species. However, in poultry it is found that glycerol is contraceptive, i.e. the semen must be washed free of glycerol after thawing [21]. The type of CPA used varies widely between species, and sometimes within one species a CPA is successfully used in one study and is found to be unsuited in another study with the same species [52]. Glycerol is used in most mammalian species. In avian species, also DMSO, Ethylene glycol (EG), DMA and DMF are frequently used. In fish species, glycerol, DMA, DMF, DMSO, and methanol are often used.

Semen is generally cryopreserved with 'slow cooling' methods. Optimal cooling rates for freezing semen are mostly found between 10 and 100 °C/min. To some extent, the reported differences may be related to the use of different types of CPA and different CPA concentrations. An extreme example is that fowl semen can be effectively frozen at a cooling rate of approximately 600 °C/min when using dimethylacetamide as CPA, but not using glycerol (Woelders et al., in preparation). CPAs may differ widely in the cell membrane permeability, and also may affect the membrane permeability to water. These parameters greatly affect the velocity of dehydration, and therewith the optimal range of cooling rates.

Oocytes
In the last 10 years, considerable progress has been made with cryopreservation of oocytes. Viable oocytes have been recovered after freezing and thawing in a great number of species [see references in 59]. Successes have been reported as to post-thaw oocyte maturation, fertilisation, and embryo development in a number of species. Live born young from embryos produced from cryopreserved oocytes have been reported in cattle [39, 1], mouse [16, 47], rat [35], horse [30] and human [46]. The present efficiency and reliability of using frozen thawed oocytes for generating offspring is still much lower compared to cryopreserved embryos. Freezing oocytes of avian and fish species is not successful [3], largely because of the large size, the high lipid content, and the polar organisation (vegetal and animal pole) of bird and fish ova.

Embryos or embryonic cells
In cattle, cryopreservation of embryos is highly successful. Both slow freezing and vitrification protocols are effective. The success of cryopreservation is dependent on the stage of the embryo; that is, especially good results are obtained with blastocysts. Cryopreservation of embryos resulting in live offspring has been reported for of the important (mammalian) livestock species [reviewed by 42; see also e.g., 8, 45]. Cryopreservation of pig embryos has long been quite problematic, due to extreme chilling sensitivity and high lipid content of the pig embryos. However, recent studies have focussed on overcoming these problems and produced successful vitrification methods for cryopreservation of pig embryos [e.g., 49, 34, 9]).
Embryo cryopreservation is not viable in birds [3] and fish [20] species, largely because of the same limitations as in the case of avian and fish oocytes, i.e. the large size, the high lipid content, and the polar organisation of the ova and the early embryos of fish and birds. However, in birds and fish species, cryopreservation of isolated embryonic cells is an option. Post-thaw survival of blastomeres was demonstrated in rainbow trout, carp and medaka [see references in 3]. Embryonic cells and recipient embryos can be used to produce chimeric embryos. Provided that the gonads become populated with primordial germ cells from the donor embryo, such chimeric embryos can be used to produce future progeny of the donor genotype [e.g., 36]. In the chicken, the primordial germ cells can be specifically harvested. Recently, improvement of the efficiency of producing chimerae with donor genotype germinal cells was achieved by depleting PGC from the recipient embryos using busulfan [44].

Somatic cells
Cryopreservation of somatic cells proved to be possible for a number of cell types. In early studies, the methods came down to adding 5 to 10% of a suitable cryoprotectant, like glycerol or dimethylsulfoxide (DMSO), to the suspension of cells in culture medium, and place tubes with a few ml of that suspension at −80 °C in a mechanical freezer. In fact this simple procedure is still effectively used today. Obviously, with this simple procedure the rate of cooling cannot be controlled; in fact in many publications the cooling rate is unknown. There are only a few studies in which controlled rate freezers were used, e.g., with skin fibroblast.

Further progress
More attention to fundamental aspects of cryobiology should enable further progress in cryopreservation methods. A fundamental approach has been taken in a number of studies concerning mammalian semen and embryos [19, 29, 7], but fewer so concerning avian and fish semen [e.g., 53]. Recently a theoretical model was presented to predict the optimal

cooling program for 'slow cooling' freezing methods [57]. The model indicated that a non-linear cooling profile could give better results than linear freezing programs. This and other models [cf. 29] also demonstrate that the optimal cooling rate can be expected to be inversely related to the CPA concentration, and in fact this is found in empirical studies. Therefore, it is important to address both factors in empirical optimisation studies. It can also mean that a lower concentration of CPA would become feasible provided that a higher cooling rate is used. Further improvement could result from preventing delayed ice formation or 'supercooling', e.g., by using so-called 'directional solidification' methods [58]. Improving the freezing methods can raise the general level such that even the semen of 'bad freezers' would have an adequate post-thaw sperm survival [58].
Attempts to vitrify spermatozoa have not been successful to date. It has recently been shown that vitrification of human spermatozoa is possible in the absence of CPA by using an extremely high cooling rate of 720,000 °C/min. [24]. In this way, damage due to the presence of the CPA, chilling injury and ice formation may be avoided. Further improvement of vitrification techniques is especially important for freezing cells that are sensitive to chilling, e.g., to 'outrun' spindle microtubule depolymerization in metaphase II oocytes. Very high cooling rates can be applied in the open-pulled straw (OPS) technique [50], or by using the cryoloop [27, 24]. However, also interrupted slow cooling methods can be highly effective, as a fully normal and functional spindle can reform after thawing [46].

State of the art in reproductive technology

Artificial Insemination

In several species, artificial insemination (AI) techniques and strategies have been improved and knowledge on the fate of sperm in the female genital tract (e.g., phagocytosis) improved during the last decades. However, there are large differences between species in insemination techniques and pregnancy rates using fresh or frozen semen. In cattle and pigs existing AI infrastructure allows easy collection and future use of semen, but only in cattle the use of frozen semen replaced the use of fresh semen. In pig production disadvantages of using frozen semen (reduced fertility, high freezing, storage and transport costs) are still larger than the advantages.
In sheep, surgical (laparoscopic) AI gives much better pregnancy rates than cervical AI. However, laparoscopic AI is more laborious and also more invasive than cervical AI. Molinia et al. [33] showed that the difference in pregnancy rates between surgical and non-surgical AI was even larger with frozen semen compared to fresh semen: 20% versus 70% pregnancy with 180×10^6 vs. 10×10^6 sperm. It is believed that frozen-thawed sperm are less motile and lack stamina to transverse the highly viscous cervical mucus, but phagocytosis of the sperm by leukocytes is also considered as a cause of the reduced fertility. Development of a non-surgical technique to reach the oviductal end of the uterine horns as closely as possible would enhance the efficiency and ease of use of cryopreserved semen in sheep. Such deep intrauterine insemination techniques have been developed in pigs [reviewed by 51] and may, in general, contribute to the more efficient use of semen (less sperm per insemination).
AI can be used successfully in poultry, but is not used extensively in any domestic avian species except turkeys where it is used almost exclusively for commercial flock production [reviewed by 12].

Embryo Transfer

Surgical embryo transfer is in principle possible in all mammalian livestock species. In contrast, non-surgical embryo transfer is only possible in cattle (routinely performed), horses and also pigs, though still not as efficient as in cattle and horses. For embryo transfer

purposes, embryos can either be flushed from donors or can be produced *in vitro*. Surgical embryo collection is in principle possible in all mammalian livestock species. In contrast, non-surgical embryo collection is only possible in cattle and horses. After surgically shortening of the long uterine horns of the pig, non-surgical recovery of embryos has been proven to be possible in pigs too [22]. Although ethical issues have prevented the further use of this method, it may be used in specific situations, e.g., to collect in a relatively short time large numbers of embryos in very rare pig breeds. The efficiency of non-surgical embryo collection in cattle, and to a lesser extent in horses, can be improved by hormonal induction of superovulation.
In vitro production of embryos by *in vitro* maturation and fertilisation of oocytes is possible for major livestock breeds, although the efficiency varies between species. Oocytes for this purpose can either be collected by aspiration of immature oocytes from ovaries from slaughtered (or deceased) animals or by the use of ovum pick-up techniques in live animals. The latter techniques are presently mainly in use in cattle and horses, but could also be used in other livestock species.

Reproductive cloning
Reproductive cloning involves collection of oocytes , culture and *in vitro* maturation of oocytes, enucleation of oocytes, transfer of (somatic) nuclei to (or fusion of the somatic cells with) enucleated oocytes, culture of the resulting embryos, and finally, embryo transfer into recipients of the same or a highly related species [reviewed by 17]. The use of nuclear transfer means that the original mitochondrial genotype of the nucleus donor is lost.
In mammals, live offspring have been obtained from embryos generated from somatic cells in a number of species, i.e. sheep, cattle, mice, pigs, goats, horse, rabbits and cats. Until now, cloning has failed in rat, rhesus monkey and dog. Amazingly, some success (embryo development but no live offspring) was even obtained when bovine oocytes were used as recipients for somatic nuclei from other mammalian species [e.g., 10]. However, it must be emphasised that current techniques are inadequate to be used safely and efficiently for procreation. In all published research, only a small proportion of embryos produced by using somatic cells developed into live offspring, i.e., typically less than 4% [41]. The low overall success rate is the cumulative result of inefficiencies at each stage of the cloning process. Many pregnancies are terminated by abortion, and full term pregnancies not seldom result in abnormal offspring. Therefore it seems that current cloning techniques introduce errors that affect prenatal development. Even apparently healthy live born offspring could have anomalies that only become apparent later in live, or in the next generation of animals. On a long time horizon it is very likely that cloning methodology will become both reliable and efficient.
In fish, successful cloning has been reported by Lee et al. [28]. In their experiments with zebrafish, a overall success rate of 2% was achieved. To our knowledge no successful cloning has been reported in poultry.

Miscellaneous emerging reproductive technologies
Transplantation of ovarian tissue and germ cells (e.g., primordial germ cells (PGCs) or spermatogonial stem cells(SSCs)) are emerging technologies with potential for future use in conservation programs. Autotransplantation of ovarian tissue has been developed to restore fertility in woman after aggressive chemotherapy resulting in ovarian failure. Successful transplantation of ovarian tissue has been reported in rodents, sheep, marmoset monkeys and humans [references in 11]. Successful whole sheep ovary cryopreservation and autotransplantation has recently been reported [43]. The potential of ovary transplantation as a

tool in genetic conservation is underlined by the work of Dorsch et al. [13]. Their experiments demonstrate that transplantation of rat ovaries can be used as tool for rescue of rat strains of which females are unable to reproduce despite having normal ovarian cycles.
Germ cell transplantation research has been developed as a unique approach for the study of gametogenesis and germ line manipulation. To date, successful germ cell transplantations have been reported in several livestock species, e.g., transplantation of SSCs in cattle [25] and goats [23], but also in poultry [e.g., 40] and fish [e.g., 48]. As far as application of this technology in fish is concerned, fascinating results of allogenic transplantation in rainbow trout have been reported [48]. By transplanting PGCs of donors into the peritoneal cavity of hatching recipient embryos, live fry with donor-derived phenotype were produced from gametes of PGC-recipients.
Although many hurdles have to be taken, in the long term these emerging technologies may enable production of gametes or offspring of rare or extinct breeds by abundantly available individuals of related common breeds. First steps to overcome limitations for homologous transfer of ovarian tissue and germ cells are now underway. The development of an effective recipient preparation protocol in mice [5] is an example of this.

Implications for *ex situ* conservation strategies

The choice of type of material to be preserved and sampling strategies depend, amongst others, on the objectives of cryopreservation programs [14]. Decisions will be different between species, because of variation in technical feasibility, costs and practical circumstances for cryopreservation of different types of material. In general, cryopreservation and associated reproductive technologies are costly and main limitations for extensive development of *ex situ* collections are high costs of collection and limited use of preserved material [38]. Costs of sampling, collection, freezing, storage and use of genetic material differ between species and optimum strategies depend on local circumstances, availability of technology and costs of labour and facilities. Gandini and Pizzi [18] reviewed the literature on conservation costs (*in situ* and *ex situ*) and concluded that published information on *ex situ* conservation costs was very limited and not very timely. Labroue et al. [26] calculated total costs for creating pig semen storage among four European countries at about 30.000 Euro per breed and 15 Euro per doses. Costs of cryopreservation of pig semen in the Netherlands (1999-2001) were estimated at approximately 10 Euro per doses, based on labour costs and costs for laboratory materials and infrastructure, assuming a freezing capacity of 6 ejaculates per day. Transport costs of the semen from the AI-centre to the freezing facility and costs for semen collection, are not included in this figure.
CGN experienced that costs of collecting and freezing of semen of different species varies from less than 1 Euro per dose (cattle) to more than 20 Euro per dose (sheep and poultry). The higher costs in sheep and poultry are due to the much higher handling, training, collection and freezing costs per dose of semen and the lack of AI infrastructure in these species. As an alternative for semen collection and freezing of ejaculated semen, CGN concluded that collection and freezing of epididymal semen of culled rams is a cost-effective method to conserve genetic diversity in sheep breeds (Woelders et al., in preparation).
Differences in generation interval and reproductive rates between species may also influence decision-making in conservation programs. In some species it is possible to regenerate a breed very quickly with inexpensive, sometimes less sophisticated, methods, compared to other species. For example, in fish the regeneration of an extinct species with stored semen is feasible through backcrossing since fish species have a low generation interval and a high annual turnover. In contrast, such a strategy in horse or cattle would be time-consuming and

extremely expensive. In these species, cryo-banking of embryos rather than sperm is highly preferable.
Costs of embryo collection and freezing are much higher than those for semen collection and freezing. However, regeneration costs using embryos are much lower compared to those for semen (repeated backcrossing). Many conservation programs focus on freezing of semen only. Taking into account the loss of mitochondrial DNA and the time lag to re-establish a breed by backcrossing, collection and cryopreservation of embryos is underrated, if the aim is to conserve breeds. In this context, cryopreservation of somatic cells does not seem to be a good alternative for cryopreservation of embryos, even if the efficiency of cloning is largely improved. Upfront costs of freezing somatic cells may be very low, but mitochondrial DNA is not conserved and the efficiency of subsequent steps in reproductive cloning can never beat the efficiency of cryoconservation and implantation of embryos. Storage of both oocytes and semen may also be efficient in terms of sampling and freezing costs. However, high costs are associated with IVF and ovum pick-up (OPU). We expect that costs will overall certainly not be lower than when using embryos instead of semen plus oocytes.
When survival of material after freezing/thawing will improve and the chance of pregnancy will increase, costs of sampling and freezing of gene bank material will drop, because less genetic material in needed in the gene bank to generate a sufficient number of live offspring. Furthermore, if freezability of semen of genetically important males can be improved substantially (especially in the case of 'bad freezers'), sampling costs will drop even more.
Cryopreservation technology strongly affected reproduction in livestock. Ironically, (unsustainable) use of cryopreservation can cause a decline in genetic diversity but at the same time its use is beneficial when applied in conservation programs. For example, in dairy cattle the combined use of genetic evaluation, AI and frozen semen and more recently several other reproductive technologies, has resulted in high genetic gain in the Holstein Friesian (HF) breed and thus stimulated their world-wide, large-scale use at the cost of local dairy breeds and a decline in effective population size of the HF breed. As a side effect, however, know-how about cryopreservation, AI and other reproductive technologies developed for use in cattle has turned out to be of major importance for the conservation of breeds of various species

Conclusions

- In *ex situ* conservation programs with the aim to conserve breeds, collection and cryopreservation of embryos is underrated, and should be given more attention.
- Advances in cryopreservation and reproductive technology have contributed and will continue to contribute to the efficiency and effectiveness of conservation programs.
- Species in which cryopreservation and reproductive technology are less developed can benefit from advances in other species.
- Decision-makers in conservation programs should regularly reconsider the balance between objectives, costs, technical feasibility and practical feasibility.

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