Food and Agriculture Organization
of the United Nations

AHPND

Acute Hepatopancreatic Necrosis Disease

ASIAN FISHERIES SOCIETY

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Through an agreement between FAO and the Asian Fisheries Society (AFS) signed in September 2017, it was mutually agreed to publish the 23 papers contained in this special volume based on the technical presentations that were delivered during two workshops, namely: (1) International Technical Seminar/Workshop: "EMS/AHPND: Government, Scientist and Farmer Responses", held from 22–24 June 2015 in Panama City, Panama; and (2) Second International Technical Seminar/Workshop on AHPND: "There is a Way Forward", held from 23–25 June 2016 in Bangkok, Thailand, as well as results of the work generated from an earlier FAO project "TCP/VIE/3304 (E) Emergency assistance to control the spread of an unknown disease affecting shrimps". This volume addresses a wide range of topics and is aimed at updating the knowledge and experiences in dealing with AHPND and related topics from the perspectives of the government, academe and producer sectors.

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FAO Technical Assistance Efforts to Deal with Acute Hepatopancreatic Necrosis Disease (AHPND) of Cultured Shrimp

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Abstract

The authors briefly describe the efforts of the Food and Agriculture Organization of the United Nations (FAO) between 2011 and 2017 in providing assistance to member countries in dealing with Acute hepatopancreatic necrosis disease (AHPND) of penaeid shrimp, through two Technical Cooperation Project (TCP), which lead to the production of this volume of collected papers. The first project TCP/VIE/3304 was an emergency TCP project, Vietnam as recipient country. The second project TCP/INT/3502 was an interregional TCP project with Colombia, Ecuador, Guatemala, Honduras, Mexico, Panama and Peru from the Latin America and the Caribbean (LAC) region, and India, the Islamic Republic of Iran, the Philippines and Sri Lanka from the Asian region as recipient countries.

A significant concern to the shrimp aquaculture sector, AHPND will continue to hamper the continuity of food supply, impact livelihoods and reduce national export earnings. This special issue of Asian Fisheries Science on AHPND contains some of the technical papers that were delivered during the Viet Nam, Panama and Bangkok EMS/AHPND events between June 2013 and June 2016. This volume contains 23 contributions on a range of topics aimed at continuously updating the knowledge and experiences in dealing with AHPND and related topics from the perspectives of the government, academe and producer sectors.

Keywords: AHPND, EMS, disease response, FAO projects, TCP/INT/3502

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Introduction

In July 2011, based on a request from the Government of Viet Nam for technical assistance, the Food and Agriculture Organization of the United Nations (FAO) fielded a Rapid Deployment Team (RDT) through the Crisis Management Centre – Animal Health (CMC-AH) and made a quick assessment of an unknown disease affecting cultured shrimp in the Mekong Delta provinces of Viet Nam. Based on epidemiological observations and other relevant field data, the CMC-AH mission confirmed that a serious disease outbreak of unknown etiology had begun in early 2010 and continued into 2011, with high mortalities among cultured giant tiger prawn (*Penaeus monodon*) and whiteleg shrimp (*P. vannamei*).

The pattern of disease spread was consistent with an infectious agent, i.e. starting in one pond in one location and subsequently spreading to several ponds within the same farm, followed by spread to neighbouring farms. The pattern of spread and the clinical signs of the disease were not similar to those of any known major shrimp viral or bacterial disease previous reported in Viet Nam or elsewhere in the world.

FAO/MARD Project TCP/VIE/3304

Following the recommendations of the RDT, the FAO project "TCP/VIE/3304 (E) Emergency assistance to control the spread of an unknown disease affecting shrimps" was developed and implemented from 2012–2013. This project provided emergency technical support to Viet Nam's Ministry of Agriculture and Rural Development (MARD). The project achieved its objectives of: (1) identifying the causative agent of the unknown disease; (2) building the capacity of farmers on biosecurity and good aquaculture practices (GAPs); and (3) developing a National Aquatic Animal Health Strategy (NAAHS) for Viet Nam. Toward the end of the project, the FAO/MARD Technical Workshop on "Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPNS) of Cultured Shrimp" was held in Hanoi, Viet Nam from 25 to 27 June 2013 (Viet Nam EMS/AHPNS June 2013, the first event).

The workshop was attended by 63 participants consisting of key personnel involved in the TCP/VIE/3304 project, members of the MARD National Task Force on Shrimp Diseases, and key experts involved in the work on early mortality syndrome/acute hepatopancreatic necrosis syndrome (EMS/AHPNS) in other countries (Peoples Republic of China, Malaysia, Thailand) and other resource experts from FAO and the United States of America. The participants concluded that the technical workshop significantly improved their understanding of AHPND, and that the process taken by the FAO/MARD TCP/VIE/3304 project could serve as a good model for conducting similar unknown disease investigations in the future.

FAO Inter-regional Project TCP/INT/3502

Upon completion of TCP/VIE/3304, the FAO continuously received requests from member countries for technical assistance in dealing with EMS/AHPNS. Thus, an interregional Technical Cooperation Project (TCP), TCP/INT/3502 "Reducing and Managing the Risks of Acute Hepatopancreatic Necrosis Disease (AHPND) of Cultured Shrimp" was developed to further generate a better understanding of the disease. The FAO inter-regional project, which was implemented from 2015 to 2017, had two major project outputs:

- Output 1: Awareness and technical knowledge on AHPND at national, regional and interregional levels enhanced.
- Output 2: Intersessional activities at country level to support the development of national strategies and/or other relevant instruments.

The project had 11 participating countries, namely: Colombia, Ecuador, Guatemala, Honduras, Mexico, Panama and Peru from the Latin America and the Caribbean (LAC) region, and India, the Islamic Republic of Iran, the Philippines and Sri Lanka from the Asian region. One of the expected outcomes of this project under Output 1 was enhanced knowledge and strengthened capacities for dealing with AHPND in the Asian and LAC regions. In view of this, the convening of two international technical seminars/workshops involving resource experts from the government, academe and the private sector was one of the mechanisms that contributed to achieving this goal.

The International Technical Seminar/Workshop: "EMS/AHPND: Government, Scientist and Farmer Responses", held from 22–24 June 2015 in Panama City, Panama (referred to as Panama AHPND June 2015 in this document) was implemented jointly with the *Organismo Internacional Regional de Sanidad Agropecuaria* (OIRSA, the International Regional Organization for Plant and Animal Health). The Panama AHPND June 2015 provided a platform to improve the understanding of the disease through the lens of governments, scientists and producers and collectively generate practical management and control measures.

Two delegates from each of the 11 FAO member countries officially participating in the interregional TCP, representatives from two FAO member countries participating on a non-FAO funded basis (i.e. Nicaragua and the Kingdom of Saudi Arabia), members of the private sector from Asian and Latin America and the Caribbean (LAC) countries, invited scientists and other experts, representatives from FAO (technical officers from FAO headquarters in Rome and the Subregional Office for Mesoamerica, and international consultants) and OIRSA, comprised a total of 105 attendees drawn from 21 countries. The Second International Technical Seminar/Workshop on AHPND: "There is a Way Forward", held from 23–25 June 2016 in Bangkok, Thailand (referred to as Bangkok AHPND June 2016 in this document)was implemented jointly with the Network of Aquaculture Centres in Asia-Pacific (NACA).

The Bangkok AHPND 2016 updated knowledge and exchanged experiences in dealing with AHPND. It also validated current concepts and models under different systems and environmental conditions, and generated actions and responsibilities targeting different sectors (i.e. government, producers and academe) as a way forward to deal with AHPND. The Bangkok AHPND 2016 event was attended by 84 participants, including attendees from ten of the project's 11 participating countries (6 participating countries from LAC: Columbia, Ecuador, Guatemala, Honduras, Mexico, Panama) and 4 participating countries from Asia (India, the Islamic Republic of Iran, the Philippines, Sri Lanka), as well as from 11 non-participating FAO member countries (Australia, Bangladesh, Brazil, Indonesia, Japan, the Kingdom of Saudi Arabia, People's Republic of China, Singapore, Thailand, the United Kingdom, Viet Nam) and representatives of four international and regional organizations (NACA, OIRSA, the World Organisation for Animal Health (OIE) and the Southeast Asian Fisheries Development Center – Aquaculture Department (SEAFDEC-AQD)).

In addition to the above, under major Output 2, the project also provided detailed guidance and a framework for the development of country National Action Plans (NAPs) on AHPND. This was facilitated by participating countries completing the FAO Aquatic Animal Health Capacity and Performance Questionaire Survey (a national self-assessment survey) which identified strengths, weaknesses and needs related to national aquatic animal health and biosecurity. This was followed by the holding of national stakeholder consultations in the majority of countries, where the national competent authorities had the opportunity to engage with relevant stakeholders from academia and the private sector regarding their draft NAPs and to disseminate the knowledge gained from participation in this project, particularly in the Panama AHPND June 2015 and Bangkok AHPND June 2016. By the end of the project, six countries (Columbia, Guatemala, India, the Islamic Republic of Iran, Philippines, Sri Lanka) had prepared NAPs for AHPND, while another four countries (Ecuador, Honduras, Mexico, Nicaragua) had prepared comprehensive documents in a different format (e.g. national aquatic animal health plans) that incorporated many of the key points contained in the FAO AHPND action plan framework.

The highlights of the three events are summarized below:

Highlights of the Viet Nam EMS/AHPND June 2013

The workshop highlighted the following:

- A period of relatively trouble-free shrimp production has resulted in complacency in the shrimp aquaculture sector. This laxity made the sector vulnerable to any newly emerging pathogen that might arise unexpectedly, as was the case of EMS/AHPNS.
- Poor management practices, including weak compliance with biosecurity standards and good aquaculture practices (GAPs) were evident at both farms and hatchery facilities.
- Shrimp aquaculture needs to improve and to continue to develop into a sector that implements responsible and science-based farming practices.

- With the current understanding that EMS/AHPNS has a bacterial aetiology and is caused by a strain of *Vibrio parahaemolyticus*, the workshop recommended that a proper name should now be given to EMS/AHPNS, i.e. acute hepatopancreatic necrosis disease (AHPND).
- The sector was asked to consider a number of recommendations for specific and generic actions and measures for reducing the risk of AHPND. These recommendations were directed to the wider community of shrimp aquaculture stakeholders in both the public and private sectors and were pertinent to important areas such as:
    - AHPND diagnosis;
    - AHPND notification/reporting;
    - international trade of live shrimp, shrimp products (frozen, cooked), and live feed for shrimp;
    - advice to countries affected and not affected by AHPND;
    - measures at farm and hatchery facilities;
    - advice to pharmaceutical and feed companies and shrimp producers;
    - actions on knowledge and capacity development;
    - AHPND outbreak investigation/emergency response; and
    - Specific AHPND-targetted research on various themes (i.e. epidemiology, diagnostics, pathogenicity and virulence, public health, mixed infections, non-antimicrobial control measures, environment, polyculture technologies).

Highlights of Panama AHPND June 2015

The Panama EMS/AHPND June 2015 event presented the latest information available at that time (June 2015) about AHPND, including the current state of knowledge about the causative agent, the host and geographical distribution, detection methods, risk factors, management efforts, and the actions taken by regional and international organizations. The information summarized below is based on the 21 technical presentations given by resource experts.

The causative agent of AHPND*:* The causative agent was discovered in 2013 as unique isolates of *Vibrio parahaemolyticus* (VP_{AHPND}) that carry a plasmid (pAP1) of approximately 69 kbp. This plasmid contains two genes that produce toxins (one 12.7 kDa and one 50.1 kDa) that are capable of acting together to cause AHPND. The Pir A/B toxin genes that code for the two toxin proteins that induce AHPND in shrimp have been reported to be similar to PirA/B toxin genes known from *Photorhabdus* spp. (Gram-negative, luminescent, rod-shaped bacteria that are members of the Family Enterobacteriaceae). In nature, *Photorhabdus* spp., which live in an obligate, symbiotic relationship with the entomopathogenic nematode *Heterorhabditis* spp. and a closely-related genus, *Heterorhabditis* spp., are parasites of insect larvae. These nematodes have a wide geographic distribution and since the 1980s, have been researched extensively for application in insect control.

Possible public health implications*:* The genome of *V. parahaemolyticus* has several clusters of genes that have been acquired by horizontal gene transfer. Some of them (the tdh and trh gene clusters) are associated with pathogenicity to humans. However, the AHPND-causing strains lack the gene clusters involved in pathogenicity to humans and thus, fortunately, the VP_{AHPND} isolates characterized so far pose no threat to human health.

Host and geographical distribution: AHPND infects mainly whiteleg shrimp (*Penaeus vannamei*), but has also been reported from giant tiger prawn (*P. mondon*) and fleshy prawn (*P. chinensis*). AHPND first appeared in the People's Republic of China around 2009 and was called covert mortality disease. It has since been recorded from Viet Nam (2011), Malaysia (2011), Thailand (2012), Mexico (2013) and the Philippines (2015), and is suspected to be present in in India. It is also suspected to be present in, but unreported from other countries in both Asia and LAC.

Current status of detection methods: The presumptive gross signs of AHPND in penaeid shrimp include an empty stomach and midgut, a pale and shrunken hepatopancreas, and mortality within approximately 35 days after stocking of postlarvae (PL). However, similar gross signs may occur with other diseases and thus, confirmation requires histological examination of the hepatopancreas to reveal the unique feature of the acute stage of AHPND, i.e. massive sloughing of cells of the tubule epithelium in the absence of any clear evidence of a causative agent. To aid in the identification of reservoirs and potential transmission routes, two interim polymerase chain reaction (PCR) detection methods based on primers designated as AP1 and AP2 were introduced at the NACA Website in December 2013 and later updated. Of these, AP2 turned out to be the better primer, with about 3 percent false-positive results. Despite this weakness, the method was used successfully to reveal a high prevalence of VP_{AHPND} in live broodstock feeds (i.e. polychaetes and bivalves), in pond-reared and hatchery broodstock and in PL used to stock shrimp farms. Testing in Thailand also provided evidence that specific pathogen free (SPF) stocks of shrimp that had tested free of VP_{AHPND} became positive after use for PL production in some local shrimp hatcheries, providing clear evidence of biosecurity failures.

To address the problem of false-positive PCR test results, an improved PCR detection method (AP3) was developed based on discovery of the two AHPND toxins and on use of the gene sequence of the smaller 12.7 kDa toxin. The AP3 method, which was released at the NACA Website in June 2014, gave no false-positive or false-negative results with 104 bacterial isolates tested. Since the AP1 to AP3 methods for VP_{AHPND} detection were one-step PCR detection methods and could not be successfully modified into nested-PCR methods, samples with low pathogen loads had to be subjected to an enrichment step by culture in broth medium for 4 hr before separation of bacterial cells to prepare DNA template for the PCR assays. To address the problems with samples that could not be subjected to the enrichment step (e.g. samples preserved in alcohol or archived DNA samples), a nested-PCR method (AP4) was then developed (introduced on the NACA Website on 20 February 2015).

It targeted the whole sequence of the 12.7 kDa toxin gene and 70 percent of the large toxin gene, and it gave 100 percent positive and negative predictive values for the same 104 isolates used to validate the AP3 method. However, it had 100 times higher detection sensitivity (down to 100 fg template DNA). By cooperation between Centex Shrimp and Sakarindrwirote University in Bangkok, antibodies have been produced against heterologously expressed AHPND toxins and used for detection by enzyme-linked immunosorbent assay (ELISA). This will allow for quantification of the toxins in feeds and the environment and for more convenient laboratory testing for therapeutic measures and resistant shrimp stocks.

***Risk factors*.** The most important risk factors for the international spread of AHPND are:

- the movement of live shrimp from a geographic region where AHPND is prevalent to an unaffected region for aquaculture (AHPND is thought to have been transmitted to Mexico from Asia by this route), and
- the importation of live animals (e.g. polychaetes, clams) as feeds for shrimp broodstock (polychaetes imported from P.R. China may have been the major route for introduction of AHPND to Thailand).

Other potential but as yet unconfirmed routes of disease transfer are by:

- crabs, crayfish and other crustaceans;
- predatory birds and mammals;
- attachment of flocs to zooplankton that are carried long distances by ocean currents;
- attachment on crustaceans and in ships' ballast waters;
- via untreated wastes from infected shrimp in processing plants; and
- via use of infected shrimp.

Environmental factors that are believed to promote infection by VP_{AHPND} in shrimp ponds include:

- high concentration of nutrients in pond water by addition of fertilizers, molasses, etc.;
- high water temperature, salinity >5 ppt and pH >7;
- low water turnover coupled with low planktonic biodiversity; and
- the presence of soluble nutrients (feed), unconsumed pelleted feed, and shrimp carcasses, leading to accumulation of organic-rich sediment.

Most cases of VP_{AHPND} have shown co-infection with other shrimp pathogens, for example, monodon baculovirus (MBV), whitespot disease (WSD), hepatopancreatic virus (HPV), *Enterocytozoon hepatopenaei* (EHP) and unidentified gregarine-like entities.

Farm-level disease management: AHPND cannot be excluded from the farm, but it can be managed. Effective farm-level management measures include:

- ensuring good farm biosecurity and best management practices (BMPs) by:
    - beginning with PL derived from broodstock verified to be free of AHPND (i.e. PL derived from SPF or high health (HH) broodstock);
    - avoiding overfeeding, as uneaten pellets are substrate for AHPND bacteria to grow;
    - removing sediment as often as possible, as it also serves as substrate;
    - ensuring that all facilities and equipment are properly desinfected before stocking of PL (e.g. implementing cyclical dry-out and clean-up routines after every production cycle, involving careful cleaning and disinfection of all facilities, including the insides of air lines, pipes, water pumps and air pumps);
    - ensuring that live and treated feeds are free of infection (e.g. by sterilization of frozen material via gamma irradiation or pasteurization or by the development of SPF lines of polychaetes and clams for use in shrimp culture);
    - modifying farm and pond designs to allow better biosecurity (e.g. use of smaller-sized ponds with plastic liners that can be fully drained, dried and disinfected between culture cycles);
    - increasing the number of reservoirs and water filtration to eliminate fish and other disease carriers;
    - using water of a salinity of 5 ppt for growing shrimp;
    - using water drawn from a deep well for growing shrimp;
    - avoiding heavy chlorination pre-treatment of water;
    - avoiding traditional fertilization schedules with commonly used products, especially if these strategies have been used previously and were found not to reduce AHPND losses;
    - avoiding stocking ponds during the high-temperature season;
    - applying “designer” pre- or probiotic preparations (if available); and
    - applying “designer” phages that specifically target the VP_{AHPND} (if available).

- where AHPND is present in the culture environment, managing culture systems to delay infections by, e.g.:
    - stocking larger-size PL;
    - co-culturing of shrimp with finfish (e.g. tilapia) or using water from tilapia ponds;
    - using appropriately designed grow-out systems which mitigate the environmental conditions that support high densities of VP_{AHPND} (i.e. central drainage);
    - stocking at appropriate density according to farm capacity;
    - monitoring of shrimp health and removal of infected animals; and
    - if diseased shrimp are found, conducting laboratory analyses to aid decision making.

Reducing the risk of international spread of AHPND: The international spread of AHPND can be prevented or at least, reduced, by moving only live penaeid shrimp broodstock or PL that have tested free from AHPND by use of the AP4 test.

Conclusions of the Panama AHPND 2015: Outbreaks of AHPND caught the entire industry by surprise and took a long time to unravel because the disease broke through all existing biosecurity measures. While the industry had been dealing with vibriosis in all phases of culture for decades, nobody thought that a *Vibrio* would become an industry game-changer. Because the pathogen is ubiquitous in the environment, the disease thus calls for new strategies in biosecurity and control. A series of important questions that may require future research include:

- Is AHPND caused solely by a strain of *V. parahaemolyticus* that has a plasmid containing some toxins; or are other strains of *V. parahaemolyticus* or even other *Vibrio* spp. involved?
- Is the disease continuing to spread to new countries and geographical regions?
- What recent improvements are there in terms of detection methods?
- What is the current thinking on what are the persistent risk factors and how these risks be can reduced, prevented or managed?
- What technologies may assist the aquaculture sector in dealing successfully with AHPND?(For example, does the solution lie in the use of relatively closed culture systems? What technological and management innovations may be required?)

Highlights of Bangkok AHPND June 2016

The highlights of the Bangkok EMS/AHPND June 2016 event are summarized below based on the conclusions of the plenary panel discussion (industry, academe and government) that followed the expert presentations that were given during the seminar/workshop:

Industry update on AHPND: Some general conclusions drawn from the Industry Panel Discussions are summarized as follows:

- There is no “silver bullet” or “quick fix” to AHPND available or on the horizon.
- The continued spread of AHPND indicates that national biosecurity measures have for the most part failed. AHPND will probably soon be enzootic in all major shrimp producing countries.
- The industry must thus adapt to the new reality.
- Highly intensive and extensive systems appear to offer the best chance of success; semi-intensive and traditional large pond systems are likely to face more difficulties.
- Several systems are being successfully used in various parts of the world. In general, to be successful, the “clear water” system requires an improved ecology-based farm and pond management to assure: (i) clean PL, (ii) clean water and (iii) clean pond bottom.

- Some actions that can contribute to success include:
    - use of smaller ponds;
    - good pond preparation;
    - use of pond liners;
    - good feed management;
    - use of clean broodstock and PLs;
    - use of water treatment systems (shift to closed, semiclosed and recirculation systems);
    - use of "shrimp toilets" or other systems allowing rapid and frequent removal of pond wastes;
    - higher stocking densities to compensate for loss of area used for shrimp toilets and settling ponds;
    - on-site nurseries can help by conditioning PLs before stocking;
    - partial harvest (when risky conditions require);
    - avoiding the use of unapproved drugs and chemicals
    - higher rates of water exchange; and
    - improved surveillance and routine monitoring for early detection.
- Conversion to the "Asian system" will be expensive for owners of traditional ponds; however, it may now be "convert or perish".
- Biofloc ("brown water") and polyculture ("green water") systems may also be successful, but come with their own issues.
- Systems allowing higher biosecurity will be better able to deal the next transboundary aquatic animal disease (TAAD) coming down the road.
- Novel bacterial strains may help to reduce organic wastes in ponds and balance microbial populations.
- Use of some oral probiotics, bacterophages, herbal extracts and toxin absorbents mixed in feed may show promise; however, researchers need to know exactly what it is they are testing and have rigorous experimental design backed by strong statistical analysis.
- Genetic improvements offer some hope.
- Countries need to "step up" and comply with the spirit of their membership in the OIE, as without timely and accurate disease reporting, the OIE system cannot function properly.

Academic update on AHPND**:** This session discussed the most recent advances in research on AHPND. A general theme throughout this session was the opportunistic nature of vibrios, their great diversity in both species and strains, and their great adaptability to environments and hosts. Some general conclusions are summarized below:

- *The pathogen(s): one or several?*
    - Two bacteria (belonging to the genera *Shewanella* and *Delftia* were identified as having synergistic effect with VP_{AHPND} on shrimp mortality.

- Genomic analysis has been done to AHPND *V. parahaemolyticus* strains from P.R. China, Thailand, Viet Nam and Mexico together with non. *V. parahaemolyticus* strains. The conclusion is that AHPND is caused by more than one strain of *V. parahaemolyticus* or even by other *Vibrio* species.
- The plasmids have mutations that suggest a fast evolutionary process.

- *Pathogenicity:*
  - There is variation of the virulence of AHPND isolates that may act to potentiate the binary toxins killing shrimp without the pathognomonic histopathology characteristics of AHPND.
  - There are bacteria of different genera that are synergistic with *V. parahaemolyticus,* resulting in infections leading to higher mortalities.
  - *Vibrio* does not colonize shrimp tissues (without chitin lining), but liberates toxin into the hepatopancreas. In broth culture, after centrifugation the toxin remains in the supernatant and not in the cells.
- *Host and geographical distribution:*
  - No new shrimp species have been reported to be naturally infected, but it is likely that there could be.
  - There are OIE reports of two outbreaks in northern Australia with Pir^{vp} toxin genes in the chromosome.
  - There may be unreported outbreaks in India and Central America other than Mexico.
  - *V. parahaemolyticus* adheres to and degrades chitin, especially in low salinities. This characteristic relates to the number of zooplankton in the environment.
  - Possible carriers include zooplankton, birds, ballast water and marine currents.
- *Risk factors observed in Viet Nam*
  - Infected PL were the major source of infection.
  - Other associated factors include contaminated water, live feeds and broodstock, high stocking density and nearby contaminated farms sharing the same water supply.
- *Diagnostic considerations*
  - Diagnosis should be done at the pond-side by equipping farm staff with the skills to recognize early signs of disease.
  - This can be enhanced by incorporating new formats for molecular diagnosis to verify and confirm pond-side observations.
- *Genetic improvement*
  - Genetic improvements could offer options for resistant or tolerant stocks.
- *Other issues: specific pathogen free, (SPF), specific pathogen tolerant (SPT) and specific pathogen resistant (SPR)*
  - Detailed definitions of SPF, SPT and SPR were offered to avoid confusion.
  - The use of SPF stocks has achieved levels of productivity never seen before, which proves that the health status of PL is a key factor for the development of the industry.

  - Selective breeding is necessary to search for resistance to the toxin in those animals that do not die.
- *Other issues*: *Enterocytozoon hepatopenaei* (EHP)
  - The other disease of high concern is hepatopancreatic microsporidiosis caused by EHP, which is enzootic in Asia.
  - This parasite causes severe growth retardation, morbidity and mortality in the infected countries. For example, losses in Thailand due to EHP could be in the order of US $32 000 per ha per culture cycle.
  - There is the danger that this parasite could be exported to different areas and regions, so it should be added to the OIE list of pathogens.
  - Polychaetes have potential risk for pathogen transmission, especially via feeding to broodstock. Epidemiological studies on EHP in polychaetes are necessary.
  - EHP and AHPND cause significant losses in P.R. China. Sensitive and more effective diagnostic tools have been developed to detect both pathogens.
  - EHP infection may cause more than two times percentage of size variation, two to three times percentage of unexpected weight fluctuation, and about 30 percent of weight losses for same-size individuals.

National and international framework update: This session informed the participants about the global and national impacts of AHPND and other emerging shrimp diseases, the progress made by several countries (i.e. Brazil, Islamic Republic of Iran, Thailand, Viet Nam) in combating these diseases, and the relevant activities undertaken by several international and regional organizations (e.g. OIE, NACA, OIRSA, the Association of Southeast Asian Nations (ASEAN) and the Andean Community of Nations (CAN)). The following summarizes the major points made during this session:

- It is important to protect the affected and unaffected small-scale shrimp sector from AHPND. Efforts in research and biosecurity should also consider opportunities for this important sector. If not, the contribution of shrimp aquaculture to alleviating poverty and increasing food security will be reduced.
- There is a hidden role that ballast waters may be playing in spreading some shrimp pathogens, including those involved in AHPND. Some consideration appears necessary.
- Establishing and implementing the comprehensive National Action Plan towards either preventing the occurrence or reducing the occurrence of AHPND in the participating countries is paramount.
- Collaboration and communication among the relevant agencies should be improved.

Conclusions of the Bangkok AHPND 2016: The following recommendations were made by the experts and participants attending the seminar/workshop:

- Countries should establish a National Taskforce on AHPND.
- Countries should conduct a national epidemiological survey for AHPND.
- Countries should establish active surveillance programmes for AHPND.
- A collaborative research programme on AHPND should be developed.
- Counties should take steps (e.g. via good aquaculture practices (GAPs) or hatchery standard operating procedures (SOPs)) to ensure better national PL quality (SPF/ SPR/SPT, PCR-tested PL, etc.).
- If possible, establish broodstock and multiplication facilities for better PL.
- Establish a national programme and develop a national better management programme (BMP) or GAP guidelines towards improving shrimp pond biosecurity.
- Improve diagnostic capacity.
- Empower farmers to detect the disease and take remedial action based on the national programme/action plan.
- Develop national guidelines and standards, including quarantine procedures, risk analysis, etc. for importation of live shrimp and shrimp products.
- Initiate an awareness campaign on AHPND.
- Improve national fisheries-veterinary dialogue.
- Improve private-public partnership.
- Link up with relevant regional and international agencies.
- The reported survival of EHP at -20 ^{o}C for 1 month contradicts previous understanding and if confirmed, has major implications for international trade in frozen product; this needs to be sorted out quickly.

Conclusion

Globally, the trend in aquaculture is that every 3 to 5 years or so a serious emerging disease (a transboundary aquatic animal disease, TAAD) appears that spreads rapidly and causes major production losses. As can be seen in the disease scenario for AHPND, there is often a long time lapse (usually years) from the time that a serious mortality event caused by an unknown and emerging pathogen is observed in the field, to its subsequent identification and confirmation, acheiving global awarenss among aquaculturists and aquatic animal health experts, establishment of surveillance systems, and disease listing and reporting/notification, up to the time when cost-effective risk management measures are identified and implemented. The aquaculture sector and all its stakeholders need to rethink critically the drivers and pathways to aquatic animal disease emergence. In cooperation with partners together with member countries, FAO is leading the process of developing a new paradigm for dealing with aquatic animal diseases, the Progressive Management Pathway for Improving Aquaculture Biosecurity (PMP/AB).

This is a framework, originally called Progressive Control Pathway (PCP), that has been used by the terrestrial animal disease sector for dealing more effectively with specific livestock diseases such as foot and mouth disease, pestes des petits ruminants (PPR), rabies and African animal trypanosomosis (AAT). The PCPs provide systematic frameworks for planning and evaluating field interventions and enable realistic control objectives to be defined and achieved. In a similar manner, the PMP/AB is a step-wise risk management framework that focusses on building management capacity through both bottom-up and top-down approaches, with strong stakeholder involvement to promote the application of risk assessment and management at the producer level, as part of a national approach. Thus, it is risk-based, collaborative and progressive.

AHPND is a disease of significant concern to the shrimp aquaculture sector that will continue to hamper the continuity of food supply, impact livelihoods and reduce national export earnings. This special issue of Asian Fisheries Science on AHPND contains some of the technical papers that were delivered during the Viet Nam, Panama and Bangkok EMS/AHPND events between June 2013 and June 2016. This volume contains 23 contributions on a range of topics aimed at continuously updating the knowledge and experiences in dealing with AHPND and related topics from the perspectives of the government, academe and producer sectors.

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Ecology, Virulence Factors and Global Spread of *Vibrio parahaemolyticus*

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Abstract

Vibrio parahaemolyticus is part of the autochthonous microflora in estuarine and coastal marine environments and is associated with water, sediment and various aquatic animals ranging from tiny zooplankton to marine mammals. The ecology of this organism is affected by temperature, salinity, turbidity, and the presence of zooplankton, crustaceans and molluscs. Most environmental strains are non-pathogenic to man and human pathogenic strains are characterized by the ability to produce a thermostable direct haemolysin (TDH) and *tdh*-related haemolysin (TRH). The *tdh* and *trh* genes are present in genomic islands that have been possibly acquired by *V. parahaemolyticus* by lateral gene transfer. Strains of *V. parahaemolyticus* causing acute hepatopancreatic necrosis disease (AHPND) harbour a 70 kb conjugative plasmid carrying pirA and pirB genes encoding a binary *Photorhabdus* insect-related toxin A and B (PirAB). Genetically diverse strains of *V. parahaemolyticus* isolated in Asia seem to have acquired the 70 kb plasmid, while Central American AHPND strains can be distinguished from Asian strains based on PCR amplification of TN-3-like transposon. All AHPND-causing strains tested so far lack virulence factors associated with human pathogenic strains, suggesting that risk to human health due to these strains is negligible. Bacteriophage therapy has shown potential for management of AHPND.

Keywords: AHPND, *Vibrio parahaemolyticus*, plasmid, genetic exchange, virulence

Introduction

Vibrio spp. are heterogenous Gram-negative, comma-shaped bacteria that inhabit freshwater, estuarine and marine environments. Over 100 species are recognized, and they are associated with water, sediment and a whole range of aquatic organisms ranging from microplankton to aquatic birds and marine mammals all over the globe. Only a few species of *Vibrio* are pathogenic to humans.

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Vibrio cholerae is a freshwater species and consists of over 200 serotypes. Of these, only two serotypes, O1 and O139, are associated with the disease cholera. While most non-O1, non-O139 are non-pathogenic to man, there are some strains that may cause gastroenteritis or even extra-intestinal infections. *Vibrio parahaemolyticus* and *V. vulnificus* are two other species that are involved in human infections, the former causing gastroenteritis and the latter causing primary septicaemia, mainly in immunocompromised people, wound infections and cellulitis.

Some *Vibrio* spp. are pathogens of aquatic animals. *Vibrio anguillarum* and *V. salmonicida* (now renamed as *Aliivibrio salmonicida*) are pathogens of finfish causing vibriosis. Others like *V. harveyi*, *V. owensii*, *V. penaecida* and *V. parahaemolyticus* are pathogens of crustaceans. Eleven closely related bacteria are referred to as comprising the Harveyi clade, and these include *V. harveyi, V. alginolyticus, V. parahaemolyticus, V. campbellii, V. rotiferianus, V. mytili, V. natriegens, V. azureus, V. sagamiensis, V. owensii* and *V. jasicida* (Urbanczyk et al. 2013). Members of this clade include important pathogens of aquatic animals and are also used as models in studies related to bioluminescence, quorum sensing and biofilm formation. Members of the Harveyi clade share some virulence-associated genes, suggesting genetic exchange between these in the natural environment (Ruwandeepika et al. 2010).

Until recently, *V. parahaemolyticus* attracted attention mostly as a human pathogen transmitted through raw or inadequately cooked seafood. Oysters and other bivalves eaten raw have been the main vehicles, but even marine finfish have been associated with human infections in some parts of the world, like Japan. Continued outbreaks of seafood poisoning in different parts of the world led the Codex Alimentarius Commission to request the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) to assess such risk to human health. The FAO/WHO risk assessment report (FAO/WHO 2011) presented models for growth of *V. parahaemolyticus* in seafood and for predicting the risk of illness. Noting the geographical variations in the levels of *V. parahaemolyticus* in seafood and considering the need to collect more data on this pathogen, an FAO/WHO expert meeting identified the possible end users of methodologies for detection/enumeration of *V. parahemolyticus*, performance characteristics of the methods and came up with guidance on selection and application of methods (FAO/WHO 2016).

Vibrio parahaemolyticus and its habitat

Vibrio parahaemolyticus is part of the autochthonous microflora of estuarine and coastal environments in tropical to temperate zones all over the world, and there is no correlation between the presence of this organism and faecal contamination of the environments (Joseph et al. 1982; Oliver and Kaper 2007). This organism has been isolated from water, sediment, plankton, various fish and shellfish species, and marine mammals (Joseph et al. 1982). Thus, *V. parahaemolyticus* is naturally present in fish and shellfish, including shrimp and molluscs, growing in harvesting areas. The level of this organism in the environment and in various fish and shellfish may vary depending on environmental and geographical factors.

Certain areas may have more favourable environmental conditions that support establishment, survival and growth, such as temperature, salinity, zooplankton abundance, tidal flushing, suspended sediments, nutrients, dissolved organic matter and dissolved oxygen (Kaneko and Colwell 1977; Garay et al. 1985; Venkateswaran et al. 1990). In temperate waters, the ecology is strongly influenced by temperature and salinity. In these environments, *V. parahaemolyticus* is often detected in warmer months, and the organism has been reported to survive in the sediment during winter (Kaneko and Colwell 1977; De Paola et al. 2003). However, in tropical waters, *V. parahaemolyticus* can be detected throughout the year with low counts being recorded during post-monsoon period, suggesting that salinity may influence the levels in tropical waters (Deepanjali et al. 2005). In the United States of America, a predictive model based on temperature and salinity has been developed, but further studies indicated that addition of chlorophyll a as an additional parameter increased the predictability (Urquhart et al. 2016).

Vibrio parahaemolyticus can adhere to chitin, and zooplankton may thus play an important role in the ecology of this organism. In a study conducted off the coast of Spain, over 80 % of *V. parahaemolyticus* biomass was associated with zooplankton. Although cnidarians accounted for only 2 % of zooplankton biomass, they accounted for 51.87 % variation in abundance of *V. parahaemolyticus* (Martinez-Urtaza et al. 2012). Unlike *V. cholerae*, copepods have a smaller effect on the abundance of *V. parahaemolyticus*, which was favoured by a decrease in primary production, possibly due to grazing pressure by enhanced abundance of zooplankton.

Vibrio parahaemolyticus can grow in sodium chloride concentrations ranging from 0.5 to 10 %, with optimal levels between 1 and 3 % (Colwell et al. 1984). Adsorption of *V. parahaemolyticus* on plankton or chitin-containing materials occurs with higher efficiency under conditions of estuarine salinity (Kaneko and Colwell 1977). Adherence to chitin seems to improve survival of the organism at low temperatures (Karunasagar et al. 1986). In tropical shrimp culture environments, *V. parahaemolyticus* is often present. This organism accounted for 0 to 27 % of the flora in water and sediment of shrimp ponds in India (Otta et al. 1999; Gopal et al. 2005). The level of *V. parahaemolyticus* in seafood may vary depending on the type of seafood and geographical location. In oysters from the Gulf Coast of the United States of America during warm months, a level such as $1.1x10^4$ cfu.100 g^{-1} has been reported, but in Pacific oysters, which grow at lower temperatures, the levels were $2.1x10^3$ cfu.100 g^{-1} (Drake et al. 2007). In Indian oysters, the levels range from 10^2–10^4 cfu.g^{-1} (Deepanjali et al. 2005). In shrimp, the levels range from undetectable to 10^4 cfu.g^{-1}, high counts being rare (Cann et al. 1981; Karunasagar et al. 1984). In finfish, levels of ~88 cfu.g^{-1} have been reported (Chan et al. 1989).

Vibrio parahaemolyticus as a human pathogen

Vibrio parahaemolyticus was first described in 1950 from an outbreak of gastroenteritis implicating “shirasu” (small semi-dried sardine) involving 272 people. Early studies in Japan indicated that human pathogenic strains induce haemolysis in a high salt blood agar medium called Wagatsuma agar, and this phenomenon was referred to as “Kanagawa phenomenon”.

The observed haemolysis has been attributed to a thermostable direct haemolysin (TDH). Further studies on a large collection of clinical and environmental strains showed that 96 % of clinical strains produce TDH, while only 1 % of the environmental strains produce this haemolysin (Joseph et al. 1982), suggesting that most of the environmental strains may not be pathogenic to man. TDH has ability to lyse erythrocytes of various species. It also exhibits cytotoxicity that is lethal to small experimental animals and causes increased vascular permeability in rabbit skin. Using isogenic mutants, it has been demonstrated that TDH has an important role in fluid accumulation in rabbit ileal loop. This has been confirmed by a more sensitive assay using rabbit ileal tissue mounted in Ussing chambers. Culture filtrate of TDH-positive strain induced an increase in short circuit current in these chambers, but this was not seen with culture filtrate of TDH-negative strains. The Ussing chamber activity was neutralized by antiserum to TDH, thus providing confirmation that the activity was due to this virulence factor.

Low prevalence of TDH-positive strains in the environment has been confirmed from different geographical regions. In the Gulf Coast of the United States of America, the percentage has been generally less than 1 %, but in the Pacific northwest, up to 3.2 % of strains could be TDH-positive (FAO/WHO 2011). Oysters from India were positive for *V. parahaemolyticus,* with 6–10 % carrying the *tdh* gene (Deepanjali et al. 2005; Raghunath et al. 2008). Some TDH-negative strains from clinical cases were found to produce a TDH-related haemolysin (TRH) (Honda et al. 1988). Presently, strains producing TDH and TRH are considered pathogenic to man. The levels of pathogenic strains in oysters have been found to be low. DePaola et al. (2003) indicated that the average number of TDH-positive *V. parahemolyticus* in oysters from Alabama was 2 cfu.g^{-1}, while in Chesapeake Bay, a level of 10 cfu.g^{-1} was noted (Parveen et al. 2008). Studies in the People's Republic of China indicated that although the average number of pathogenic *V. parahaemolyticus* in oysters was 0.5 cfu.g^{-1}, the number of pathogens increased to 10 cfu.g^{-1}in oysters that harboured more than 10^4 cfu.g^{-1} total *V. parahaemolyticus* (Han et al. 2015).

There are five sequence variants of the *tdh* gene (*tdh*1 to *tdh5*) and two sequence variants of the *trh* gene (*trh*1 and *trh*2) (Nishibuchi and Kaper 1995). Kanagawa phenomenon has been attributed to the expression of *tdh*2 gene, but most such strains contain both *tdh*1 and *tdh*5 genes. Sixteen percent of environmental Kanagawa-negative strains may contain *tdh*1 gene or other *tdh* genes. The *tdh*4 gene was found in a plasmid, and some *tdh* genes have been detected, albeit rarely, in *V. hollisae, V. mimicus,* and in non-O1/O139 *V. cholerae*.

There is about 69 % nucleotide similarity between *tdh* and *trh* genes. The latter may occasionally be found in *V. alginolyticus* and in *Aeromonas veronii* (Raghunath et al. 2010). Analysis of whole genome sequence of several environmental and clinical strains in this study indicate that all *V. parahaemolyticus* strains carry Type Three Secretion System (TTSS). While TTSS-1 is present in both clinical and environmental strains, TTSS-2 is associated with strains carrying *tdh* (TTSS-2α) and *trh* (TTSS-2β).

Diverse serotypes may be associated with human infections, but at the beginning of 1996, an ongoing surveillance in Kolkata indicated an increase in the incidence of gastroenteritis due to *V. parahaemolyticus*. Subsequent studies indicated that 50–80 % of the isolates belonged to the O3:K6 serotype and were genetically very similar. Within a few months, cases due to the same or closely related serotype were reported in Bangladesh, Viet Nam, Laos, Indonesia, Thailand, Republic of Korea and Japan. These isolates could be identified based on nucleotide sequence variation in *toxRS* region. Today, 21 serotypes are recognized as "serovariants" of O3:K6 serotype, and these have been found to be the causative agents of several outbreaks in Europe, Mozambique, and countries of North and South America (Nair et al. 2007). Although several publications refer to these strains as "pandemic", Nair et al. (2007) pointed out that this is misleading in the epidemiological sense because outbreaks have not affected an exceptionally high proportion of the population. Nevertheless, strains belonging to this group show clonality in molecular typing methods like arbitrarily primed (AP) polymerase chain reaction (PCR), ribotyping or pulse field gel electrophoresis (PFGE). Strains are characterized by presence of only the *tdh* gene (and not *trh* gene), some mismatches in nucleotides in the *tox*RS gene and an open reading frame ORF8 derived from a filamentous bacteriophage f237 (Nair et al. 2007).

Genome Plasticity in Vibrio parahaemolyticus

Analysis of the nucleotide sequence of the *tdh* and *trh* genes of several strains of *V. parahaemolyticus* indicates that their G+C content of about 30 % is much lower than the average G+C content of *Vibrio* chromosomes (46–49 %). This suggests that *tdh* and *trh* genes might have been acquired by *V. parahaemolyticus.* This is further supported by the observation that the *tdh* genes are flanked by insertion sequence-like elements (ISV) that are related to *IS*903 (Nishibuchi and Kaper 1995). Although *IS*903 is known to encode an active transposase, there has been no evidence to demonstrate that actual transposition of *tdh* gene occurs. However, this could be due to base changes and deletions that have occurred in the ISVs, which show only about 50 % identity with *IS*903. Park et al. (2000) demonstrated that the *trh* gene cluster also has lower (41 %) G+C content than *Vibrio* chromosome. This gene cluster contained *trh* gene, nickel transport operon and urease genes. The first gene in this cluster is a transposase gene flanked by an 18 bp inverted repeat on both sides. The next gene is the *trh* gene, followed by urease and nickel transport genes. Analysis of the whole genome sequence of *V. parahaemolyticus* O3:K6 strain RIMD 2210633 and other clinical strains has provided more insights into the genome plasticity of this organism. It is now well established that *V. parahaemolyticus* has two circular chromosomes. So far, nine genomic islands, VPaI-1 to VPaI-9, ranging in size from 10–81 kb have been identified in *V. parahaemolyticus* (Ceccarelli et al. 2013).

Generally, genomic islands are characterized by having G+C content that is different from the other portions of the chromosome and are flanked by transposase or integrase gene or direct repeat regions that suggest they have been acquired through lateral gene transfer. Features of genomic islands of *V. parahaemolyticus* are listed in Table 1.

Table 1. Features of genomic islands of *Vibrio parahaemolyticus.*

Genomic Island	Size	Features
VPaI-1	22.79 kb	• Has 24 open reading frames encoding proteins involved in DNA replication, transcription regulation, signal transduction, general metabolism, type 1 restriction modification complex and DNA methyltransferase (VP0394), which may be an additional colonization factor • 8 kb region containing genes VP0389-VP0392 is syntenic with chromosomal region in *V. vulnificus* CMCP6 and *Shewanella* sp MR7 • Has been reported to be unique to post-1995 pandemic strains, though this pathogenicity island may be missing in some pandemic strains • Inserted adjacent to tRNA-Met
VPaI-2	10 kb	• Encodes outer membrane proteins and revolvases • Present in both pandemic and non-pandemic strains • Inserted adjacent to tmRNA
VPaI-3	32 kb	• Encodes methyl-accepting chemotaxis proteins, considered unique to post-1995 pandemic strains • Inserted adjacent to tRNA-Ser
VPaI-4	17 kb	• Encodes putative pore-forming cytotoxin integrase and M proteins involved in bacterial surface virulence factors • Inserted adjacent to tRNA-Ser
VPaI-5	12 kb	• Encodes a phage-like protein
VPaI-6	27 kb	• Encodes putative colicin
VPaI-7	81 kb	• Located in chromosome-2 • Encodes Type Three Secretion System (TTSS) and *tdh* gene or *trh* gene, homologue of *E. coli* cytotoxic necrotizing factor, ADP ribosyl transferase, enterotoxin, proteins inhibiting MAPK signaling pathway, which prevent cytokine induction
VPaI-8	17 kb	• Encodes hypothetical proteins, integrases and homologues of KAP proteins
VPaI-9	22 kb	• Encodes excisionase, helicase, type 1 restriction modification system

In addition to genomic islands, mobile genetic elements have been reported to be associated with *V. parahaemolyticus*. Mobile genetic elements like plasmids and bacteriophages may contribute to expansion of the ecological niche of this organism by enhancing the environmental fitness. Plasmids ranging in size from 3.5 to 70 kb have been described in *V. parahaemolyticus*. While smaller plasmids are coding for hypothetical proteins, a 28.8 kb plasmid contained a gene with 98 % sequence identity with a gene found in the genomic island VPaI-6 associated with pandemic strains (Hazen et al. 2010). This protein has close similarity to a protein found in *V. harveyi* and the *rep* gene encoding the replication protein in this 28.8 kb plasmid (p22702B) has 85 % identity with *rep* of *V. campbelli* plasmid p09022, suggesting that plasmids may have roles in genetic exchange and lateral gene transfer in *Vibrio* spp. The 70 kb plasmid of *V. parahaemolyticus* carries virulence genes involved in causing acute hepatopancreatic necrosis disease (AHPND).

Filamentous bacteriophage f237 has been found in the genome of pandemic strain of *V. parahaemolyticus*. Some of the prophages detected in chromosome 1 or 2 of *V. parahaemolyticus* have similarity to prophages found in the genome of *V. cholerae* (Kalburge et al. 2014).

Vibrio parahaemolyticus as a shrimp pathogen

Strains of *V. parahaemolyticus* implicated in acute hepatopancreatic necrosis disease (AHPND) are unique in carrying a 70 kb plasmid (pVA1) harbouring the virulence genes, *pirA* and *pirB* encoding the binary protein *Photorhabdus* insect related (Pir) toxin (Lee et al. 2015). All AHPND-causing strains tested harboured this plasmid, although size may vary marginally. The plasmid pVA1 described by Lee et al. (2015) contains 45 open reading frames (ORF) with known function. These include five putative transposases, one putative ORF with homology to the toxin-antitoxin gene *pndA* associated with post-segregational killing (PSK) system, operon that encodes proteins (~30 % homology) to PirA and PirB toxins, a cluster of conjugative transfer genes and two plasmid mobilization genes. The *pirAB* operon has transposases both upstream and downstream suggesting that the operon can be acquired by lateral gene transfer. The PSK system ensures that only progeny containing the plasmid survive, since the stable PSK mRNA in a plasmid-negative strain will be translated into bactericidal *pndA* toxin.

The detection of conjugative elements in the plasmid pVA1 suggests the possibility of mobilization to other strains or even other *Vibrio* spp. Kondo et al. (2015) reported *pirA* and *pirB* genes and related plasmid sequences in a *Vibrio* causing AHPND that is close to *V. harveyi*. These genes have also been detected in AHPND-causing *V. owensii* strain SH14 (Liu et al. 2015). Furthermore, strains of *V. parahaemolyticus* carrying the 70 kb AHPND plasmid are not clonal, but genetically diverse, suggesting that the virulence plasmid has been acquired by several genotypes of *V. parahaemolyticus* by lateral gene transfer (Chonsin et al. 2016).

The copy number of the plasmids has been reported to range from 7–121 in different strains from Viet Nam and Mexico (Han et al. 2016). However, virulence of the strains did not correlate with plasmid copy number (Tinwongger et al. 2016). All the AHPND strains studied so far are negative for *tdh* and *trh* genes and the associated TTSS2 present in genomic island VPaI-7 of human pathogenic strains. Thus, AHPND strains are not considered to possess the ability to cause human infections. Genetic variations in the virulence plasmid from different geographical regions have been reported (Han et al. 2015).

The isolates from Mexico and Central American countries had a 4.2 kbp TN-3-like transposon, which was absent in isolates from Asia. A 9b p repeat region (small sequence repeat, SSR) was found within the ORF in the plasmids. Isolates from the People's Republic of China, Viet Nam and Thailand had 4SSR, and one isolate from Viet Nam had 5SSR, while Mexican strains had 6SSR. Analysis by PCR based on the sequence of TN-3-like transposon could distinguish Asian from American strains causing AHPND.

Global spread of pathogenic Vibrio parahaemolyticus

One of the most common explanation for the global spread of coastal pathogens is the role of ballast waters, but the spread of pandemic *V. parahaemolyticus* from Asia to South America cannot be explained by this theory. Association of *V. parahaemolyticus* with zooplankton has been shown to influence the distribution and population dynamics of this organism in offshore areas. Genetically related populations of *V. parahaemolyticus* were found in zooplankton from estuarine and offshore areas dispersed along 1500 km, suggesting that zooplankton may play a role in oceanic dispersal of this organism (Martinez-Urtaza et al. 2012). Further studies on pandemic strains support this view. The O3:K6 strain that caused a large number of cases in India in 1996 was previously described in Indonesia in 1995. In 1996 and 1997, several cases were reported from different countries in Asia, but the first cases by this serotype outside Asia was observed in Chile in 1997. Using El Niño data and genetic typing of *V. parahaemolyticus* strains involved in human cases in Peru and Chile, Martinez-Urtaza et al. (2008) presented evidence that the pandemic clone of O3:K6 arrived in Peru in 1997 from Asia with El Niño currents. The inflow of foreign zooplankton trapped in El Niño currents in different areas in Chile and Peru in 1997 was also reported by Sanchez et al. (2000). The studies of Martinez-Urtaza et al. (2008) showed that the emergence and pattern of dissemination of *V. parahaemolyticus* showed close correlation with the arrival and propagation of 1997 El Niño. The pandemic strain belonging to O3:K6 serovar arrived in Peru in 1997 and infections were reported in the northern part of the country, but spread southward along more than 1500 km of the coast until it reached the Chilean city of Antofagasta. The peaks of infection corresponded with the arrival of equatorial Kelvin waves. In 1997, El Niño affected the South American coast for about 6 months, and it has been suggested that recurrent invasion of tropical masses of water might have resulted in repetitive sources of populations of *V. parahaemolyticus* that would have established there. There was another episode of outbreaks in Chile in 2004. Genetic analysis of strains from Asia and South American strains from 1997 and 2004 outbreaks showed that the 1997 strains are very closely related to Asian strains (Ansende-Bermejo et al. 2010). Once established in the region, the strains may undergo genetic variations and be involved in genetic recombination with local strains.

Further evidence for the transcontinental spread of *V. parahemolyticus* has been presented by genetic analysis of strains involved in an outbreak in Galacia, Spain in 2012 (Martinez-Urtaza et al. 2016). The outbreak involved 100–114 people travelling in a food banquet cruise boat. Epidemiological studies using questionnaire identified the most probable vehicle as shrimp that was subjected to a short boiling time of 1–2 min then to rapid cooling using water and ice. The shrimp was imported from Argentina and was negative for *V. parahaemolyticus,* and this suggests that water used for cooling was the source of contamination. The strains isolated in this outbreak were positive for both *tdh* and *trh* genes that have never been reported from Europe before. Genetic typing of the strains using PFGE indicated a profile identical to that of strains involved in an outbreak in New York just a couple of months earlier. The mechanistic route of migration of strains from the United States of America to Europe has not yet been explained, but this study supports the hypothesis that *V. parahaemolyticus* can spread globally through oceanic routes.

Potential for control of Vibrio parahaemolyticus using bacteriophages

The emerging problem of antibiotic resistance in both human and animal pathogens has been driving the search for alternatives to antibiotics. Recently, there has been a surge of interest in using bacteriophages as therapeutic agents in human medicine, animal husbandry, aquaculture, agriculture and for biocontrol of food-borne pathogens to improve food safety. Bacteriophages are abundant in nature and have been found in both terrestrial and aquatic environments, and in association with plants and animals. In non-polluted waters, 2 x 10^8 bacteriophages per ml have been found (Bergh et al. 1989). The life cycle of a bacteriophage may include a lytic stage, and some bacteriophages have their genome inserted into the host chromosome and enter a lysogenic stage. Lysogenic bacteriophages are involved in gene transfer. Some of the virulence factors found in bacteria, such as the ability to produce cholera toxin by *Vibrio cholerae* O1, have been associated with bacteriophages inserted into the bacterial genome.

Soon after the discovery of bacteriophages in 1917, the potential to use them against bacteria was realized. However, the interest in bacteriophages declined after the discovery of antibiotics, the subsequent scaling up of antibiotic production to industrial levels and their effectiveness in treating infections in soldiers during World War II. However, treatment failures due to bacteria showing resistance to multiple antibiotics led to renewed interest in bacteriophage therapy. Bacteriophages are host-specific, hence they lyse only the target bacteria, unlike antibiotics, which suppress most members of the bacterial groups. Bacteriophage therapy would not suppress useful commensal bacterial flora that are required for the health of the animals, an action that would be a great advantage in aquaculture.

Use of bacteriophages for control of bacterial diseases in shrimp aquaculture has been documented. Vinod et al. (2006) tested bacteriophage therapy of larvae and postlarvae of giant tiger prawn (*Penaeus monodon* Fabricius 1798) in both laboratory microcosms as well as in hatcheries during a natural outbreak of luminous bacterial disease by adding bacteriophages to rearing water. In microcosms, larval survival was 25 % in the control compared with 85 % in bacteriophage treatment. In hatchery trials, the survival was 86 % with bacteriophages, 40 % with antibiotics and 17 % in controls (Vinod et al. 2006). Bacteriophage treatment brought down counts of luminous bacteria in the tanks. In another hatchery trial during a natural outbreak of luminous bacteria disease, 86–88 % survival was obtained with bacteriophage treatment compared to 65–68 % with antibiotics (Karunasagar et al. 2007). These studies show the potential for bacteriophages to be effective alternatives to antibiotics in shrimp larval health management. One of the problems in shrimp larval health management is the persistence of *V. harveyi* in the hatchery environment by forming a biofilm that is resistant to antibiotics and disinfection (Karunasagar et al. 1996). The ability of bacteriophages to bring about a 3-log reduction in *V. harveyi* growing in biofilms on high density polyethylene (HDPE) surfaces was demonstrated by Karunasagar et al. (2007). However, considering that the host range for selected phages was 65–70 % and the possibility that bacterial strains may develop resistance to bacteriophages, phage therapy with a consortium of phages would be necessary to ensure efficacy against a wide range of opportunistic bacterial strains.

One of the concerns regarding the use of bacteriophage therapy has been the possibility that certain phages may go into a lysogenic state and may be involved in gene transfer. Virulence genes have been associated with lysogenic bacteriophages. Bacteriophages against the shrimp pathogen *V. harveyi* may belong to the family Siphoviridae or Myoviridae (Oakey and Owens 2000; Shivu et al. 2007; Crothers-Stomps et al. 2010). Generally, members of Siphoviridae have been reported to be lytic phages (Vinod et al. 2006; Shivu et al. 2007; Karunasagar et al. 2007; Crothers-Stomps et al. 2010). A *V. harveyi* myovirus-like phage (VHML) has been reported to be temperate and confer virulence to the host strains (Pasharawipas et al. 2005). Shivu et al. (2007) tested the host range of a collection of *V. harveyi* phages against 180 isolates from different geographical regions. Three phages from the family Siphoviridae were able to lyse 65–70 % of the strains, indicating a broad host range. Bacteriophages used by Vinod et al. (2006) and Karunasagar et al. (2007) lacked the putative virulence gene carried by VHML; hence, concern regarding carriage of virulence gene would be minimal.

Application of bacteriophages to control human pathogenic *V. parahaemolyticus* has been attempted. Rong et al. (2014) reported reduction in *V. parahaemolyticus* population in oysters by use of bacteriophages during depuration. Jun et al. (2014) demonstrated reduction of levels of antibiotic-resistant pandemic strain of *V. parahaemolyticus* from 8.9 x 10^6 cfu.mL^{-1} to 1.4 x 10 cfu.mL^{-1} in spiked oysters after 72 h treatment with bacteriophage by bath immersion. Application of bacteriophages on oyster surface led to reduction of *V. parahaemolyticus* counts from 1.44 x 10^6 cfu.mL^{-1} to 1.94 cfu.mL^{-1} in 12 h. Lomeli-Ortega and Martinez-Diaz (2014) reported that two bacteriophages could reduce mortality of larval whiteleg shrimp (*Penaeus vannamei* Boone 1931) challenged with *V. parahaemolyticus*. However, these were not AHPND strains. Jun et al. (2016) demonstrated that bacteriophage pVp-1 lysed 20 of 22 AHPND strains from Asia and America, showing potential for the control of this disease in aquaculture systems.

Conclusion

Vibrio parahaemolyticus is a very versatile organism adapting to new ecological niches and new hosts by acquiring genes. In the case of human pathogenic strains that spread rapidly from Asia to South America, oceanic currents associated with El Niño might have played a role. These strains are genetically highly related and demonstrate even clonal features. All AHPND-causing strains so far tested lack virulence factors required to cause human infections, indicating negligible human health risk from these strains. These strains have unique 70 kb plasmid carrying virulence genes that have features of acquired genes. Genetically diverse strains of *V. parahaemolyticus* seem to have acquired AHPND virulence plasmid, and Asian strains can be distinguished from South American strains based on PCR amplification of TN-3-like transposon. Bacteriophages have potential applications in the management of AHPND.

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Asian Shrimp Production and the Economic Costs of Disease

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Abstract

Using FAO aquaculture production statistics, the global production of cultured crustaceans for 2018 is predicted to be ~8.63 million tonnes. The growth of the shrimp industry, however, is impacted by episodes of disease resulting in huge national income losses (despite compensatory price rises in response to supply shortage), amounting to billions of dollars annually. To illustrate this, the current study reviews losses over the past 40 years and then focuses on current disease problems in Asia, notably AHPND (acute hepatopancreatic necrosis disease caused by pathogenic isolates of *Vibrio parahaemolyticus*), the microsporidian *Enterocytozoon hepatopenaei* (EHP), and WSSV (white-spot syndrome virus). The impacts of AHPND in affected countries, with particular focus on Thailand and the changes in the number of farm operators, land use and production, is investigated. The economic loss from decreased production is followed through the volume of product traded through Mahachai Market, one of Thailand's principal seafood markets, throughout 2010–2017 and is estimated to be US$ 7.38 billion with a further US$ 4.2 billion in export losses. Shrimp disease-related losses within the Vietnamese Mekong Delta were, in the absence of detailed production data, estimated using an assumption-based exercise. Losses due to AHPND in 2015 were determined to be >US$ 26 million, while the costs of WSSV in the same year were >US$ 11 million.

Keywords: acute hepatopancreatic necrosis disease, economic losses, *Enterocytozoon hepatopenaei*, shrimp disease, *Penaeus vannamei*, white-spot syndrome virus

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Introduction

Asia's combined aquaculture production currently exceeds 99.10 million tonnes and yet despite its 6.58 % per annum growth, it is marred by episodes of disease that continue to result in major economic loss. Asia's farmed shrimp and prawn industry, which consists of at least 18 classes of product as categorized by FAO (2017), is expected to exceed 5.37 million tonnes in 2018 (ave. 2010-2015; 4.91% year-on-year growth) compared to a global estimated production of 6.25 million tonnes based on a 5.15 % year-on-year growth (ave. 2010–2015; 22 shrimp-prawn groups).

However, national feed sales and aquaculture statistics, as independently verified by industry specialists, would suggest that the figures submitted for the People`s Republic of China, Indonesia and Viet Nam are over-estimated. By adjusting the production figures for these countries by using feed sales data, then global production in 2018 looks set to be ca. 4.15 million tonnes, of which 3.26 million tonnes would be produced in Asia. The disagreement in production figures lies in the manner in which the figures are compiled and edited by the different agencies. It is also dictated by local interpretations of what constitutes "production"; who is registered, who is required to register and what percentage of the industry is captured by registration; and the return rate and accuracy of submission data regarding output and land use versus submissions based on forecasting.

Shrimp production over the last four decades has been erratic and characterized by a series of apparent boom-bust cycles which have been shaped by a plethora of major disease events. Figure 1 presents year-on-year growth for both global and Asian production (1970–current) and additionally, begins to summarize some of the major disease outbreaks with the associated estimates of economic loss. Using the feed sales adjusted production data, it can be seen that there has been negative growth since 2010 (ave. -4.69 ± 2.16 %; 2010–2015), with the year-on-year growth notably plummeting to -8.10 % in 2014 at a time when the Thai production of whiteleg shrimp (*Penaeus vannamei* Boone 1931) was at its very nadir.

The graph highlights the effect of viral pathogens (e.g. yellow head virus – YHV, Taura syndrome virus – TSV and white-spot syndrome virus – WSSV) on production throughout the 1980s and 1990s, and then during the last five years, the impact of bacterial agents (e.g. isolates of *Vibrio parahaemolyticus* with a toxin gene-bearing plasmid responsible for acute hepatopancreatic necrosis disease (VP_{AHPND}); 2010–present) and the fungal microsporidian *Enterocytozoon hepatopenaei* (EHP). Although the final production statistics for 2016 and 2017 are still outstanding, the year-on-year growth for Asia for the period between 2010 and 2015 has been consistently negative. While globally, production averages are at -2.87 % per annum, for Asia, the annual growth appears to be -4.69 %.

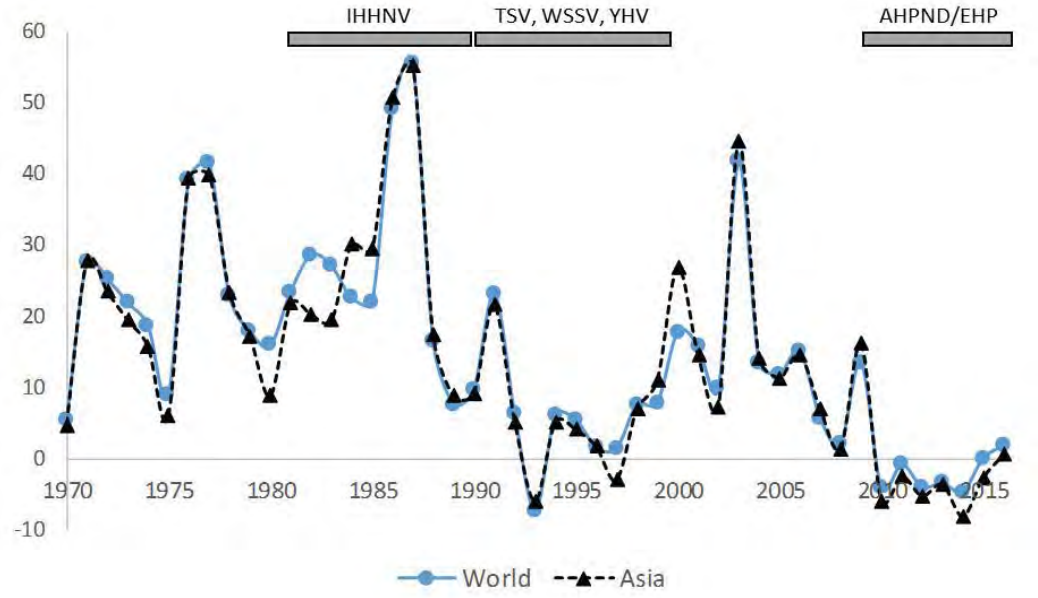


Fig. 1. The percentage year-on-year change in the growth of Asian and global shrimp production. Episodes of disease that have been a key factor in shaping growth of the shrimp aquaculture sector are summarized in Annex 1.[1,]

[1] Data from FAO FishStatJ (2017) and national feed sale figures (where available) are used.

[2]AHPND = acute hepatopancreatic necrosis disease, EHP = *Enterocytozoon hepatopenaei*, IHHNV = infectious hypodermal and haematopoietic necrosis virus, TSV = Taura syndrome virus, WSSV = white-spot syndrome virus, YHV = yellow head virus.

Using FAO FishStatJ statistics, Asia's entire crustacean aquaculture production, not just shrimp and prawns, is ca. 6.56 million tonnes (46 categories) of which 3.16 million tonnes is *P. vannamei* (based on the latest data available which is for 2015), an industry worth an estimated US$ 14.00 billion and employing >2 million people including casual or seasonal labour. It is estimated that the value of Asia's *P. vannamei* industry will rise to US$ 19.15 billion in 2018 when assuming an average year-on-year increase of 11.00% (2010-2015), while the value of the global whiteleg shrimp industry is expected to be US$ 26.48 billion when an average year-on-year growth of 11.90% is applied. Shrimp disease has, however, resulted in huge national income losses despite compensatory price rises in response to supply shortage, amounting to billions of dollars annually (Fig. 1).

AHPND and EHP-ASSOCIATED losses in Asia

The current disease status within Asia's shrimp aquaculture industry is summarized in Table 1, however, this study will focus principally on some of the economic losses associated with AHPND and, to a lesser degree, on EHP.

Table 1. Summary of the last known report of each significant shrimp disease in countries and territories in the Asia-Pacific Region.[1,2]

	Shrimp disease [4]										
	AHPND	**ASDD**	**ATM-WFD**	**BMGN**	**CMNV**	**EHP**	**HPD**	**HPH**	**IHHNV**	**IMNV**	**LSNV**
Australia	2016 [3]					2001?	2007		2015/16*		
Brunei Darussalam									2010		
China PR	2018		2014		2015	2018	2007		2016	2015	
Fiji									2007		
French Polynesia									2009		
India	2018?		2018	2003	2015	2018	2017		2017	2017	
Indonesia		2006	2014?			2016?	2007	2009/2016?	2015	2015	
Iran									2011		
Israel							2007				
Japan				2000							
Kuwait							2007				
Malaysia	2011/17*	2006	2014?			2017	2007		2015/16*	2012/16*	
Myanmar									2012/15*		
New Caledonia									2013		
Papua New Guinea									2010		
Philippines	2016						2007		2016	2015	
Republic of Korea				2000			2007		2015*	2015	
Singapore							2007				
Sri Lanka									2013	2007?	
Taiwan POC	2016*						2007		2013/16*		
Thailand	2018	2008	2018		2015	2018	2007		2015		2011
Viet Nam	2018		2018?			2018					

Table 1. Continued.

	MSGS	MVD	NHP	NPB	SHIV	SIMS	SB (PMTB)	TBV (BP)	TSV	WSSV	WTD (MrNV)	YHV
Australia		2007		2000		2006	2009			2018	2008/16*	2008
Bangladesh										2018		2015
Brunei Darussalam										2013	2013	2010
China PR					2018				2013	2015	2013	2015
Fiji		2007					2007	2007				
Hong Kong SAR										2013/16*		
India	2016*						2017		2017	2017	2012/16*	2006
Indonesia							2006		2013	2015		2011
Iran										2013		
Japan										2015/16*		
Malaysia		2007	2012/16*				2004	2004	2012/16*	2015/16*	2013/16*	2012/16*
Myanmar									2012/16*	2012/16*	2017	2012/16*
New Caledonia							2006					
Philippines						2006	2009			2016		2010/16*
Republic of Korea									2013?	2013/15*		
Saudi Arabia										2013		
Singapore							2008			2016		
Sri Lanka							2010			2013	2007	2013
Taiwan POC							2008	2008	2016	2013/16*	2012/16*	2013
Thailand	2011	2007			2018		2008		2013	2018	2015	2015
Viet Nam	2016*	2007	2013				2008			2018	2016*	2013/16*

[1]No data are available for other countries and territories in the region.

[2]Data are drawn from a variety of resources including NACA-OIE-FAO (2015–2017), Cefas (2018), and white and grey literature.

[3]*Abbreviations:* In Australia known as *Penaeus monodon* mortality syndrome (PMMS); * = not officially reported but the disease is known to occur.

[4]AHPND = acute hepatopancreatic necrosis disease; ASDD = abdominal segment deformity disease; ATM-WFD = aggregated transformed microvilli and white faeces disease; BMGN = baculoviral midgut gland necrosis; EHP (HP) = *Enterocytozoon hepatopenaei* (hepatopancreatic microsporidiosis); CMNV (CMD) = covert mortality nodavirus (covert mortality disease); HPD = hepatopancreatic parvovirus disease; HPH = hepatopancreatic haplosporidiosis; IHHNV = infectious hypodermal and haematopoietic necrosis virus (also now known as *Penaeus stylirostris* densovirus, PstDNV); IMNV = *Penaeus monodon*-type baculovirus; LSNV = Laem Singh virus; MSGS = monodon slow growth syndrome; MVD = mourilyan virus disease; NHP = necrotising hepatopancreatitis; NPB = nuclear polyhedrosis baculoviroses (reference here is made to MBV or *Penaeus monodon*-type baculovirus or more accurately to PmSNPV or singly enveloped nuclear polyhedrosis virus from *P. monodon*); SB (Pmtb) = spherical baculovirus (*Penaeus monodon*-type baculovirus); SHIV = shrimp hemocyte iridescent virus; SIMS = spawner-isolated mortality syndrome; TBV (Bp) = tetrahedral baculovirosis (*Baculovirus penaei*); TSV = Taura syndrome virus; WSSV = white-spot syndrome virus; WTD (MrNV) = white tail disease (also known as (*Macrobrachium rosenbergii* nodavirus, *Mr*NV); YHV = yellow head virus.

Acute Hepatopancreatic Necrosis Disease

AHPND of *P. vannamei* and of giant tiger prawn (*Penaeus monodon* Fabricius 1798) has had a devastating impact on shrimp production (see Pakingking et al. 2016) and is now reported from at least eight Asian territories (see Table 1), and from Costa Rica, Honduras and Mexico (Shinn et al. 2018). The impact of infection on production in the People's Republic of China, Malaysia, Mexico, Thailand and Viet Nam is presented in Fig. 2, with losses estimated on the farm-gate value of stock (i.e. values do not include losses to feed, processing, export and other support industries).

In Thailand, production fell from a peak of 611 194 tonnes in 2011 (Table 2) to ca. 200 000 tonnes by 2014/15; productivity fell from a peak of 11.19 tonnes.ha^{-1} in 2010 to only 6.14 tonnes.ha^{-1} in 2014 following the AHPND outbreak (Table 2). When pre-AHPND (i.e. 2009) production is compared to that in 2014 (last date for which comprehensive figures are available), then it can be seen that in addition to total production having dropped by 53.91 %, there were also 16.16 % fewer farms in production, and the land area used for shrimp culture was down by 10.45 % (Table 3; Fig. 3). To determine whether the losses estimated and presented for Thailand in Fig. 2 were accurate, the value of lost product was independently assessed by evaluating the volume and value of *P. vannamei* passing through Mahachai Market in Samut Sakhon Province, Thailand throughout 2010–2017.

Figure 4 shows the number of six-tonne containers passing through the seafood market on a daily basis, which when summarized and presented as annual trends (Fig. 5) shows that there has been a consistent yearly decrease in volume. This decline is particularly marked when the average for 2010–2012 is compared to that for 2013–2017 (Fig. 6). The daily price of the shrimp (number per kg; Thai baht (THB) per kg) was also tracked by following a LINE (a communication app) group hosted by the Thai Shrimp Centre and then used to define an accurate average farm gate price of product (Fig. 7), and consequentially the loss from the decreased volume of container traffic moving through Mahachai Market. From this, the consequential impact of the AHPND outbreak on the volume of shrimp passing through Mahachai Market (2010-2016) is estimated to be US$ 7.38 billion.

It should be stressed that not all this decreased trade can be attributed to only AHPND, and while AHPND may be responsible for a significant proportion of the losses, other concurrent shrimp infections also account for a percentage of the losses. The additional revenue lost on exported shrimp products was also determined by calculating the yearly difference in exported volume from its peak in 2010, and by looking at trend data relating to the value added to export data and the percentage of product that was exported (Table 4). From the data, the additional Thai export losses are determined to be US$ 4.2 billion. National losses to date are put at more than US$ 11.58 billion (2010–2016), with an estimated 100 000 jobs lost as a result of infections. A recent study conducted by Flegel and co-workers (Flegel 2016) looked at the causes of early mortality in 196 shrimp ponds in Thailand and confirmed AHPND by histology and polymerase chain reaction (PCR) within 21.4 % of ponds.

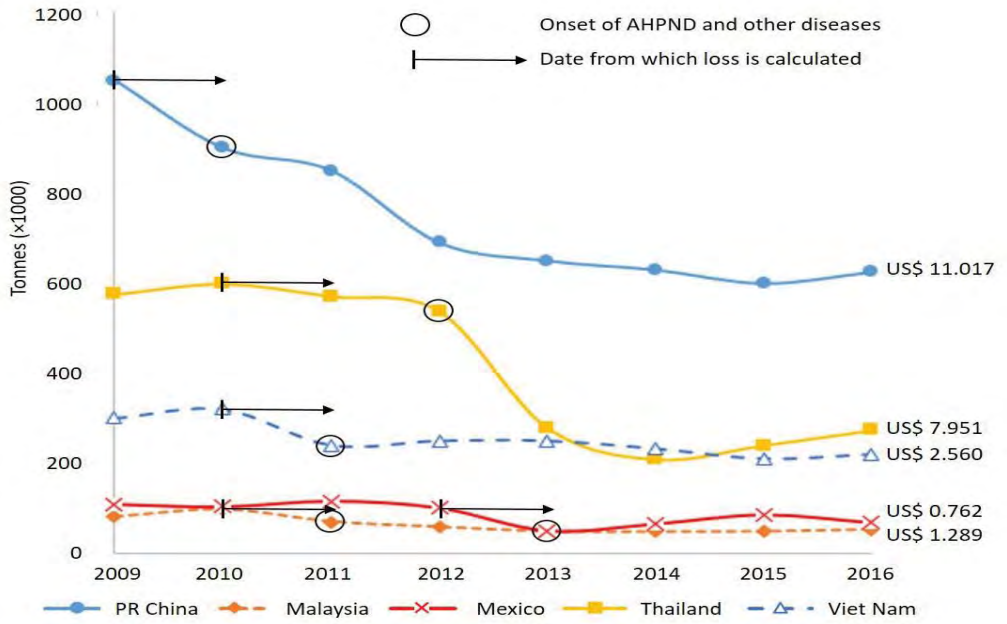


Fig. 2. Whiteleg shrimp (*Penaeus vannamei*) production and the subsequent losses (US$ billion) due to shrimp disease in countries in which AHPND has been reported. The first date of reported losses attributable to AHPND for each country is marked as a black circle, while losses are calculated from the black arrow. Data are drawn and averaged from three independent sources.

Table 2. Shrimp industry production data for Thailand over the period 2000 to 2014 giving the number of farms in operation, total land area used for culture (ha) and total shrimp production (tonnes) with details given for *Penaeus monodon* (*P.m.*) and *P. vannamei* (*P.v.*).[1,2]

Year	Farms	Area (ha)	Production (tonnes)	Tonnes.ha^{-1}	*P. m.* (tonnes)	*P. m.* tonnes.ha^{-1}	*P. v.* (tonnes)	*P. v.* (tonnes.ha^{-1})
2000	34 979	81 120	309 862	3.82	304 988	3.82		
2001	31 839	76 941	280 007	3.64	274 330	3.64		
2002	31 179	74 381	264 923	3.56	260 573	3.56		
2003	34 977	82 019	330 725	4.03	194 909	3.67*	132 365	4.58
2004	33 411	71 200	360 289	5.06	106 884	3.67*	251 697	5.98
2005	33 444	71 825	401 250	5.59	26 055	3.67*	374 487	5.79
2006	30 732	67 772	494 401	7.30	13 986	3.67*	480 061	7.51
2007	30 311	68 402	523 226	7.65	14 317	3.67*	508 446	7.88
2008	25 041	54 758	506 602	9.25	4 745	3.67*	501 394	9.38
2009	25 131	52 811	575 098	10.89	3 533	3.67*	571 189	11.02
2010	23 333	50 911	559 644	10.99	5 105	3.67*	553 899	11.19
2011	23 675	58 023	611 194	10.53	6 514	3.67*	603 227	10.72
2012	23 832	58 820	609 552	10.36	20 558	3.67*	588 370	11.06
2013	21 668	49 854	325 395	6.53	14 279	3.67*	310 705	6.76
2014	21 071	47 291	279 907	5.92	16 292	3.67*	263 245	6.14

[1]an asterisk indicates that no figures relating to the total area used for each species are available and so for 2003–2014, an average figure of 3.67 tonnes.ha^{-1} is assumed for *P. monodon* culture so that approximate production figures for *P. vannamei* (*P.v.* tonnes.ha^{-1}) can be determined.

[2]Data are extracted from the Fisheries Statistics of Thailand Yearbooks 2000–2014.

Table 3. A comparison of the number of farms, the land area used and the tonnage resulting from shrimp production in the main shrimp-producing provinces of Thailand in 2009 (pre-AHPND) and 2014 (on-going AHPND infection and for which the latest complete figures are available). Part of the data is drawn from the Fisheries Statistics of Thailand Yearbooks for 2009 and 2014. The "map code" follows that given in Fig. 3a.

2009					2014							2009 versus 2014			
Map code	**Province**	**No. farms**	**Area (ha)**	**Total shrimp (tonnes)**	**Map code**	**Province**	**Farm no. (% diff)**	**Area (ha) (% diff)**	***P. vannamei* production (tonnes) (% diff)**	***P. monodon* production (tonnes)**	***P. vannamei* production (tonnes)**	**Map code**	**Province**	**Farm no. (% diff)**	**Area (ha) (% diff)**	***P. vannamei* production (tonnes) (% diff)**
	Coastal zone 1	2 565	7 635	132 271		Coastal zone 1	10.64	8.71	-49.91	305	65 749		Coastal zone 1	10.64	8.71	-49.91
1	Trat	690	2 029	46 935	1	Trat	-18.70	-26.92	-54.21	-	21 400	1	Trat	-18.70	-26.92	-54.21
2	Chanthaburi	1 500	3 916	64 336	2	Chanthaburi	28.33	47.49	-45.77	214	34 455	2	Chanthaburi	28.33	47.49	-45.77
3	Rayong	375	1 690	21 000	3	Rayong	-6.13	-38.34	-52.89	91	9 894	3	Rayong	-6.13	-38.34	-52.89
	Coastal zone 2	10 234	14 200	62 361		Coastal zone 2	-8.87	51.59	-21.55	2 792	47 882		Coastal zone 2	-8.87	51.59	-21.55
4	Chon Buri	146	168	600	4	Chon Buri	118.49	100.67	122.39	-	1 321	4	Chon Buri	118.49	100.67	122.39
5	Chachoengsao	7 350	5 810	32 617	5	Chachoengsao	-56.03	-50.42	-42.68	149	18 409	5	Chachoengsao	-56.03	-50.42	-42.68
6	Prachin Buri	400	800	2 970	27	2 943	431	758	4 408	-	4 408	6	Prachin Buri	7.75	-5.30	49.78
7	Samut Prakan	600	2 400	2 584	432	2 152	1 928	5 506	5 018	890	4 070	7	Samut Prakan	221.33	129.43	89.13
8	Bangkok Metropolis	197	515	106	29	48	936	2 525	951	508	443	8	Bangkok Metropolis	375.13	390.16	822.92
9	Samut Sakhon	800	2 317	10 071	50	9 774	1 291	4 233	10 968	191	10 771	9	Samut Sakhon	61.38	82.68	10.20
10	Samut Songkhran	291	1 326	1 215	0	1 215	423	2 838	1 529	145	1 296	10	Samut Songkhran	45.36	114.01	6.67
11	Phetchaburi	450	864	12 198	9	12 189	766	2 449	8 173	909	7 164	11	Phetchaburi	70.22	183.29	-41.23
	Coastal zone 3	3 521	12 788	128 154	546	127 608	1 360	3 679	41 641	2 117	39 524		Coastal zone 3	-61.37	-71.23	-69.03
12	Prachuap Khiri Khan	786	3 990	32 239	92	32 147	537	1 079	13 247	178	13 069	12	Prachuap Khiri Khan	-31.68	-72.96	-59.35
13	Chumphon	500	1 600	34 116	47	34 069	303	766	9 272	1 019	8 253	13	Chumphon	-39.40	-52.13	-75.78
14	Surat Thani	2 235	7 198	61 799	407	61 392	520	1 834	19 122	920	18 202	14	Surat Thani	-76.73	-74.52	-70.35

Table 3. Continued.

2009					2014							2009 versus 2014			
Map code	**Province**	**No. farms**	**Area (ha)**	**Total shrimp (tonnes)**	**Map code**	**Province**	**Farm no. (% diff)**	**Area (ha) (% diff)**	***P. vannamei* production (tonnes) (% diff)**	***P. monodon* production (tonnes)**	***P. vannamei* production (tonnes)**	**Map code**	**Province**	**Farm no. (% diff)**	**Area (ha) (% diff)**	***P. vannamei* production (tonnes) (% diff)**
	Coastal zone 4	4 205	7 864	97 059	405	96 654	2 757	3 442	31 100	1 592	29 508		Coastal zone 4	-34.44	-56.23	-69.47
15	Nakhon Si Thammarat	2 000	4 000	37 909	60	37 849	1 383	1 903	13 891	592	13 299	15	Nakhon Si Thammarat	-30.85	-52.42	-64.86
16	Songkhla	1 800	2 880	47 989	330	47 659	1 243	1 239	14 156	808	13 348	16	Songkhla	-30.94	-56.99	-71.99
17	Phatthalung	200	320	2 127	0	2 127	35	68	698	19	679	17	Phatthalung	-82.50	-78.85	-68.08
18	Pattani	200	640	8 624	15	8 609	90	203	2 183	173	2,010	18	Pattani	-55.00	-68.23	-76.65
19	Narathiwat	5	24	410	-	410	6	29	172	-	172	19	Narathiwat	20.00	20.00	-58.05
	Coastal zone 5	2 406	6 964	134 887	497	134 390	1 548	3 619	50 349	9 486	40 863		Coastal zone 5	-35.66	-48.03	-69.59
20	Ranong	150	694	8 032	0	8 032	111	553	9 264	1 012	8 252	20	Ranong	-26.00	-20.28	2.74
21	Phang Nga	376	1 285	30 329	39	30 290	239	666	9 026	1 298	7 728	21	Phang Nga	-36.44	-48.14	-74.49
22	Phuket	100	304	4 202	90	4 112	65	144	2 382	553	1 829	22	Phuket	-35.00	-52.79	-55.52
23	Krabi	330	1 010	24 274	58	24 216	290	591	8 661	2 463	6 198	23	Krabi	-12.12	-41.46	-74.41
24	Trang	850	1 769	34 000	293	33 707	475	746	11 219	2 851	8 368	24	Trang	-44.12	-57.86	-75.17
25	Satun	600	1 901	34 050	17	34 033	368	919	9 797	1 309	8 488	25	Satun	-38.67	-51.68	-75.06
	Others	2 200	3 360	20 366	130	20 236	3 242	6 725	39 719	-	39 719		Others	47.36	100.14	96.28
	Total	25 131	52 811	575 098	3 533	571 189	21 071	47 291	279 907	16 292	263 245		Total	-16.16	-10.45	-53.91

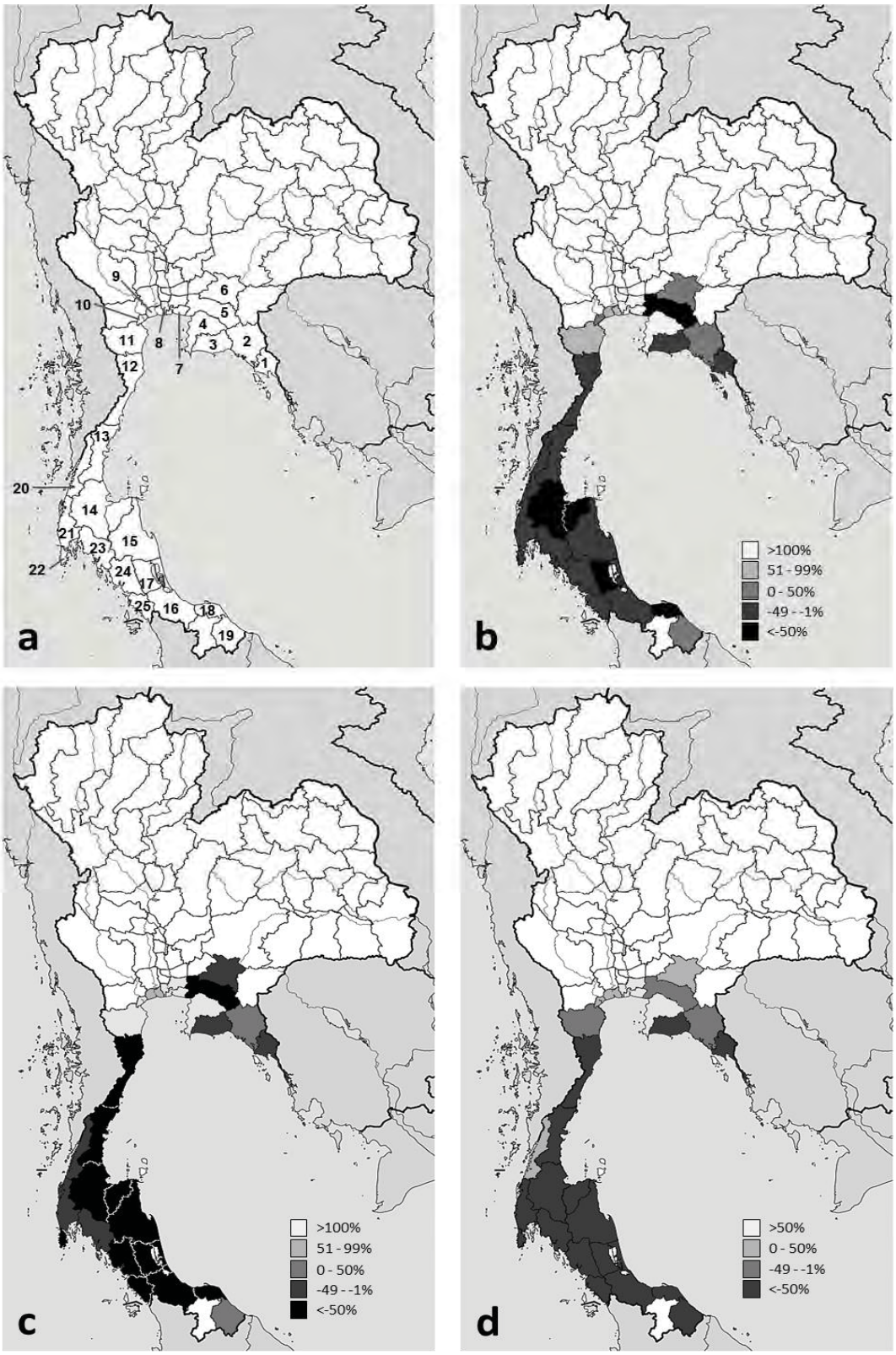


Fig. 3. Changes to the production of whiteleg shrimp (*Penaeus vannamei*) over the period of 2009 (i.e. pre-outbreak of AHPND) to 2014 (i.e. peak of infection) in the main farming provinces of Thailand. (a) The main provinces producing *P. vannamei.* (b) The percentage change in the number of registered farms producing *P. vannamei* in 2009 compared to that in 2014. The percentage change in the land area used for the culture of *P. vannamei* between that used in 2009 and that used in 2014.

For both (b) and (c) the observed changes range from over a 100 % increase to a 50+ % decrease in the number of farms or land area used for shrimp culture in 2009. (d) The percentage change in the total tonnage of *P. vannamei* produced in 2009 with that in 2014. Provincial figures range from a 50+ % increase to 50 % cut or lower in production.[1,2]

[1]1 = Trat; 2 = Chanthaburi; 3 = Rayong; 4 = Chonburi; 5 = Chachoengsao; 6 = Prachinburi; 7 = Samut Prakan; 8 = Bangkok Metropolis; 9 = Samut Sakhon; 10 = Samut Songkhran; 11 = Phetchaburi; 12 = Prachuap Khiri Khan; 13 = Chumphon; 14 = Surat Thani; 15 = Nakhon Si Thammarat; 16 = Songkhla; 17 = Phatthalung; 18 = Pattani; 19 = Narathiwat; 20 = Ranong; 21 = Phang Nga; 22 = Phuket; 23 = Krabi; 24 = Trang; 25 = Satun.

[2] Data presented in Table 3 are used to construct the maps; the raw data are taken from the Fisheries Statistics of Thailand (2000–2014).

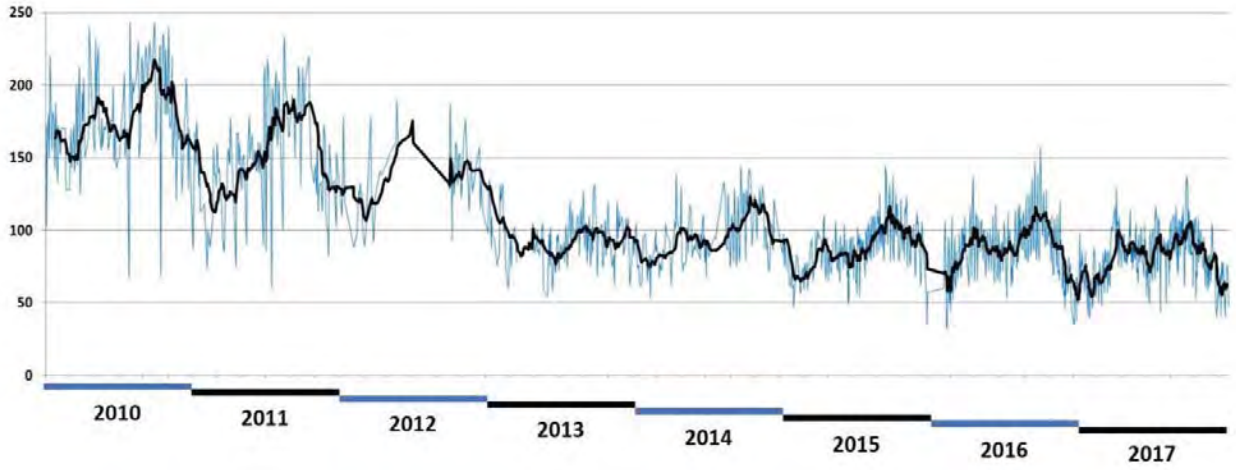


Fig. 4. The number of six-tonne containers passing through Mahachai Market in Thailand each day from January 2010 to December 2017.

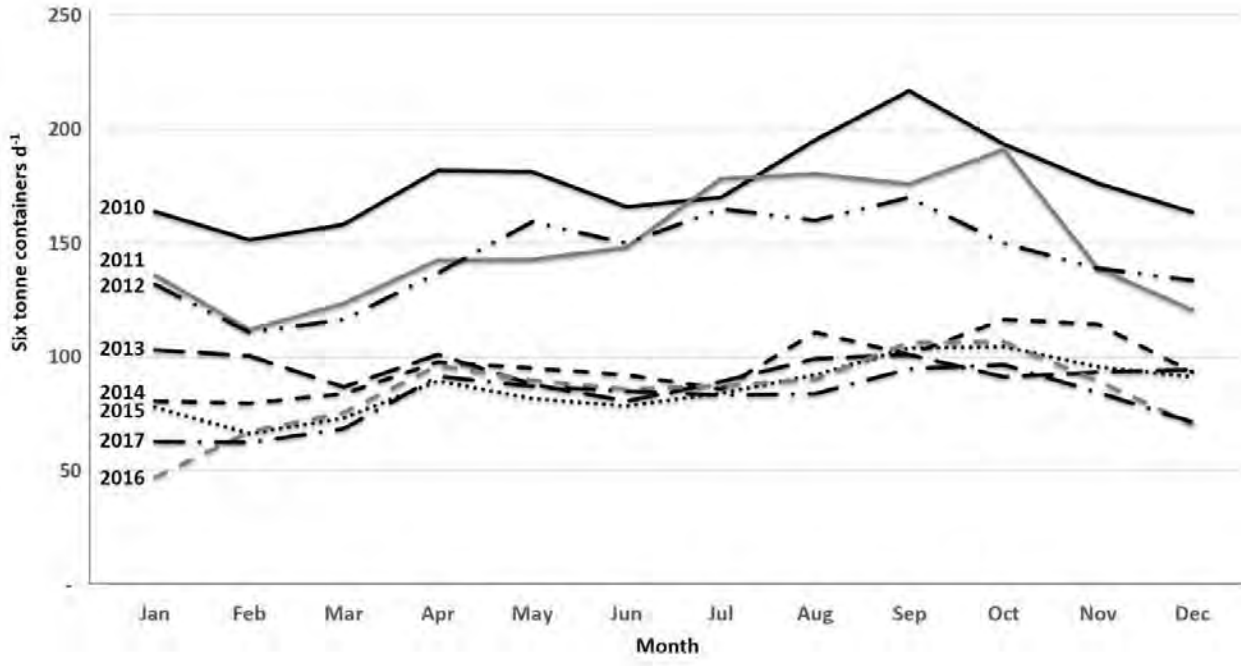


Fig. 5. Monthly summary of the number of six-tonne containers passing through Mahachai Market, Thailand throughout the period January 2010 to December 2017.

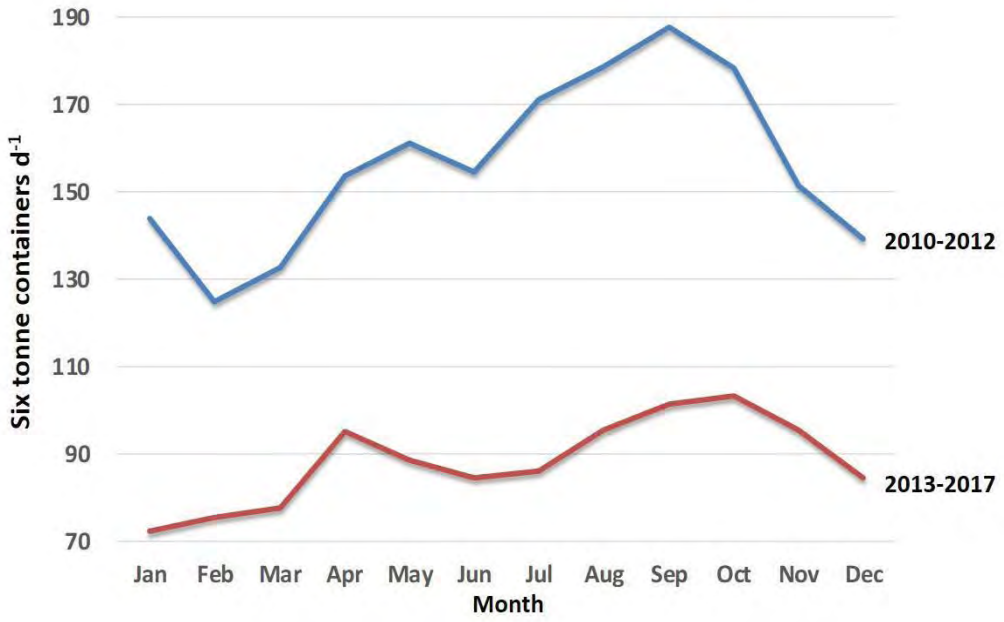


Fig. 6. The volume of six-tonne containers passing through Mahachai Market in Thailand in 2010 to 2012 compared to the decreased volume in 2013 to 2017.

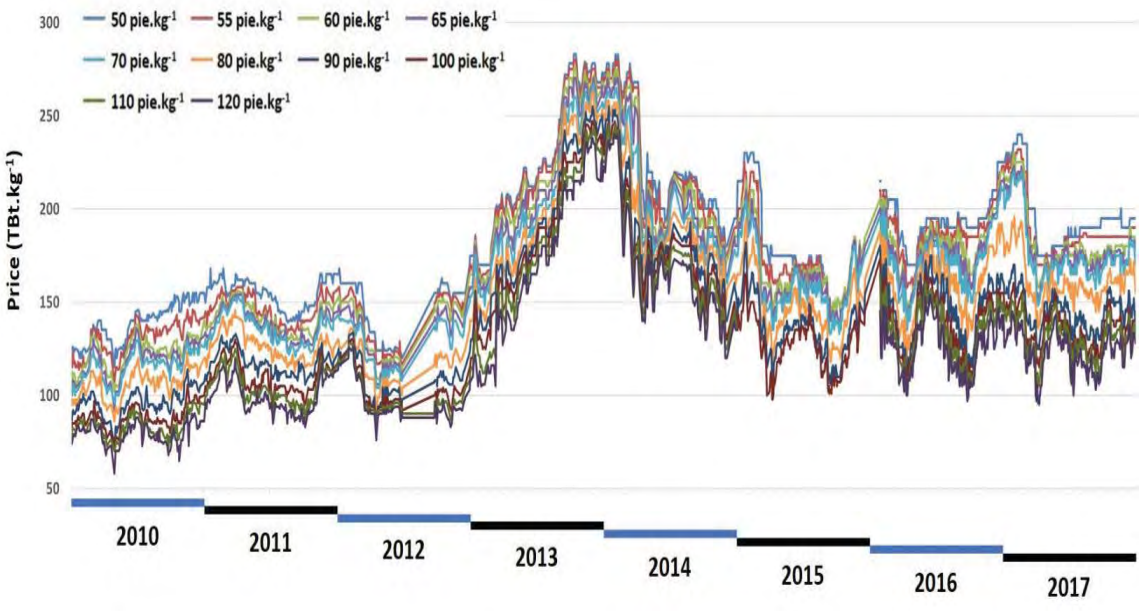


Fig. 7. Value of the shrimp pond bank in Thailand (US$ 1 = THB 31). The graph shows the daily price of the shrimp product (given as $TBt.kg^{-1}$; $pie.kg^{-1}$) fetched at Mahachai Market in Thailand throughout January 2010 to December 2017.

Table 4. The value of Thailand's national shrimp and prawn production and exports for the period 2005 to 2016. For each year, the volume (tonnes) of shrimp/prawns harvested from commercial fisheries and from aquaculture is given together with their total value and price per tonne. Following the outbreak of AHPND in 2012, the fall in production (i.e. volume and value) from aquaculture is given. The volume and value of exported product is also given and the subsequent loss in revenue following the AHPND outbreak and peak in trading in 2010 is given.[1]

	Year					
	2005	**2006**	**2007**	**2008**	**2009**	**2010**
Capture fisheries (tonnes)	81 569	75 782	63 201	53 991	54 682	54 420
Aquaculture (tonnes)	401 250	494 401	523 226	506 602	575 098	559 644
Tot. production of shrimp & prawns (tonnes)	482 819	570 183	586 427	560 593	629 780	614 064
Total value (US$ x 1000)	1 496 160	1 607 396	1 763 509	1 784 055	1 958 997	2 073 397
Value (US$ / tonne)	3 099	2 819	3 007	3 182	3 111	3 377
Lost tonnage from 2011 peak						
Value of lost tonnage (US$ x 1000)						
Exports						
Total volume of product exported (tonnes)	159 117	180 116	196 372	197 787	211 615	242 724
Total value of exports (US$ x 1000)	1 013 034	1 142 486	1 327 816	1 316 927	1 337 334	1 688 911
Value of export (US$ / tonne)	6 367	6 343	6 762	6 658	6 320	6 958
Percentage of production that is exported	32.96	31.59	33.49	35.28	33.60	39.53
Value added on exports (US$ / tonne)	3 268	3 524	3 755	3 476	3 209	3 582
Lost export tonnage from 2010 peak						
Add. revenue lost on exports (US$ x 1000)						
Exchange rates applied (1 US$ = x THB)	37.5	37.9	32.2	32.6	34.6	31.5

	Year					
	2011	**2012**	**2013**	**2014**	**2015**	**2016**
Capture fisheries (tonnes)	48 646	45 479	41 327	40 339	40 339	40 339
Aquaculture (tonnes)	611 194	609 552	325 395	279 907	240 000	273 000
Tot. production of shrimp & prawns (tonnes)	659 840	655 031	366 722	320 246	280 339	313 339
Total value (US$ x 1000)	2 702 321	2 647 785	2 041 367	1 773 972	1 552 911	1 735 711
Value (US$ / tonne)	4 095	4 042	5 567	5 539	5 539	5 539
Lost tonnage from 2011 peak		1 642	285 799	331 287	371 194	338 194
Value of lost tonnage (US$ x 1000)		6 637	1 590 907	1 835 132	2 056 193	1 873 393
Exports						
Total volume of product exported (tonnes)	202 339	178 850	92 062	75 447	70 085	78 335
Total value of exports (US$ x 1000)	1 740 168	1 458 605	918 860	880 734	818 137	914 444
Value of export (US$ / tonne)	8 600	8 155	9 981	11 674	11 674	11 674
Percentage of production that is exported	30.66	27.30	25.10	23.56	25	25
Value added on exports (US$ / tonne)	4 505	4 113	4 414	6 134	6 134	6 134
Lost export tonnage from 2010 peak	40 385	63 874	150 662	167 277	172 639	164 389
Add. revenue lost on exports (US$ x 1000)	181 928	262 729	665 076	1 026 101	1 058 993	1 008 387
Exchange rates applied (1 US$ = x THB)	30.2	31.1	31.3	32	34	35

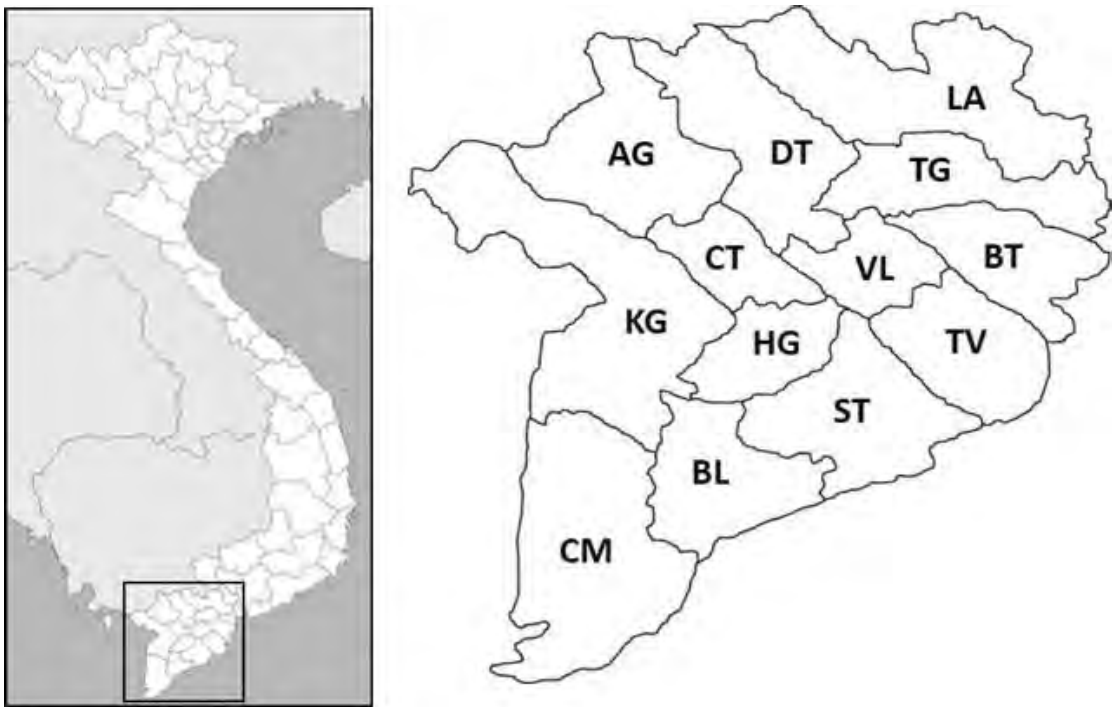
[1] Data drawn from various sources including the Fisheries Statistics of Thailand Yearbooks 2000–2014 and FAO (2016). No data for 2015–16 are available (shaded columns); predictions are estimated on selected parameters given for 2014.

Elsewhere, in the Vietnamese Mekong Delta, 8.72 % of *P. monodon* ponds (i.e. 47 574 ha) and 32.48 % of *P. vannamei* ponds (i.e. 18 966 ha) in 2014 were reported to have been affected by shrimp disease (Table 5). In 2015, the area of *P. monodon* ponds affected by AHPND was reported to be 5 875 ha, while a further 5 509 ha of ponds used for *P. vannamei* culture were infected. In the absence of detailed production data and information relating to losses, a series of assumptions based on national husbandry practices were used to estimate national losses (see Table 6).

In calculating the value of lost stock, it was assumed that only a single crop of shrimp was lost from any one pond in the year. From this, the combined AHPND-associated losses for both species were estimated to be over US$ 25.98 million for the year. Hien et al. (2016) also reported on the occurrence of AHPND within Viet Nam and suggested that losses were of the order of US$ 97.96 million (i.e. US$ 10 352 per tonne), however, no details relating to how the losses were calculated were provided.

White-spot syndrome virus (WSSV) still remains the most significant viral pathogen of cultured shrimp – infections are rapid and typically result in an 80–100 % loss of stock. Infections in 5 370 ha of *P. monodon* and *P. vannamei* culture ponds in the Mekong Delta in 2015 were determined using a similar series of assumptions based on local practices and estimated at US$ 11.02 million (Table 6). Again, these differ from those provided by Hien et al. (2016), who suggested WSSV associated losses were US$ 55.58 million.

Table 5. The area of shrimp ponds (ha) reported to be affected by shrimp disease in 2014 in the key shrimp-producing provinces of the Vietnamese Mekong Delta.



Code	Province	*Penaeus monodon*			*Penaeus vannamei*		
		Infected (ha)	**Total (ha)**	**%**	**Infected (ha)**	**Total (ha)**	**%**
AG	An Giang						
BL	Bạc Liêu	13 485	119 996	11.24	2 054	8 076	25.43
BT	Bến Tre	3 314	27 000	12.27	993	5 113	19.42
CM	Cà Mau	11 802	262 915	4.49	1 027	6 600	15.56
CT	Cần Tho						
DT	Đồng Tháp						
HG	Hậu Giang						
KG	Kiên Giang	7 325	88 648	8.26	14	1 915	0.73
LA	Long An	303	1 000	30.30	1 622	5 700	28.46
ST	Sóc Trăng	7 103	19 736	35.99	11 704	27 017	43.32
TG	Tiền Giang	151	2 599	5.81	414	1 380	30.00
TV	Trà Vinh	4 091	23 841	17.16	1 138	2 600	43.77
VL	Vĩnh Long						
Total		454	545 735	8.72	18 966	58 401	32.48

The figures presented here, however, are in marked contrast to the losses presented in Fig. 3 for Viet Nam, which indicates that since 2011, when AHPND was first reported in the country, shrimp disease including AHPND has cost the country US$ 2.56 billion (ca. US$ 437 million per annum). As already stated, caution must be exercised in that not all the reported losses can be ascribed to a single pathogen, i.e. VP_{AHPND}. The case study presented here begins to calculate losses for ponds where an infection of VP_{AHPND} was confirmed, using a conservative, very simple series of assumptions (i.e. only one crop from any one pond is lost; loss occurs within 40 days post-stocking; that the percentage of semi-intensive versus extensive ponds infected follows the national ratio; that the number of ponds with an infection is close to the actual situation; that the values assigned for each parameter are accurate). From this, however, there appears to be a huge underestimation in the calculation of loss. Such models, however, are dependent on the quality of farm data available.

With the addition of further details relating to, for example, the number of crops lost, confirmed diagnosis of the pathogen responsible for the loss, the stocking density used/degree of farm intensification, the point at which infection occurred and, the impact on the crop that was harvested etc., only then can we begin to design better models to compute the value of loss. For some studies, loss is simply estimated on the total pond area affected by pathogen "×" multiplied by the value of the expected tonnage to be harvested, which overestimates loss. Part of the challenge in calculating disease losses lies in: the sheer magnitude of the problem; the complexity of the data; untangling the complications of co-infections and assigning loss to particular pathogens; and, respectfully, a lack of resources to collate and process the data. While the approach used in this study attempts to provide loss estimates linked to primary production, calculating the additional losses through the value chain and to satellite industries adds further complexity.

Table 6. Losses due to acute hepatopancreatic necrosis diseases (AHPND) and white-spot syndrome virus (WSSV) in the Vietnamese Mekong Delta for 2015. Calculations are based on the assumptions provided and that only a single crop was lost. [1]

AHPND	
Penaeus monodon	***Penaeus vannamei***
5 875 ha affected	5 509 ha affected
Assumptions	Assumptions
38 % of production is semi-intensive; 62 % extensive	52.7 % of production is semi-intensive.; 47.3 % extensive
100 % of one crop up to 40 d poststocking lost	100 % of one crop up to 40 d poststocking lost
Stocking 15 PL.m^{-2} (semi.); 8 PL.m^{-2} (exten.)	Stocking 100 PL.m^{-2} (semi.); 70 PL.m^{-2} (exten.)
110 d production cycle	110 d production cycle
1 000 PL = $6.30	1 000 PL = $4.30
$Tmort_{50}$ = 20 days	$Tmort_{50}$ = 20 days
10 % per day increment in feed/growth	10 % per day increment in feed/growth
Feed = US$1.5 kg^{-1}	Feed = US$1.3 kg^{-1}
Labour = ca. 12.5 % of total production (US$5.77 ha.d^{-1})	Labour = ca. 12.5 % of total production (US$5.77 ha.d^{-1})
Farm gate price = US$7.65 kg^{-1}	Farm gate price = US$3.83 kg^{-1}
Harvest = 2.74 tonnes.ha^{-1} (semi.); 1.5 tonnes.ha^{-1} (exten.)	Harvest = 12 tonnes.ha^{-1} (semi.); 9 tonnes.ha^{-1} (exten.)
Loss = US$4 675 709	Loss = US$21 303 962
WSSV	
Penaeus monodon	***Penaeus vannamei***
3 447 ha affected	1 923 ha affected
Assumptions	Assumptions
38 % of production is semi-intensive; 62 % extensive	52.7 % of production is semi-intensive; 47.3 % extensive
2 % loss per day between 9 and 109 d poststocking	2 % loss per day between 9 and 109 d poststocking
Stocking 15 PL.m^{-2} (semi.); 8 PL.m^{-2} (exten.)	Stocking 100 PL.m^{-2} (semi.); 70 PL.m^{-2} (exten.)
110 d production cycle	110 d production cycle
1 000 PL = US$6.30	1 000 PL = US$4.30
10 % per day increment in feed/growth	10 % per day increment in feed/growth
Feed = US$1.5 kg^{-1}	Feed = US$1.3 kg^{-1}
Labour = ca. 12.5 % of total production ($ 5.77 ha.d^{-1})	Labour = ca. 12.5 % of total production ($5.77 ha.d^{-1})
Farm gate price = US$7.65 kg^{-1}	Farm gate price = US$3.83 kg^{-1}
Harvest = 2.74 tonnes.ha^{-1} (semi.); 1.5 tonnes.ha^{-1} (exten)	Harvest = 12 tonnes.ha^{-1} (semi.); 9 tonnes.ha^{-1} (exten.)
Loss = US$3 250 775	Loss = US$7 770 624

[1]Data are drawn from various sources including the Directorate of Fisheries Viet Nam, from Fistenet and from industry contacts.

Enterocytozoon hepatopenaei

Spreading infections of the fungal microsporidian parasite *Enterocytozoon hepatopenaei* (EHP), the causative agent of hepatopancreatic microsporidiosis (HPM) in shrimp, which is reported to result in severe growth retardation, morbidity and, in heavily infected individuals, mortality, is also causing serious concern within the industry. Infections in *P. vannamei* and *P. monodon* are reported from Australia, the People's Republic of China, India, Indonesia, Malaysia, Thailand and Viet Nam, where the parasite infects not only the tubule epithelial cells of the hepatopancreas as previously reported but is also found within the intestinal cells (J. Jiravanichpaisal unpublished data). Despite the growing number of reports of EHP, details relating to the economic impacts that this parasite has on production are scant. EHP spores are extremely small (ca. 1 × 0.67 μm), persistent and can be readily transmitted horizontally between shrimp. A Thai survey of 196 ponds reporting early mortality in stocked shrimp found EHP in 119 of the ponds (i.e. prevalence at 60.7 %; Flegel 2016).

The consequential slowed growth or growth arrest in heavily infected stock means that either the entire stock is lost (i.e. culled out if found to be heavily infected and ca. 7–9 g) or forces an early harvest, in which event the production costs may not be covered. Under normal production, a typical 110-day culture (i.e. PL10 to 18 g) with an anticipated harvest of 12 tonnes.ha^{-1} and a value of ca. US$ 5.30 kg^{-1} against production costs of ca. US$ 3.58 kg^{-1} might be assumed. For EHP-infected sites with poor or arrested growth, the shrimp may not grow beyond 12 g, resulting in a lower than anticipated harvest yield of 9 tonnes.ha^{-1}. The value of the harvested shrimp (e.g. at 12 g may fetch US$ 3.50 kg^{-1}) may be lower than the costs invested at this point in production, e.g. US$ 4.00 kg^{-1}. Under such circumstances, production costs are not covered and so losses per hectare may range between US$ 4 500 and US$ 32 100 over normal practice economics, depending on the proportion of stock affected and at what point the decision to harvest is made.

If, however, a 60.7 % level of infection remains a reflection of current infection levels and is applied across the Thai industry and an anticipated production for 2018 of 345 000 tonnes of *P. vannamei* is attained, then approximately 209 415 tonnes could be infected. If, however, it is assumed that 20 % of the value of this is lost as a consequence of undersized stock and a value of US$ 5 539 per tonne (see Table 4) is applied, then losses to the Thai economy, not including the additional consequential losses of feeding stock that have arrested growth, could be in the order of US$ 232 million per annum.

This estimate, however, requires substantiating through a structured survey and a comparative evaluation of industry production data between infected and uninfected sites. Elsewhere, an interview conducted with a farmer from India reported losses of ca. US$ 5 000 per ha, while another from Indonesia suggested that his EHP-related losses were US$ 7 538 ha^{-1}. The management and containment of EHP lies in strict biosecurity practices and regular disease testing.

The faeces from broodstock and/or larger shrimp can be screened for EHP spores either by molecular and/or by histochemical means, while batches of smaller-sized shrimp should be regularly screened by PCR at key steps in commercial production, e.g. on leaving the hatchery/nursery and before entering on-growing sites. Preventative measures against the acquisition and/or the establishment of EHP infections are discussed in Pakingking et al. (2016) and elsewhere in this volume.

International Movement of Live Stocks

The global aquaculture production of crustaceans for 2018 is estimated to be 8.63 million tonnes and includes 40 categories. Production for 2018 is forecasted from FAO FishStatJ data by applying an average 5.49 % year-on-year growth as seen for the period 2010–2015. This production is dominated by *P. vannamei,* which represents over 50 % of the volume produced, while *P. monodon,* ranking fourth behind Chinese mitten crabs, *Eriocheir sinensis* H. Milne-Edwards 1853, and red swamp crayfish, *Procambarus clarkii* (Girard 1852), represents less than 10 % of global production. *Penaeus vannamei* is currently produced in 35 territories (16 in Asia; 81.37 % by volume) based on countries providing returns to FAO in 2015 (FAO 2017) and is truly a pantropical species of major significance. Figure 8, which provides somewhat of a summary of its current distribution, maps some of the historical international movements linked to the culture of *P. vannamei*.

As key aquaculture species, i.e. those for human consumption and the ornamental trade, are exchanged globally, the inherent risks of pathogen transfer and introduction also increase with the number of translocation events and the volume of live species that are moved (Fig. 9). This is demonstrated by the global movement of Nile tilapia (*Oreochromis niloticus* Linnaeus 1758) and its parasite fauna into new territories (García-Vásquez et al. 2011).

There remains, therefore, concern regarding the movement of any shipment of non-native species into new environments and the rigour of biosecurity practices in detecting potential pathogen threats. Within the Sub-phylum Crustacea, crayfish are notorious invasive species (Ahjong and Yeo 2007; Gherardi and Acquistapace 2007). Worryingly, crayfish, e.g. *Procambarus* spp., are known to be hosts to chytrid fungus, *Batrachochytrium dendrobatidis*, a pathogen of global significance that is decimating amphibian populations (Crawford et al. 2010).

Their significance as potential vectors are evidenced by a recent survey of farmed and native populations of *Procambarus clarki* in Louisiana, United States of America where *B. dendrobatidis* was found at a low prevalence and intensity of infection that mirrored the seasonal patterns of infection seen within the local amphibian population (Brannelly et al. 2015). *Batrachochytrium dendrobatidis* has also been reported as an infection of the Malaysian giant freshwater prawn *Macrobrachium rosenbergii* (De Man 1879) (see Paulraj et al. 2016), however, this finding has been rejected after the results were found to be incomplete and inconsistent with previous descriptions of the pathogen (Pessier and Forzan 2017).

Fig. 8. International movement of *Penaeus vannamei*. The graphic summarizes the information listed in DIAS (2016) and is supplemented by additional reports from the literature. The information provided, however, is by no means complete and most likely captures less than 50 % of the movements that have taken place. The direction of the translocation is given, as is the natural range of *P. vannamei* – denoted by a red line.

Although there are no statistics on crayfish production for Thailand listed within the FAO FishStatJ database, there is an active aquaculture and ornamental industry. Two species have been introduced for aquaculture, namely *P. clarkii* (introduced from the United States of America ca. 1987, and the Australian red claw crayfish (*Cherax quadricarinatus* Von Martens 1868), which was brought in from Australia in 1995. Numerous other species of *Procambarus* and *Cambarellus* are commonly encountered on sale as ornamentals. Populations of *C. quadricarinatus*, for example, were introduced and raised in rice fields in Chiangmai Province as an initiative under a royal project (Srisaad and Thinkhaonoi 2015). Crayfish are now cultivated in several Thai provinces including Chiangmai, Chonburi, Khonkaen, Nakhon Ratchasima, Pathumthani, Prae, Srakaew and Supanburi. From these culture activities, however, wild populations of *C. quadricarinatus* have already established in Buriram, Chiangmai, Kanchanaburi, Sisaket and Sra Keaow provinces, while wild communities of *P. clarkii* are reported from the Kwaiyai and Kwainoy rivers in Kanchanaburi Province (Wanjit and Chaichana 2013). While FAO FishStatJ does not provide the details of which live species are being exported and imported, in general terms it would appear that the volume of live products being imported into Asia (see Fig. 9) is falling, while exports are rising. While this particular study does not enter into a discussion on the mechanisms and routes of pathogen introduction, the results presented in Fig. 9 highlight that there is still active traffic in the movement of live crustaceans and that vigilance and strict biosecurity measures at the regional, national and international levels (Galli et al. 2014a, b, 2015) must be upheld.

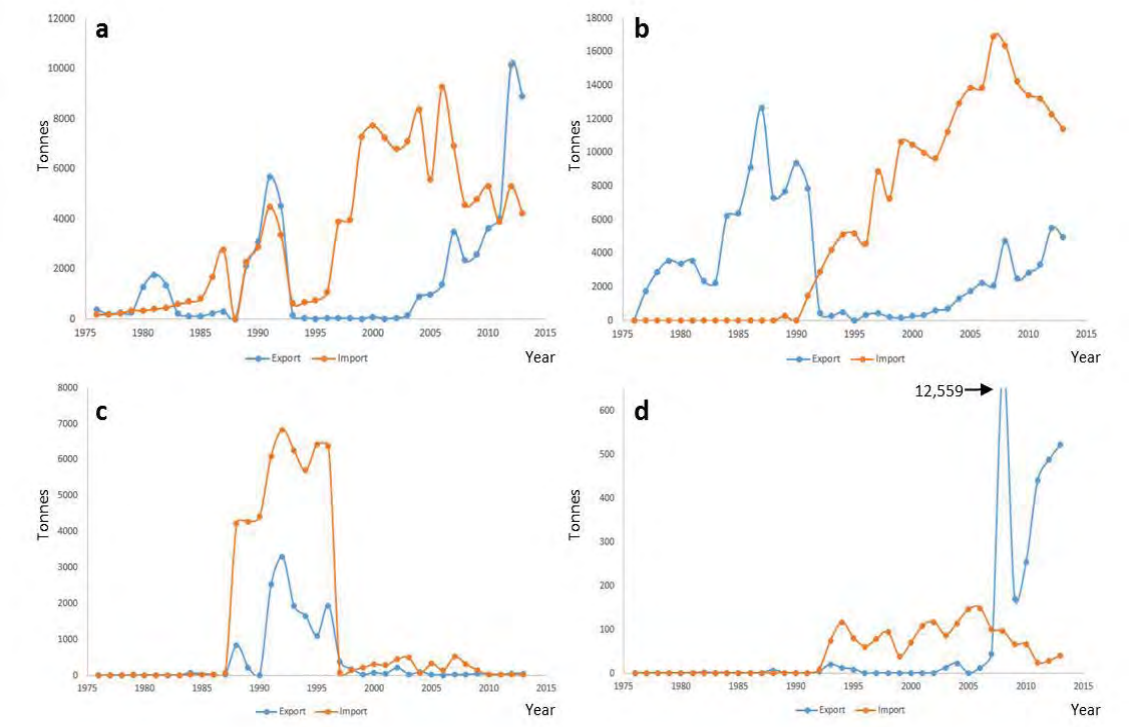


Fig. 9. The export-import trade of live crustaceans throughout Asia. (a) Trade in live prawns and shrimp; (b) trade in live crabs; (c) trade in live crustaceans for breeding etc.; and (d) trade in live crustaceans for human consumption. Data are drawn from FAO (2016).

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Annex 1

Some of the Disease Episodes that have been a Key Factor in Shaping Growth of the Shrimp Aquaculture Sector

Since 1981: Infectious hypodermal and haematopoietic necrosis virus (IHHNV) in the Americas (including the fishery in the Gulf of California) has cost the collective economies US$ 0.5–1 billion (Lightner et al. 2012). **1987–89:** Taiwanese *P. monodon* production crashes from 78 500 to 16 600 tonnes due to various factors including viral agents (Briggs et al. 2005). **1988:** *Penaeus monodon*-type baculovirus (MBV) infection of Malaysian *P. monodon* PL results in up to 100 % mortality (Yang et al. 2001); similar mass mortalities are reported in the Philippines (Albaladejo 2001). **1988:** MBV in Sri Lanka results in a 64 % drop in production (from 5.3 tonnes.ha^{-1} to 1.9 tonnes.ha^{-1}) with a US$ 6 million loss in foreign income (Siriwardena 2001).

1990s: Taura syndrome virus **(**TSV) in Latin America results in losses of US$ 1–1.3 billion in the first three years (Briggs et al. 2005). **1990–1991:** Yellow head virus (YHV) is reported as causing extensive losses of *P. monodon* in Thailand (Briggs et al. 2005). **Since 1991:** YHV in Asia has resulted in US$ 0.5 billion of loss (Lightner et al. 2012). **Early 1990s:** YHV and white-spot syndrome virus (WSSV) result in losses in Indonesia, the Philippines and Thailand (Briggs et al. 2005). **1991–92:** TSV in the Americas results in ca. US$ 1–2 billion (Lightner 2003; Lightner et al. 2012). **1992:** TSV results in 30 % drop in Ecuadorian production from 100 000 to 70 000 tonnes; losses are estimated at US$ 400 million (Lightner, 1996). **1992:** Southern Thailand reports significant losses to YHV (Briggs et al. 2005). **1992:** WSSV reported from farmed *P. japonicus* (Spence Bate 1888) in Japan using PL imported from the People's Republic of China (Nakano et al. 1994). **1992/3:** Losses due to WSSV in Asia put at >US$ 6 billion and at US$ 1–2 billion throughout the Americas in 1999 (Lightner et al. 2012). Total collective losses, however, are estimated at US$ 15 billion (i.e. US$ 0.8 billion per annum – 5 % of the total harvest value of US$ 16.7 billion in 2010) (Lightner et al. 2012). **1992–1993/4:** Chinese production falls from 207 000 to 64 000 tonnes due to WSSV (Briggs et al. 2005). **1993:** WSSV affects 85–90 % of the *P. chinensis*, *P. japonicus* and *P. monodon* culture area in the Chinese provinces of Wenzhou, Xiamen, Jiangsu and Shanghai and 70–80 % of that in Shangdong, Liaoning and Hebei; national production falls by 60 %, i.e. a loss of 123 000 tonnes valued at US$ 250 million. WSSV epizootic in the People's Republic of China affects 1 million people (Jiang 2001). **1993:** MBV, WSSV and YHV induced losses in Viet Nam put at US$ 100 million (Khoa et al. 2001). **1993:** TSV infection at two Peruvian sites receiving PL from Ecuador results in US$ 2.5 million loss (Talavera and Vargas 2001). **1993:** Necrotising hepatopancreatitis (NHP) affects two-thirds of Peruvian shrimp culture area resulting in a ca. 50 % loss of sales valued at US$ 20 million. Five farms (450 ha) close (Talavera and Vargas 2001). **1994:** WSSV and TSV losses in Thailand put at US$ 240 million per annum but estimate of annual loss in 1997 rises to US$ 650 million per annum (Chanratchakool et al. 2001). **1994:** WSSV causes Chinese shrimp production to fall to 53 000 tonnes (Jiang 2001).

1994: Rosenberry (1993, 1994) and Lundin (1996) calculate and summarize shrimp disease losses (tonnes × 1 000; US$ million) as: Bangladesh (5; 25); People's Republic of China (180; 900); Ecuador (34; 170); India (25; 125); Indonesia (50; 250); Mexico (1; 5); Philippines (57; 284); Thailand (130; 650); Taiwan POC (100; 500); United States of America (4.5; 60); and Viet Nam (10; 50). **1994 *et seq*.:** WSSV suggested to cost Asian production US$ 1 billion per annum (Briggs et al. 2005). **1994–1995:** Two outbreaks of WSSV and YHV throughout India result in loss of 10 000–12 000 tonnes; the second episode valued at US$ 17.6 million (Mohan and Basavarajappa 2001). **1994–1996:** TSV in Honduras causes shrimp survival to drop to 15 %, reducing production by 18 % in 1994, 31 % in 1995 and 25 % in 1996 (Corrales et al. 2001). Honduran losses are calculated as a loss of 1 943, 1 868 and 3 278 tons in the three years priced at US$ 6.61, 6.61 and 7.02 kg^{-1} which equates to losses of US$ 12.84, 12.35 and 23.01 million. Losses resulted in an 18 % cut in labour costs (Corrales et al. 2001). **1995:** Viral infections throughout Andra Pradesh and Tamil Nadu, India result in a US$ 64 million loss (Hugh-Jones 1995). **1994–1996:** 80 % of Malaysian farms are hit by WSSV (Yang et al. 2001). **1994–1996:** Widespread WSSV infection in Bangladesh in almost all semi-intensive farms in Khulna (37 400 ha) and Cox's Bazaar; losses are estimated at 3 400 tonnes, US$ 10 million and 500 jobs. PL imported from Taiwan POC and Thailand are implicated as the source of infection (Rahman 2001). **1994–1998:** Mid Crop Mortality Syndrome (MCMS) costs the Australian industry US$ 32.5 million (Walker 2001). **1995:** WSSV outbreak in Nicaragua results in 5–10 % survival of stock (Drazba 2001). Assuming a 10 % survival, 2 305 tonnes were produced in 1995 valued at US$ 15 213 000 (i.e. US$ 6.60 kg^{-1}. Loss is estimated at 20 745 tons valued at US$ 136.92 million. **1995–1999:** Malaysian losses to WSSV are US$ 25 million per annum (Yang et al. 2001). **1996:** TSV detected in Panama. Infection results in a 285 tonne (i.e. 30 %) decrease in production (Morales et al. 2001). Panamanian losses are calculated at US$ 1.85 million (i.e. US$ 6.50 kg^{-1}). **1996:** WSSV puts 90 % of Sri Lankan farming units out of production valued at US$ 18.5 million in foreign income (Siriwardena 2001). **1996:** Lundin (1996) suggests total disease-related losses are 540 000 tonnes valued at US$ 3 billion; i.e. 40 % of total tropical production per annum (Stentiford et al. 2012). **1998–1999:** WSSV and YHV in Sri Lanka reduce the area for production to 9.5 % (i.e. 264 ha), of which 55.5 % was infected (Siriwardena 2001). **1998–2000:** WSSV causes shrimp exports from Ecuador to fall from 115 000 to 38 000 tonnes (Cámara Nacional de Acuacultura; Briggs et al. 2005). Ecuadorian losses are 77 000 tonnes with an av. shrimp price of US$ 5.28 kg^{-1} (1998-2000) which represents a loss of US$ 406.56 million. **1999:** WSSV in Panama results in loss of 4 400 tonnes (40 % of production) of *P. vannamei*; export loss is ca. US$ 40 million (Morales et al. 2001). The direct losses are estimated at US$ 23.23 million based on US$ 5.28 kg^{-1}. Nauplii production falls to 45 % of that in 1998. Only 29 % (2 638 ha) of ponds in operation. Infection results in direct loss of 1 500 jobs and a further 3 500 in ancillary services (Morales et al. 2001). **1999:** WSSV in Ecuador in first year causes loss of 63 000 tonnes (42 % of production) of *P. vannamei* and *P. stylirostris* (Stimpson 1871) worth US$ 280 million (Alday de Graindorge and Griffith 2001). **1999:** In Latin America, TSV in 1993 and then WSSV from 1999 result in direct losses of ca. US$ 0.5 billion per annum (Briggs et al. 2005). Losses to WSSV throughout the Americas are estimated at US$ 1–2 billion (Lightner et al. 2012). **1999:** WSSV infections in Honduras result in a 13 % reduction in the workforce (Corrales et al. 2001). **1999:** WSSV results in closure of 87.5 % of Peruvian ponds (i.e. 2 800 of 3 200 ha) (Talavera and Vargas 2001). Survival in affected ponds drops to 6–52 %

(Talavera and Vargas 2001). Peruvian production of whiteleg shrimp fell from 6 080 tonnes in 1997 to just 614 tonnes in 2000. If the entire loss is attributed to WSSV, then losses were 2 618 tonnes in 1998, 1 768 tonnes in 1999 and 5 466 tonnes in 2000. Using prices of US$ 6.83, 9.57 and 7.32 kg^{-1} respectively for the three years, then losses were US$ 74.76 million. **1999:** Kunang kunang disease ("fireflies' disease") caused by *Vibrio harveyi* affects 70 % of Indonesian PL production with resultant losses of ca. US$ 8.75 million (Rukyani 2001). **1999:** Indonesian shrimp crop failures are estimated at US$ 300 million to date. Approximately 90 % of hatcheries are affected by *V. harveyi,* with losses estimated at US$ 100 million per annum (Rukyani 2001).

1999: Shrimp disease losses in India are valued at US$ 100 million (Mohan and Basavarajappa 2001). **1999:** TSV imported from Latin America causes mortalities in Taiwan POC (Tu et al. 1999; Yu and Song 2001). **Since 1999:** TSV infections throughout Asia are estimated at US$ 0.5–1.0 billion (Lightner et al. 2012). **1999–2003:** WSSV losses in Ecuador are ca. 267 000 tonnes valued at US$ 1.8 billion. Results in loss of 26 000 jobs (13 % of labour force), closure of 74 % of hatcheries, 68 % reduction in sales and production for feed mills and packaging plants and 64 % lay off at feed mills. Indirect losses result in the loss of 150 000 jobs in the sector (Alday de Graindorge and Griffith 2001).

2000s

2001–02: Monodon slow growth syndrome (MSGS) infections in Thailand result in a loss of *P. monodon* valued at US$ 400 million (this study). **2001:** The Global Aquaculture Alliance estimates yearly losses of 22 % due to shrimp disease (60 % due to viruses, 20 % due to bacterial infections), i.e. US$ 1 billion per annum. **2002:** Shrimp disease costs the Asian shrimp industry an estimated US$ 400 million in direct losses (Briggs et al. 2005). **2002:** WSSV outbreaks in Cambodia result in losses of ca. US$ 14.5 million per annum (Tana and Todd 2002). **2002–2006:** Infectious myonecrosis virus (IMNV) in Brazil results in a US$ 100–200 million loss (Lightner 2011). **2004:** Losses due to IMNV throughout the Americas are put at US$ 100–200 million (Lightner et al. 2012). **1990–2005:** Losses due to shrimp disease are estimated at US$ 15 billion (Flegel et al. 2008). **2006:** Losses due to IMNV in Indonesia are estimated at US$ 1 billion (Lightner et al. 2012). **2008:** Kuruma shrimp losses in Japan are estimated at US$ 8.8 million (53 % due to vibriosis; 31 % due to WSSV; 16 % due to fusariosis) (Yuasa et al. 2016). **2009:** Early mortality syndrome/acute hepatopancreatic necrosis disease (AHPND) results in an 80 % loss of production in the Chinese provinces of Fujian, Guangdong, Guangxi and Hainan (Leaño and Mohan 2012). **2010**: IMNV-associated losses in Brazil to date were expected to exceed US$ 1 billion (Lightner et al. 2012).

2010 onwards

2011: AHPND infections in Malaysian *P. vannamei* are estimated at US$ 100 million (FAO 2013). **2011:** Vietnamese Mekong Delta, unprecedented losses in 40 000 ha of *P. monodon* ponds. In Bac Lieu, over 11 000 ha used for shrimp culture are destroyed. The loss of 330 million shrimp in 6 200 ha of ponds in Tra Vinh is valued at US$ 0.6 million. Loss of ca. US$ 75 million worth of *P. monodon* stock in 20 000 out of 25 000 ha of ponds in Soc Trang Province,

Viet Nam is attributed to AHPND (Lyon et al. 2013). **1981–2012:** Estimate of disease losses in cultured shrimp is US$ 12–19 billion (Lightner et al. 2012). **2012:** Stentiford et al. (2012) estimate that the top five viral pathogens (i.e. IHHNV, IMNV, TSV, WSSV and YHV) result in an annual loss of 15 % of production valued at US$ 1.5 billion. **2011–2013**: Prevalence of AHPND in Malaysia throughout 2011–2013 was 50 %, 26 % and 73 % (Kua et al. 2016). **2011–2014:** Total production losses in Malaysia to AHPND are estimated at US$ 490 million (Kua et al. 2016). **2010–2016:** Shrimp disease in Thailand results in direct losses of ca. US$ 7.4 billion with a further US$ 4.2 billion in export losses (this study).

2010–2016: Collective losses due to AHPND and other shrimp diseases throughout the People's Republic of China, Malaysia, Mexico, Thailand and Viet Nam are estimated at US$ 23.6 billion (i.e. 4.8 million tonnes; this study) with a further loss of US$ 7 billion in feed sales (this study); export losses are estimated at US$ 13.4 billion (this study). Infection losses account for 40 % of the value of the total harvest in 2016, i.e. US$ 10.3 billion (this study). **2014:** Data for the Mekong Delta indicate that 8.72 % (i.e. 47 574 ha) and 32.48 % (i.e. 18 966 ha) of the area used for the culture for *P. monodon* and *P. vannamei*, respectively, were affected by episodes of disease (Directorate of Fisheries Viet Nam; this study). The production of *P. monodon* is 17 561 tonnes lower than in 2013, valued at US$ 8.78 million (this study). **2015:** Vietnamese Mekong Delta – Hien et al. (2016) estimate losses due to AHPND to be US$ 97.96 million and US$ 55.58 million for WSSV. **2015:** In the Vietnamese Mekong Delta, 5 875 ha used for *P. monodon* culture and 5 509 ha used for *P. vannamei* are recorded as being infected with AHPND (Hien et al. 2016). Losses are put at US$ 4.7 million and 21.3 million, respectively (this study). A further 3 447 ha used for *P. monodon* and 1 923 ha for *P. vannamei* experience episodes of WSSV (Hien et al. 2016). Losses are estimated at US$ 3.3 million and 7.8 million, respectively (this study). **2016:** Annual shrimp losses in Indonesia are estimated at US$ 298.4 million (US$ 191 million for WSSV; US$ 95.6 million for IMNV; US$ 7.6 million for vibriosis) (Hastuti and Haryadi 2016). **2016:** Losses due to *Enterocytozoon hepatopenaei* (EHP) in Thailand are not known but it is speculated that they could be as high as US$ 180 million per annum (this study). **1980–2016:** Collective losses due to shrimp disease to date are estimated at US$ 42 billion (direct losses), US$ 24 billion (export losses) and US$ 13 billion (feed sales) (this study). **2018:** EHP-related losses in Thailand could be as high as US$ 232 million per annum (this study). Indian state authorities report that 21% of farms in Andhra Pradesh are infected with EHP.

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Experience with Mortalities of Cultured Catfish *Ictalurus punctatus* (Rafinesque 1818) and *I. punctatus* X *I. furcatus* (Valenciennes 1840) caused by Highly Virulent Strains of *Aeromonas hydrophila*

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ABSTRACT

Epizootic outbreaks of motile aeromonad septicaemia (MAS) due to *Aeromonas hydrophila* infections in channel catfish *Ictalurus punctatus* (Rafinesque 1818) and hybrid catfish *I. punctatus x I. furcatus* (Valenciennes 1840) spread through West Alabama and East Mississippi, United States of America in 2009 and have been seasonally recurrent, with losses in the millions of pounds. While *A. hydrophila* is typically considered an opportunistic pathogen, no other primary aetiologic agent has been found. Mortalities are as high as 60 % in ponds and primarily affect larger fish, causing diseases characteristic of MAS infections that include severe skin ulceration and haemorrhage, generalized petechiation, ascites, marked splenomegaly with necrosis, and gastric haemorrhage. A multistate research group (Alabama, Arkansas, Louisiana and Mississippi) examined *A. hydrophila* isolates, which were found to be highly clonal by phylogenetic analyses of gene sequences (*atpD, dnaJ, dnaX, gyrA, gyrB, recA, rpoD*). A representative strain of the hypervirulent *A. hydrophila* pathotype (vAh), ML09-119, was found to be more virulent in channel catfish than historical *A. hydrophila* strains associated with traditional, opportunistic infection (tAh). Bar coded sequencing of numerous vAh and tAh isolates identified unique genomic regions associated with the epizootic isolates. A diagnostic polymerase chain reaction (PCR) targeting a unique vAh-associated genetic locus has been developed to differentiate vAh strains from tAh strains.

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Some of these genes were associated with *myo*-inositol metabolism, which corresponds with the ability of epizootic strains to utilize *myo*-inositol. Genes from lysogenic bacteriophage, O-antigen biosynthesis genes and transposases were also uniquely present in the epizootic strains. Collectively, these data support the conclusion that lateral gene transfer has contributed to the pathogenicity of epizootic *A. hydrophila* strains. Further research will need to be conducted to determine the specific contribution of the unique genetic loci to *A. hydrophila* virulence.

Keywords: catfish, motile *Aeromonas* septicaemia, United States of America, virulent *Aeromonas hydrophila*

Introduction

The role of horizontal genetic transfer, as it relates to enhanced bacterial virulence in aquaculture, is the focus of this contribution, which highlights the effect that one plasmid can have on an industry as well as trade worldwide (Lee et al. 2015). Mobile genetic elements, consisting of plasmids, insertion sequences, transposons, phages and integrons are ubiquitous in bacteria and figure prominently in horizontal gene transfer between bacteria. By enabling the acquisition of genes to promote survival in various environments, these mobile genetic elements have profound implications for human and animal health, where the ability of bacteria to acquire antimicrobial resistance and additional virulence factors leads to enhanced pathogenicity, disease and mortality. Such a situation has occurred internationally with *Vibrio parahaemolyticus* in farmed shrimp, as well as in *Aeromonas hydrophila* in farmed catfish in the United States of America and in farmed carp in the People's Republic of China (Lee et al. 2015; Rasmussen-Ivey et al. 2016b). The following is a brief review of our experience and understanding of this disease in the farmed catfish industry.

Currently, there are 47 recognized species of *Aeromonas* (Euzéby 1997). The members of this genus are ubiquitous in the environment, particularly in aquatic habitats, and thrive over a wide range of temperatures, pH and turbidities (Janda and Abbott 2010). Within *Aeromonas*, motile aeromonads are a group of loosely associated species (*A. hydrophila*, *A. sobria*, *A. veronii*, *A. caviae*, among others) that have similar biochemistry, genetics and serology (Cipriano and Austin 2011). This mesophilic group is typified by *A. hydrophila*, which has an optimal temperature range of 35–37 ^{o}C and is well known to cause disease in multiple species of fish. *Aeromonas hydrophila* was the first motile aeromonad species named; in addition to causing fish disease, this species can be associated with opportunistic infections in many vertebrates, including humans (Janda and Abbott 1998). Disease caused by *A. hydrophila* and other motile aeromonads is often referred to as motile *Aeromonas* septicaemia (MAS), which is characterized by widespread systemic infection (Grizzle and Kiryu 1993). The primary species responsible for MAS are *A. hydrophila*, *A. sobria* and *A. caviae* (Plumb 1999).

Natural infection in catfish has four forms: septicaemia, focal visceral lesions, cutaneous, and asymptomatic (Meyer 1975). Fish with MAS typically have marked external hyperaemia and haemorrhage, swollen abdomens and exophthalmia/ panophthalmitis that may progress to ocular rupture (Plumb 1999). Internally, there is generalized hyperaemia and petechiation, severe multifocal necrosis in the liver and kidney, renomegaly, splenomegaly, variable splenic necrosis, bloody ascites and flaccid intestines containing haemorrhage and yellow mucus (Plumb 1999; Cipriano et al. 2001).

Disease attributable to *A. hydrophila* has been reported in many fish species including channel catfish *Ictalurus punctatus* (Rafinesque 1818) (Miller and Chapman 1976; Grizzle and Kiryu 1993; Baumgartner et al. 2016), minnows and baitfish, common carp *Cyprinus carpio* (Linnaeus 1758), gizzard shad *Dorosoma cepedianum* (Lesueur 1818) (Rock and Nelson 1965), grass carp *Ctenopharyngodon idella* (Valenciennes 1844) (Deng et al. 2009), striped bass *Morone saxatilis* (Walbaum 1792), largemouth bass *Micropterus salmoides* (Lacépède 1802) (Miller and Chapman 1976) and tilapia (Abu-Elala et al. 2015). *Aeromonas hydrophila* is found abundantly in almost all freshwater environments including domestic tap water, sediment and sewage, as well as being part of the normal flora on the skin and in the intestines of fish (Hazen et al. 1978; MacMillan and Santucci 1990; Brandi et al. 1996). Therefore, the mere presence of *A. hydrophila* by itself is not indicative of poor environmental quality or impending disease outbreaks in fish populations.

Disease potential of the opportunistic, traditional *A. hydrophila* strains (tAh) is based on complex interactions between multiple biotic (host and bacterium) and abiotic (climate, water chemistry, etc.) factors (Janda 1991; Cipriano et al. 2001). *Aeromonas hydrophila* is considered to be an opportunistic or secondary pathogen, where pre-existing diseases, weakened immune systems, injury, crowding or poor water quality (e.g. low oxygen, high ammonia or extreme temperatures) provide the bacterium an opportunity for tissue infection (Miller and Chapman 1976; Walters and Plumb 1980; Plumb 1999). In culture ponds, where environmental stressors abound, it is common to find more than one pathogen in a single moribund fish. For example, catfish with motile aeromonad infections are often co-infected with *Flavobacterium columnare* or *Edwardsiella ictaluri*, giving credence to the idea that motile aeromonads are opportunistic (Rock and Nelson 1965; Hawke and Thune 1992). In catfish pond disease outbreaks, it is uncommon to find MAS without the presence of another significant bacterial, fungal or parasitic pathogen (W. Baumgartner, personal observation). Despite the historical assignation as an opportunistic pathogen, *A. hydrophila* can indeed act as a primary pathogen. Within the hypervirulent *A. hydrophila* pathotype (vAh), strain J-1 (categorized as sequence type 251 [ST251]) was first reported in 1989 in association with epizootic outbreaks in Chinese carp (Chen and Lu 1991). Since then, vAh strains (NJ-35, ZC1) have been recognized as the causative agents of severe MAS outbreaks (MASv) in farmed grass carp in the People's Republic China, resulting in losses exceeding US$74 million annually (Deng et al. 2009; Rasmussen-Ivey et al. 2016b).

Aeromonas hydrophila reference isolates (vAh and tAh) are equipped with an arsenal of virulence factors, which cumulatively contribute to disease; they include secretion systems (type II, and some have a complete type VI), biofilm formation, flagella, haemolysins, O-antigens, S-layers, collagenase, elastase, lipase, metalloprotease and serine protease, among others (Rasmussen-Ivey et al. 2016a). Furthermore, the genus *Aeromonas* has a complex array of mobile genetic elements with the ability to transfer resistance and virulence genes between strains and different species (Piotrowska and Popowska 2015).

Since 2009, pond outbreaks caused by an emergent clade of vAh have resulted in the loss of more than 20 million pounds of market-size farmed catfish (*Ictalurus punctatus* and *I. punctatus* x *I. furcatus*) in Alabama and in Mississippi. Outbreaks of MASv began in West Alabama in April 2009 and continued through September. That year, MASv was documented on at least 48 farms with an estimated loss of 3 184 000 lbs of catfish. In the spring and summer of 2010, the disease re-emerged and spread to at least 60 farms (including the 48 affected in 2009), with estimated losses of 2 400 000 lbs of catfish. Data from subsequent years are similar, with losses of over 2 million lbs of catfish per year (W. Hemstreet, Alabama Fish Farming Center, personal communication). Contaminated fish, water and seining/hauling equipment are likely sources for spreading this disease.

This disease has continued to spread through West Alabama farms and is now the most commonly diagnosed pathogen at the Alabama Fish Farming Center (diagnosed in 35 % of case submissions) (Hemstreet 2015). In East Mississippi, the same trend has occurred; MASv was the most commonly diagnosed disease from 2012 to 2015, and it was diagnosed in 35 % of case submissions in 2015. From 2013 to 2015, MASv cases have also occurred on catfish farms in the Mississippi delta. In 2015, at least five West Mississippi catfish operations and two Arkansas operations reported having MASv outbreaks. In affected farms, MASv was characterized by an acute onset of anorexia followed by high mortality rates of up to 50 to 60 %, predominantly in the large market-size fish. Affected fish had clinical signs typically seen in MAS caused by other aeromonad strains and species, with dermal haemorrhage and ulcers, and multi-organ necrosis.

Because *A. hydrophila* was initially considered a secondary pathogen, efforts focused on the identification of an underlying primary pathogen or condition. Through cooperative efforts (Auburn University; Mississippi State University, College of Veterinary Medicine in east Mississippi (MSU-CVM) and Thad Cochran National Warmwater Aquaculture Center in the Mississippi delta, University of Arkansas-Pine Bluff, Louisiana State University, and the United States Department of Agriculture Agricultural Research Service, (USDA ARS)), underlying primary bacterial disease agents (including known pathogens such as *E. ictaluri*, anaerobic bacteria or obligate intracellular bacteria), viruses, parasites, environmental conditions, genetic factors and other known causes of disease were ruled out.

At the same time, research was conducted to determine if the *A. hydrophila* isolates represented an emerging primary pathogen of catfish. The MASv isolates from channel catfish ponds had been previously typed as *A. hydrophila* based on biochemistry (API 20E assay) and 16S rRNA gene sequencing (100 % identity to known *A. hydrophila* strains). Interestingly, phenotypic characterizations by API 20E assay indicated that vAh strains had a unique biochemical profile within *A. hydrophila*, in that they ferment inositol. Based on phylogenetic analysis of the DNA gyrase B-subunit gene (*gyrB*) (Yanez et al. 2003), virulent strains of *A. hydrophila* were grouped together as a single clade that could be distinguished from all other *A. hydrophila*.

Aeromonas hydrophila Genomics

The 4.7 Mb genome of *A. hydrophila* type strain ATCC 7966 reveals considerable metabolic versatility, reflecting its ability to cause disease in multiple hosts and persist in aquatic environments (Seshadri et al. 2006). It appears able to use a large number of substrates for growth and is capable of inactivating a large number of toxic compounds. A large number of potential virulence genes were detected, several of which encoded secreted proteins. A functional repeat in toxin (Rtx) with an actin cross-linking domain was also found (Suarez et al. 2012). Pan-genome analysis of *Aeromonas* species revealed higher pathogenic potential and antimicrobial resistance in *A. hydrophila* compared to *A. veronii* and *A. caviae* (Ghatak et al. 2016). Recently the genomes of many *A. hydrophila* isolates, including vAh strains, have been completed (Tekedar et al. 2013, 2015; Pridgeon et al. 2014a,b; Pang et al. 2015; Rasmussen-Ivey et al. 2016b; Yang et al. 2016), which include AL06-06 from goldfish, virulent Chinese carp strain JBN2301, vAh strain AL09-71 and catfish pond isolates pc104A and ML90-119^{T} (type strain for initial studies).

Analysis of genome sequences from ML09-119 and other vAh strains, including those isolated from Asian carp, revealed that the vAh strains are highly similar whereas the tAh genomes were highly variable and lacked many of the gene sequences present in the vAh strains (Hossain et al. 2014; Rasmussen-Ivey et al. 2016b). This result was supported from a concatenated phylogeny based on seven evolutionarily conserved gene sequences (Hossain et al. 2014) and from a core genome phylogenetic analysis of *Aeromonas* spp. (Rasmussen-Ivey et al. 2016b), which demonstrated the highly clonal nature of these recently emerged *A. hydrophila* isolates. Interestingly, many vAh isolates obtained recently from MASv outbreaks in Mississippi are more closely affiliated with Asian carp isolates compared to vAh strains isolated from MASv outbreaks in Alabama (Rasmussen-Ivey et al. 2016b). It is possible that vAh emerged from a common *A. hydrophila* ancestor carried by grass carp or other carp species, which have been used in aquaculture ponds in the United States of America for weed control since the 1960s (Hossain et al. 2014).

Analysis of the vAh-associated unique genetic regions revealed genes that are present in vAh and absent from tAh strains, including genes located within predicted genomic islands, suggesting their acquisition through lateral gene transfer (Fig. 1 and 2) These vAh-associated genes are predicted to be involved in many functions, including *myo*-inositol catabolism, prophage structure and regulation, transposases and other genes with low percent similarity to known *A. hydrophila* gene sequences.

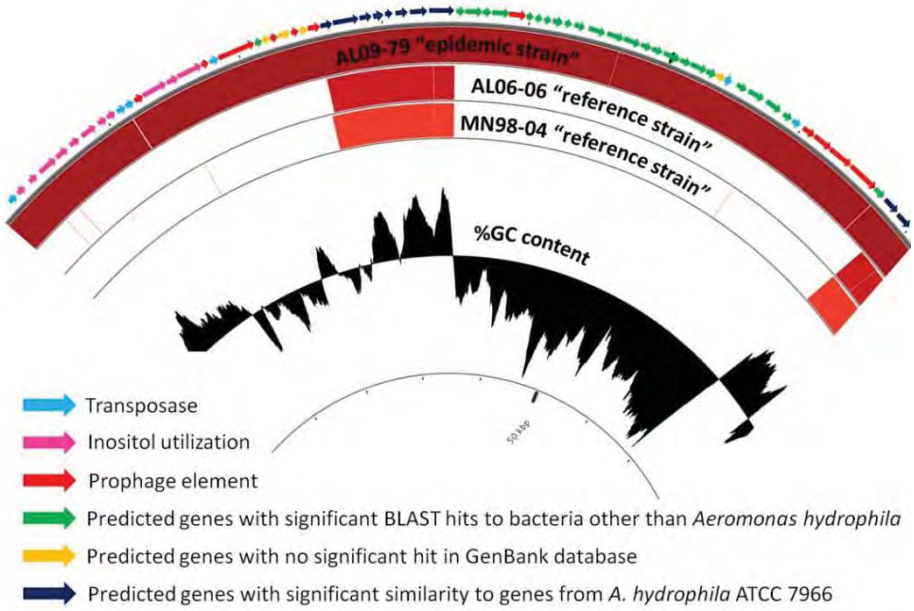

Fig. 1. Comparative genomic analysis of a contig from vAh strain ML09-119 against vAh strain AL09-79 and two tAh strains (inner rings) along with percent GC content. Graph represents BLASTn comparison using CGView (Grant and Stothard 2008).

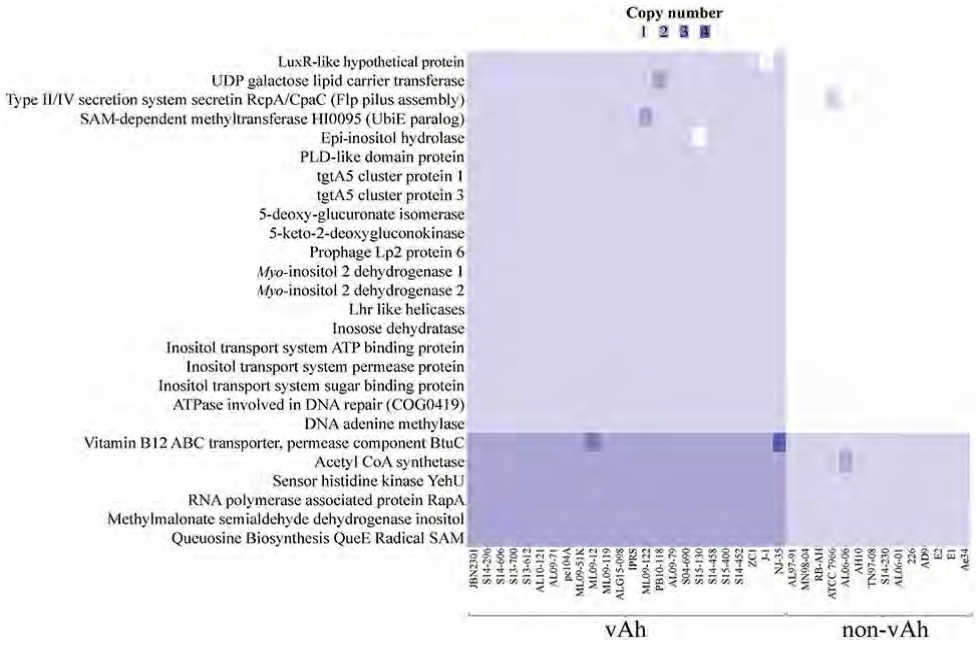

Fig. 2. Comparative whole genome predicted gene-based analysis of all confirmed vAh (n=26) and tAh isolates (n=15).[1]

[1]Source: Rasmussen-Ivey et al. (2016b), used under Creative Commons Attribution License (CC BY).

One of the important genotypic differences in the vAh isolates concerns the organization of the type six secretion system (T6SS) operon. Chinese carp isolates (J-1, NJ-35, ZC1) contain a majority of the proteins necessary for a functional T6SS, while Alabama and Mississippi vAh isolates form a subclade where the majority of core proteins (9 of 13) are lacking (Rasmussen-Ivey et al. 2016b). Immersion challenge data in catfish (see following) found that Asian strain ZC1 was less virulent than American catfish isolates, a finding that seems counter-intuitive given the assumption that T6SS should engender greater pathogenicity. However, the functionality of the T6SS in catfish vAh isolates is currently unknown; the reduced T6SS may somehow allow enhanced evasion of the fish immune system (Rasmussen-Ivey et al. 2016b) (Fig. 3).

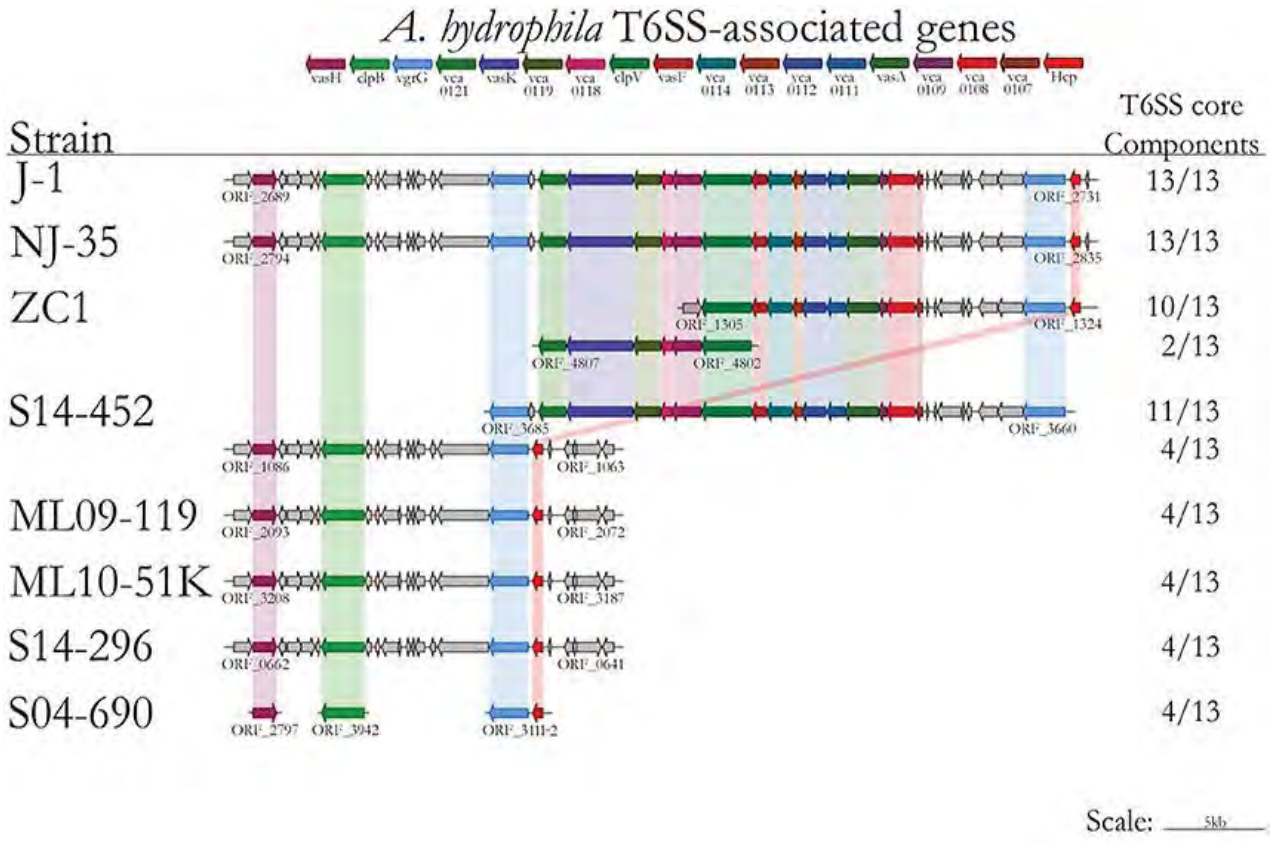

Fig. 3. Type VI secretion system gene prediction using T346 Secretion System Hunter, with results including strains included in the immersion catfish challenge (ML09-119, MNL10-51K, S04-690, S14-296, S14-452 and ZC1) and representatives from Chinese strains (J-1, NJ-35 and ZC1).[1]

[1]Source: Rasmussen-Ivey et al. (2016b), used under Creative Commons Attribution License (CC BY).

Myo-Inositol Catabolism

Phenotypic experiments showed that vAh isolates are capable of growth in a M9 minimal medium containing inositol (M9I) as the sole carbon source (Hossain et al. 2013). Growth of vAh strains on M9I has been consistently observed for all strains isolated from diseased fish in ponds experiencing a vAh epizootic (n>129). By contrast, almost all *A. hydrophila* isolates taken from pond water, pond sediment or from fish in a processing plant (n=31) were not able to grow on M9I.

Furthermore, strains that were identified as positive on the M9I medium were also tested positive as vAh strains based on the vAh-specific quantitative polymerase chain reaction (qPCR) assay. The ability of vAh isolates to use *myo*-inositol as a sole carbon source may reflect pathogen adaptation to the catfish host. In 1989, it was discovered that catfish do not require *myo*-inositol as a dietary additive because catfish are capable of *de novo* synthesis of *myo*-inositol with high endogenous levels of *myo*-inositol in tissues (Burtle and Lovell 1989). However, the significance of *myo*-inositol catabolism as it pertains to virulence remains uncertain.

Virulent A. hydrophila Strains Have a Unique O-Antigen

The vAh strain ML09-119 and other sequenced catfish vAh strains from the United States of America contain a unique 26.5 kb O-antigen biosynthesis gene cluster that ATCC 7966 and Asian carp vAh isolates lack (Pang et al. 2015). There are 25 total predicted genes within the United States catfish cluster that are all organized in the same transcriptional orientation, which suggests that this is an O-antigen biosynthesis operon. The O-antigen biosynthesis gene clusters of other sequenced *A. hydrophila* strains vary in the number of genes in this cluster (22 in the Asian carp cluster and 34 in ATCC 7966), as well as in the relative organization of the genes and in the gene sequences.

There are 96 recognized serogroups within the motile mesophilic *Aeromonas* species (Thomas et al. 1990). Given the unique O-antigen biosynthesis operon in vAh, it is likely that this region has been introduced into vAh via lateral gene transfer, and that these isolates comprise a unique serotype. O-antigen contributes to the virulence of other *A. hydrophila* strains by increasing adhesion to host cells (Merino et al. 1996a, b); however, the contribution of the vAh O-antigen towards virulence needs to be investigated. In summary, our analysis of available genome sequence data strongly supports that Asian and American vAh strains share a common ancestor and that these vAh strains share many genotypic and phenotypic characteristics (e.g. use of *myo*-inositol as a sole carbon source). In addition, these data strongly suggest that lateral gene transfer has contributed to the pathogenicity of vAh strains.

Virulent A. hydrophila Strain-Specific Primer Design and Evaluation

The initial set of consensus sequences present in catfish-associated vAh strains was used to develop primer sets that were specific to these strains and be useful for diagnostic and epizootilogical studies. From the collective results of these studies, three primer sets were selected as providing the best specificity for known vAh strains while maintaining robust PCR conditions. The most promising primers (2986L and 2986R) were refined to provide better results in real-time PCR assays (Griffin et al. 2013). This has proven more useful and specific than the previous methods that were based on sequencing a portion of the *gyrB* gene. The assay is repeatable and reproducible with a linear dynamic range covering eight orders of magnitude and a sensitivity of approximately seven copies of target DNA in a 15 µl reaction.

In addition, the assay was able to detect and quantify the epizootic strain from catfish tissues (0.025 g), pond water (40 ml) and pond sediments (0.25 g) with a sensitivity limit of approximately 100 bacteria in a sample. Recent examination of 26 vAh isolates (now including Asian strains) found that many would not produce an amplicon. Touchdown PCR was performed on all isolates, and vAh specific primers were developed not only to differentiate vAh from tAh (targeting a serine protease), but to discriminate different vAh lineages (Table 1). A new vAh specific primer set (vAh-SerF and vAh-SerFR) has now been developed to include Asian and American isolates, while excluding non-vAh isolates (Rasmussen-Ivey et al. 2016b).

Table 1. Oligonucleotide primers specific to members of the vAh pathotype (vAh-SerF and vAh-SerR), previously described qPCR vAh primers (2986F and 2986R) and primers used to screen for unique isolates used in this study (ML09-119F, ML09-119R, S14-452F, S14-452R, ZC1F and ZC1R).

Primer name	Direction	Sequence	Amplicon size (bp)
vAh-SerF	Forward	5′-AG′CATCACCAGCGTTGGCCC-3′	502
vAh-SerR	Reverse	5′-GCCGGGCTGAACTTCCGCAT-3′	
2986F	Forward	5′-CTATTACTGCCCCCTCGTTC-3′	167
2986R	Reverse	5′-ATTGAGCGGTATGCTGTCG-3′	
ML09-119F	Forward	5′-GTTCCGTTCCATCTGTTCGTGA-3′	246
ML09-119R	Reverse	5′-CAACCATCTTGGTCGCAATC-3′	
S14-452F	Forward	5′-CAGAACGTGCTGCAGAGATTGA-3′	350
S14-452R	Reverse	5′-TCCGAGAATTCGATGACGAAGG-3′	
ZC1F	Forward	5′-GCAATTCTGCGGTCACTTCTCG-3′	400
ZC1R	Reverse	5′-AGCGTACCGTCTCGTCGATATG-3′	

[1]Source: Rasmussen-Ivey et al. (2016b), used under Creative Commons Attribution License (CC BY).

Pathology of MASV

Natural Pond Outbreaks (Case Material from East Mississippi)

Catfish pond outbreaks were most often seen when temperatures were in the 30 to 32 ^{o}C range, i.e. in the summer months. Both channel and hybrid catfish ponds were affected equally. Externally, MASv pond catfish exhibited signs typical of motile aeromonad infection, with petechiation, periocular cellulitis, exophthalmia, panophthalmitis (Fig. 4A) progressing to ocular rupture, and endomeningitis with cellulitis overlying the brain (hole-in-the-head lesions, Figure 4A), as well as ulceration of the skin (particularly around the anus) and fins (Fig. 4B), (Baumgartner et al. 2016). Skeletal muscle was characteristically deep red throughout (Fig. 4C). Similar findings were seen in vAh challenge models (Rasmussen-Ivey et al. 2016b) (Fig. 5).

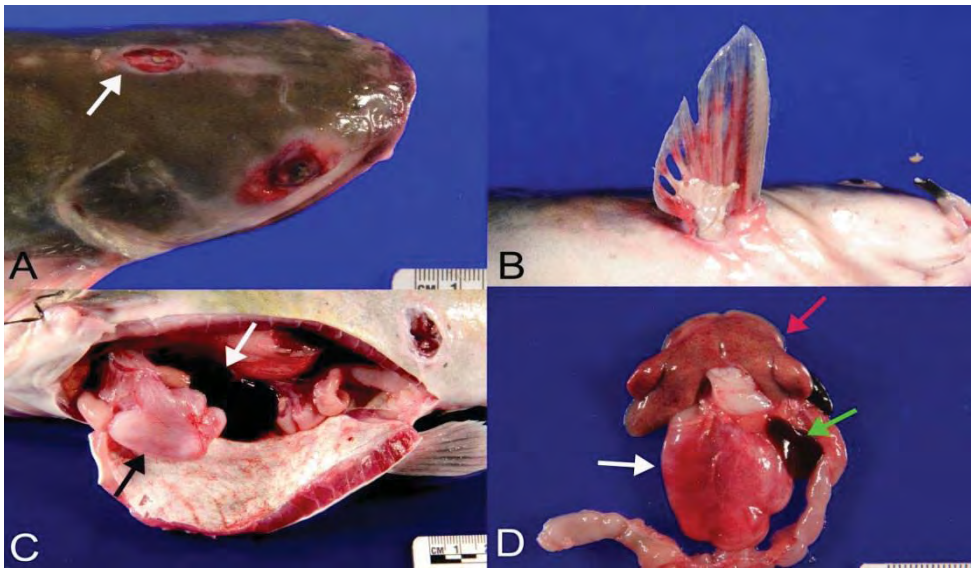

Fig. 4. Channel/hybrid Mississippi pond catfish naturally infected with virulent *Aeromonas hydrophila*. (A): The eye and surrounding soft tissues are swollen, haemorrhagic and ulcerated. The soft tissues overlying the brain are ulcerated with tan necrotic material (white arrow). (B): The right pectoral find exhibits severe necrosis of the skin and soft tissues between the fin rays. (C): Within the abdomen, tissues are hyperaemic and petechiated, with scant haemorrhagic fluid. Splenomegaly (white arrow) and gastric edema with petechiation (black arrow) are evident. The musculature is haemorrhagic and a deep ulcer is present at the right. (D): Viscera. The stomach (white arrow) is flabby with marked haemorrhage in the wall. The spleen (green arrow) is mildly enlarged and the liver (pink arrow) is petechiated with an enhanced reticular pattern. The intestine is hyperaemic.

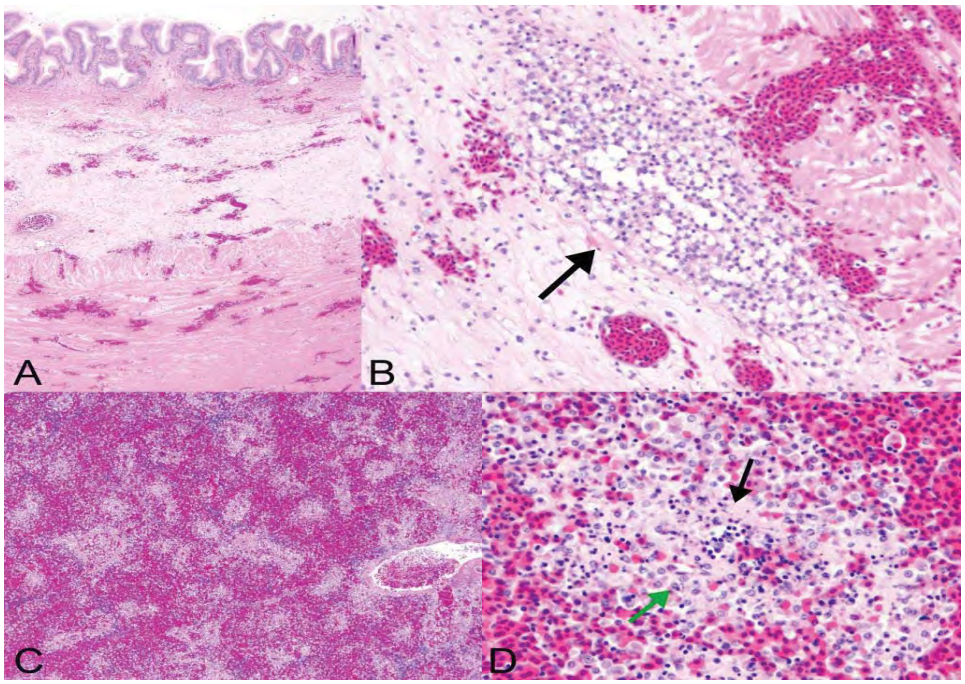

Fig. 5. Channel/hybrid Mississippi pond catfish naturally infected with virulent *Aeromonas hydrophila*. Photomicrographs, haematoxylin and eosin stain. (A): Spleen. Ellipsoid sheaths are enlarged (pink areas) and the red pulp is expanded by myriad erythrocytes. 40x. (B): Spleen. An ellipsoid sheath wall (black arrow) is expanded and obscured by pink fluid, fibrin, pyknotic cell debris and macrophages (green arrow). 400x. (C): Stomach, mucosa at the top, muscularis at the bottom. Multifocal haemorrhage spans the lamina propria, submucosa and muscularis. Edema expands the submucosa, with evenly dispersed leucocytes. 40x. D: Stomach, submucosa on the left, muscularis at the right. Edema and haemorrhage expand the submucosa. A dilated lymphatic (black arrow) is effaced by macrophages and karyorrhectic cells. 200x.

Internal tissues were hyperaemic and petechiated, with small amounts of haemorrhagic fluid in the coelom (Fig. 4C). Large bloody spleens were characteristic, occasionally with large infarctions. Microscopically, splenic ellipsoids exhibited severe necrosis with karyorrhectic debris, very few bacteria and variable numbers of large macrophages peripherally (Fig. 5A, B). The red pulp was markedly expanded by erythrocytes in the majority of cases. Thrombosis of large blood vessels was not uncommon. In cases where splenic infarctions had progressed beyond the early stages, the rarefied, friable tissues were filled with bacteria; aeromonads are facultatively anaerobic, and thus may thrive in devitalized tissue. Also, stomachs were often edematous with petechiation or mural haemorrhage in a paintbrush pattern (Fig. 4D). Microscopically, changes were largely confined to the submucosa and muscularis, where edema and acute haemorrhage were abundant (Fig. 5C). Often, lymphatics were filled with macrophages, neutrophils, karyorrhectic debris and small numbers of bacteria. Frequently, lymphatic walls were obscured by inflammation (lymphangitis and gastritis, Fig. 5D). The mucosa exhibited mild to moderate gland atrophy/apoptosis, with occasional erosion and inflammation. Changes in the spleen and stomach were often some of the first to appear grossly, occasionally prior to external signs of disease. The intestines often had mild, occasionally moderate, random ecchymosis. Microscopically, haemorrhage was common in the lamina propria and submucosa, with variable epithelial necrosis and inflammation. Livers had scant (Fig. 5D) to abundant petechiation but often lacked appreciable inflammation, and in general had only mild hepatocellular necrosis. Perivascular hepatic pancreatic tissue frequently exhibited mild to moderate necrosis, which can be seen in bacterial sepsis from various causes. Renomegaly was inconsistent; in severely diseased fish (possibly in a later stage of infection), mild granulomatous inflammation and haematopoietic hyperplasia were seen, particularly in the pronephros (head kidney). Mild random acute haemorrhage was often seen in the brain (Baumgartner et al. 2016).

The pond outbreak descriptions are taken from case materials at the MSU-CVM aquatic diagnostic laboratory in Starkville, Mississippi; descriptions of MASv from Alabama, Arkansas or the Mississippi delta (Stoneville, Mississippi) catfish may be different, depending on the infectious strain in those areas. There are some differences in pond outbreak lesions versus experimental disease; the gastritis, remarkably severe splenic necrosis and relatively mild involvement of the liver and kidneys in the case materials are interesting and may be significant. The natural route of infection for MAS and MASv is not well understood; skin, gill and gastrointestinal tract are all possible routes, and all may potentially contribute to disease in a pond outbreak. The stomach lesions seen in natural MASv outbreaks are not typical of Gram-negative septicaemia in catfish, including aeromonad infections; the significance of this finding is uncertain. The pathology in the stomach is largely that of vasculitis in the submucosa and muscular tunics. There are various possible explanations for this, some of which include: the stomach may be an early entry point for the organism, other disease mechanisms may cause an accumulation of bacteria/toxin-laden fluids into the stomach tissues, or that ligands for the bacteria are present within the stomach. Aeromonad bacteria have numerous virulence factors, and can produce a toxaemia in fish that gives rise to multi-organ necrosis.

When considering the amount of organ damage, extent of haemorrhage and relatively few bacteria seen microscopically in catfish cases, it seems probable that toxaemic tissue damage plays a large role in the progression of disease. Further understanding of the additional virulence factors acquired by vAh strains may, in time, explain these changes.

Experimental Challenge

Virulent MAS strain ML09-119 demonstrated high mortality (greater than 90 %) within 24 hr in channel catfish after intraperitoneal injection of 10^5 colony forming units (cfu), in contrast to lower mortality observed by tAh strain AL06-06, which had 10 % mortality after 1week post-intraperitoneal injection of an equivalent bacterial inoculum (Fig. 6). In contrast, much lower mortality was observed for ML09-119 in grass carp (Hossain et al. 2014).

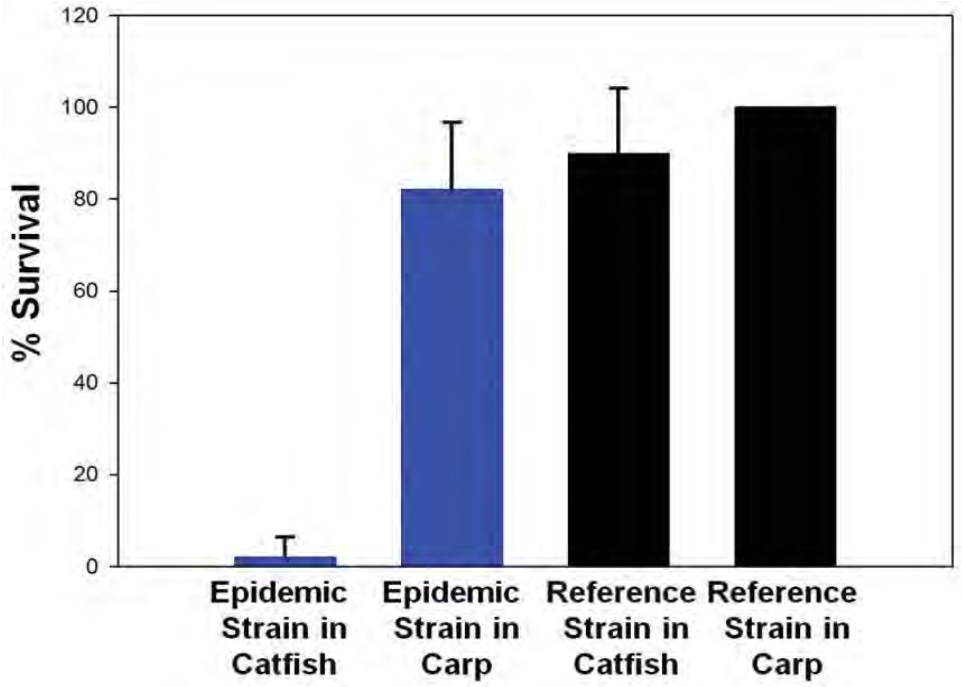

Fig. 6. Percent survival of channel catfish or grass carp after interperitoneal injection of approximately 10^5 cfu of the vAh (epizootic) strain ML09-119 or reference strain AL06-06 (n=5 tanks containing 20 fish per treatment group).

Immersion challenges in catfish infected with Asian and United States isolates using a fin-clip model found that the grass carp isolate (ZC1) was significantly less virulent in catfish (Fig. 7), with only 26.7 % mortality versus that in United States isolates, which was greater than 60 % in 48 hr. Necropsy of these challenges found large haemorrhagic spleens with prominent ellipsoid necrosis, similar to that in pond outbreaks. However, unlike the pond cases, experimentally diseased fish had prominent liver and kidney changes, relatively mild intestinal disease and no stomach pathology; this is typical of descriptions of the pathology of *A. hydrophila* in the literature (Ventura and Grizzle 1987, 1988; Rasmussen-Ivey et al. 2016b).

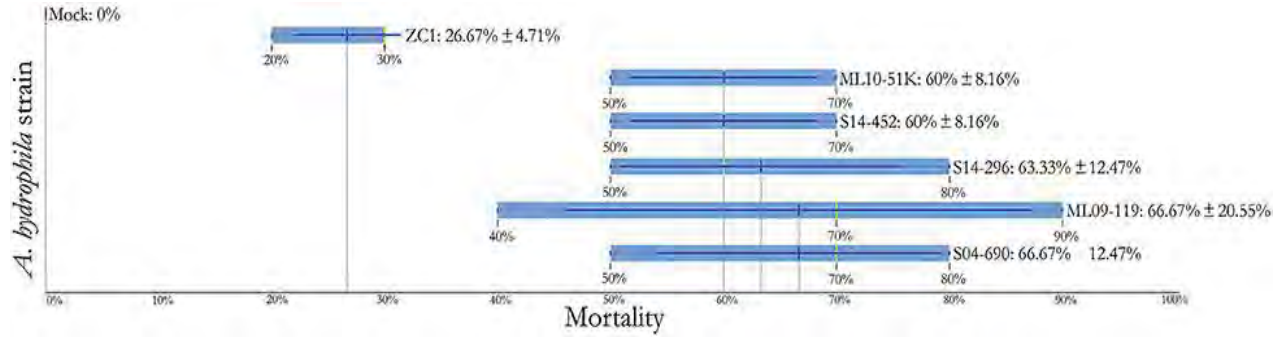

Fig. 7. Comparative assessment of the relative virulence of vAh isolates (ZC1 from Chinese grass carp, all others from American channel catfish) in channel catfish using 1 hr immersion exposure with fin clip (ANOVA = 7.628, p-value = 0.001).[1]

[1]Source: Rasmussen-Ivey et al. (2016b), used under Creative Commons Attribution License (CC BY).

Conclusion

In the last few years, a hypervirulent pathotype of *A. hydrophila* has emerged in the United States catfish industry, with severe consequences. Evidence indicates that catfish isolates share a recent common ancestor with Asian carp strains, giving this pathotype an international distribution associated with economically important disease (Fig. 8). This pathotype has unique metabolic activities, an expanded suite of virulence factors, and characteristic lesions that distinguish it from traditional *A. hydrophila* strains.

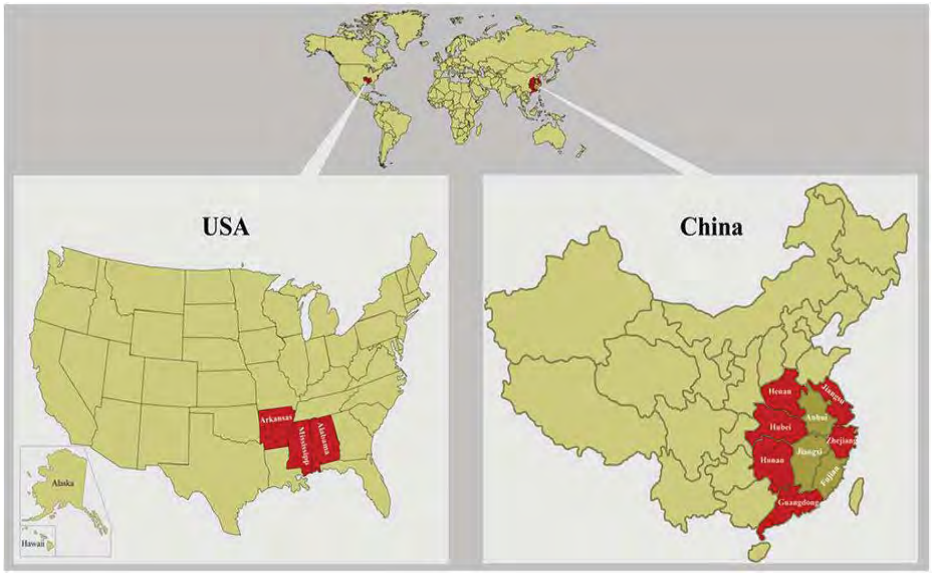

Fig. 8. Geographical distribution of the ST251 clonal group *Aeromonas hydrophila*. The regions filled in with red represent the distribution of the ST251 clonal group.[1]

[1]Source: This map was modified based on the maps obtained from PowerPoint Toolkit (http://ppt-toolkit.com/); Pang et al. (2015), used under Creative Commons Attribution 4.0 International License.

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Shrimp Bacterial Infections in Latin America: A Review

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Abstract

Shrimp aquaculture is an important industry that experiences significant losses from *Vibrio* species and intracellular bacteria, especially at the larval and juvenile stages. This review, which covers the period from 2000 to 2015, summarizes the bacterial diseases in farmed shrimp in Latin America based on information obtained for 12 countries with semi-intensive and intensive farming systems. The presence of five diseases with variable prevalence was determined. The most prevalent disease was septic hepatopancreatic necrosis (SHPN) or "vibriosis", caused by *Vibrio harveyi, V. parahaemolyticus, V. alginolyticus* and other species; followed by necrotising hepatopancreatitis (NHP), with an intracellular bacterium as the etiological agent; and then by three emerging diseases, streptococcosis, acute hepatopancreatic necrosis disease (AHPND) and spiroplasmosis.

Keywords: acute hepatopancreatic necrosis disease, bacterial diseases, Latin America, necrotising hepatopancreatitis, *Penaeus vannamei,* septic hepatopancreatic necrosis, spiroplasmosis, steptococcosis, vibriosis

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Introduction

Shrimp farming in Latin American countries contributes about 15 % of world production, with Ecuador, Mexico, Honduras, Nicaragua and Brazil as the countries with the largest productions and having about 180 000, 65 000, 27 397, 23 980 and 20 000 ha of shrimp farming ponds, respectively. Production is destined for export, mainly to markets in the United States of America, Europe and Japan. Although Latin American shrimp production has been increasing, the last decade has been characterized by high prevalence of viral and bacterial diseases in farmed shrimp, probably due to fluctuations in temperature, salinity, oxygen, pH and water nutrients (Lightner 1993). Environmental factors and confinement are two important triggers for the rapid multiplication of opportunistic bacteria, located in the digestive tract, gills and cuticle of shrimp and in the water, feed and pond sediment (Jayabalan et al. 1982; Cuéllar-Anjel et al. 1998). The exoskeleton provides an effective physical barrier against pathogens that try to penetrate the outer surface of crustaceans, while the intestine is another defense barrier to keep out pathogens. However, some chitinoclastic (or chitinolytic) bacteria of the genus *Vibrio* are associated with exoskeleton disease and can also enter through wounds (Álvarez et al. 2000; Barrientos 2010). The gills appear to be susceptible to bacterial penetration because they are covered by thin exoskeleton; however, they are naturally cleaned by the permanent movement of water through the gill chambers and by frequent molting. The midgut and hepatopancreas are not lined by exoskeleton and therefore seem to be likely sites for pathogen penetration via water, feed and sediment. Bacterial diseases reported in shrimp farming are mainly caused by *Vibrio harveyi, V. parahaemolyticus, V. alginolyticus, V. nigripulchritudo, V. campbellii, Pseudomonas* spp., *Aeromonas* spp., *Micrococcus* spp., *Candidatus Hepatobacter penaei* and *Streptococcus* spp. Thus, the aim of this study is to compile the available information on bacterial diseases affecting shrimp-producing countries in Latin America during the past 15 years (Varela 2013; SIVE 2014; Marroquín 2015). A general review conducted to obtain information from 12 Latin American countries with semi-intensive and intensive shrimp farming systems determined the presence of five diseases with variable prevalence. The following are the main bacterial diseases reported, along with their distribution and prevalence in Latin America and their clinical signs.

Previously Known Bacterial Diseases

Septic Hepatopancreatic Necrosis (SHPN), Vibriosis, Systemic Vibriosis or "Seagull Syndrome"

This shrimp disease was reported in all 12 Latin American countries during 2000 to 2015. The average prevalence was 45.5 % (Table 1), with infections being most prevalent in Mexico and Costa Rica (80 %). SHPN has been reported in hatcheries (Lightner and Redman 1994; Cuéllar-Anjel et al. 2000) and grow-out shrimp farms. The main *Vibrio* spp. isolated from shrimp with SHPN were *V. harveyi, V. parahaemolyticus, V. alginolyticus* and *V. campbellii. Photobacterium damselae* (formerly classified as *V. damselae), V. fluvialis* and *Vibrio* sp. were also reported in sick animals (Lightner and Lewis 1975).

Table 1. Prevalence of bacterial diseases infecting farmed shrimp (%) in 12 Latin American countries during the period 2000 to 2015.[1]

Country	SHPN or Vibriosis	NHP	Streptococcosis	AHPND	Spiroplasmosis
Mexico	80%	75%		60%	
Guatemala	45%	25%	10%		
Belize	35%	40%			
El Salvador	30%				
Honduras	60%	35%			
Nicaragua	30%	20%			
Costa Rica	80%	67%			
Panama	35%	40%			
Colombia	30%	10%			10% (outbreak in 2002) [2]
Venezuela	30%	10%			
Ecuador	55%	70%			
Peru	35%	80%			

[1] AHPND = acute hepatopancreatic necrosis disease, NHP = necrotising hepatopancreatitis, SHPN = septic hepatopancreatic necrosis.

[2] Source: Nunan and Lightner (2005).

SHPN has been diagnosed by classical bacteriology, quantifying the presence of bacteria in haemolymph ($1x10^4$ cfu.mL^{-1}) and hepatopancreas ($1x10^5$ cfu.g^{-1}). The wet-mount method was also used for the detection of progressive tubular deformation (Fig. 1), bacterial colonization, cell sloughing, haemocytic nodules and melanized necrotic tubules (Lightner, 1993).

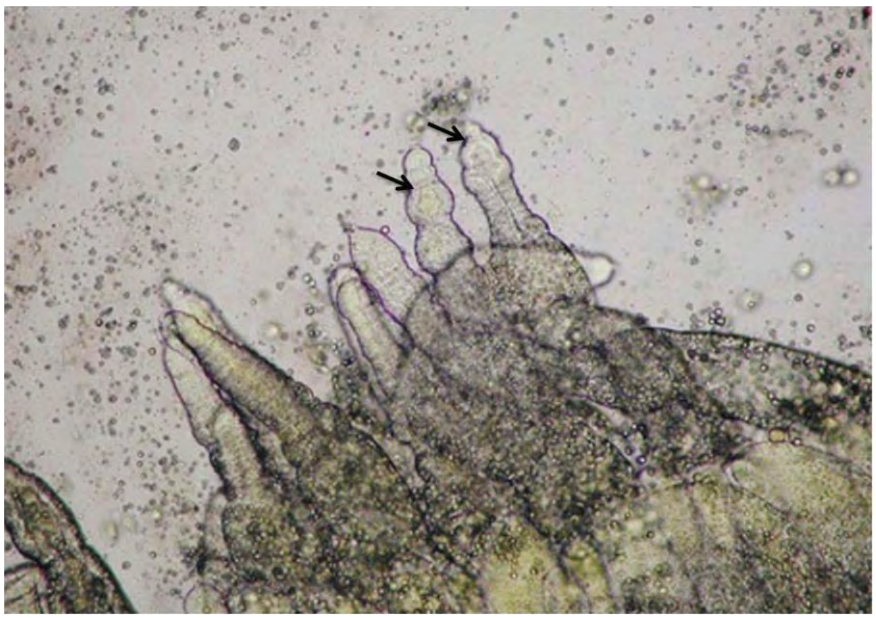

Fig. 1. Wet mount of hepatopancreatic tubules taken from *Penaeus vannamei* showing tubular deformation (arrows) and cellular desquamation.

Histopathological studies complemented the diagnostic process, by which the presence of haemocytic infiltration and nodules with bacterial clusters; hepatopancreatic tubule lumen hypertrophy; cell sloughing; and tubular melanization, necrosis and atrophy (moderate to severe) were confirmed. Haemocyte infiltration and haemocytic nodule formation with bacterial colonies were also reported in the heart (Fig. 2), gills, lymphoid organ, connective tissue, antennal gland, musculature, and caeca, with moderate to high severity.

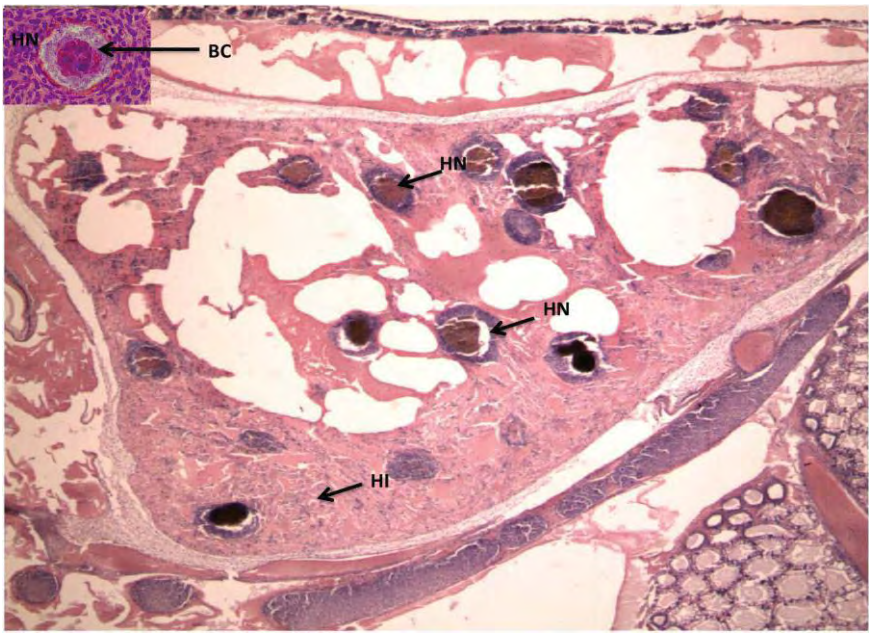

Fig. 2. Histological sections of the heart of *Penaeus vannamei* showing haemocyte infiltration (HI) and haemocytic nodule formation (HN) with bacterial colonies (BC).

Necrotizing Hepatopancreatitis (NHP)

NHP is a disease caused by an intracellular rickettsia-like bacterium recently named as *Hepatobacter penaei.* It has been reported by most Latin American countries, with an average prevalence of 39.27 % (Morales-Covarrubias et al. 2011); Mexico, Peru, and Ecuador reported the highest prevalence (see Table 1). Diagnosis of NHP is done by using a polymerase chain reaction (PCR) test. This was complemented by analysis of wet mounts, through which empty (non-vacuolated) hepatopancreatic tubules, capsule formation (Fig. 3) and melanized tubules with haemocyte infiltration (inflammatory response) were observed (Morales-Covarrubias 2010).

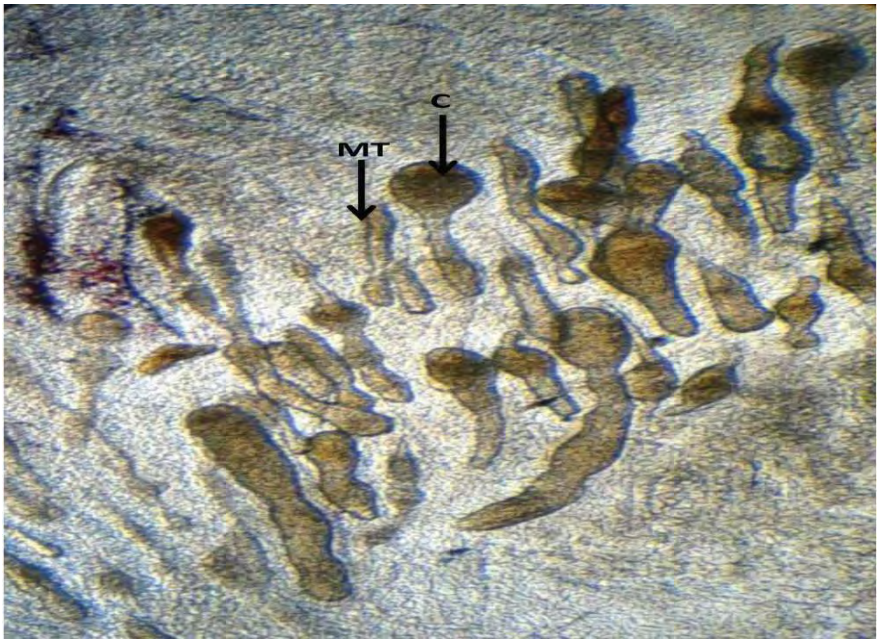

Fig. 3. Wet mount of hepatopancreatic tubules taken from *Penaeus vannamei* showing capsule formation (C) and melanized tubules (MT).

NHP was also diagnosed using histopathology with haematoxylin and eosin (H&E) staining, quantifying damage in the hepatopancreatic tubules such as cell sloughing, tubular atrophy and melanization, the formation of multifocal haemocytic capsules (Fig. 4) involving one or more affected tubules, haemocytic infiltration, presence of epithelial cells within the tubules, and in some cases, clusters of intracytoplasmic bacteria (Morales-Covarrubias et al. 2012).

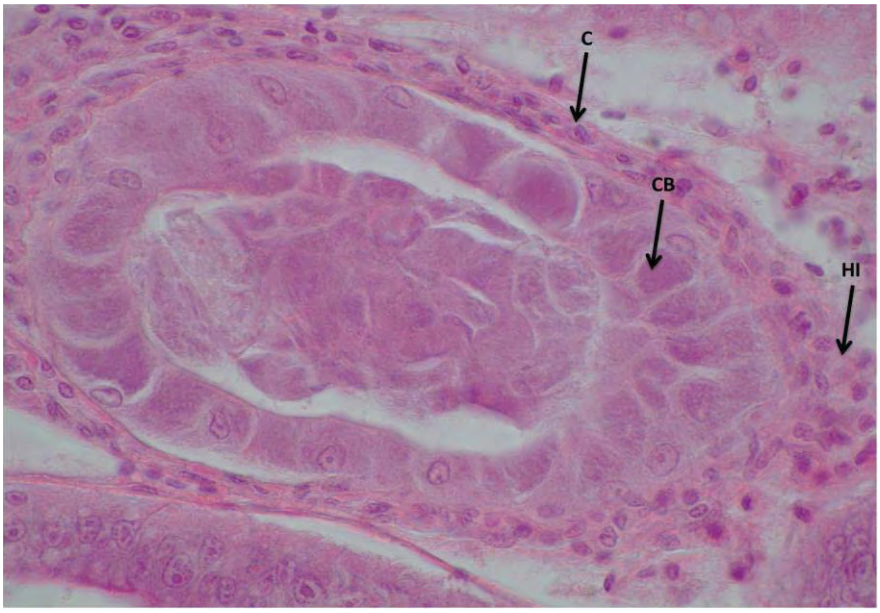

Fig. 4. Histological section of the hepatopancreas of *Penaeus vannamei* showing capsule formation (C) containing intracytoplasmic bacteria (CB) and haemocytic infiltration (HI).

Newly Emerging Diseases

Streptococcosis

This disease has been reported only in Guatemala from 2009 to 2012. It was initially diagnosed using histopathology with H&E staining and by observing haemocytic infiltration, cell sloughing, tubular necrosis and haemocytic nodules (Fig. 5) in the hepatopancreas. Haemocyte infiltration, liquefying necrosis and bacterial mass in the musculature were also observed. Classical bacteriology has been performed to quantify the number of colony forming units (cfu) in haemolymph (10^4 cfu.mL^{-1}) and hepatopancreas (10^6 cfu.g^{-1}) in blood agar. Mortality during the first two years reached 80 %, and *Streptococcus uberis* and *S. parauberis* were identified as causative agents (Hasson et al. 2009; Cazares 2012).

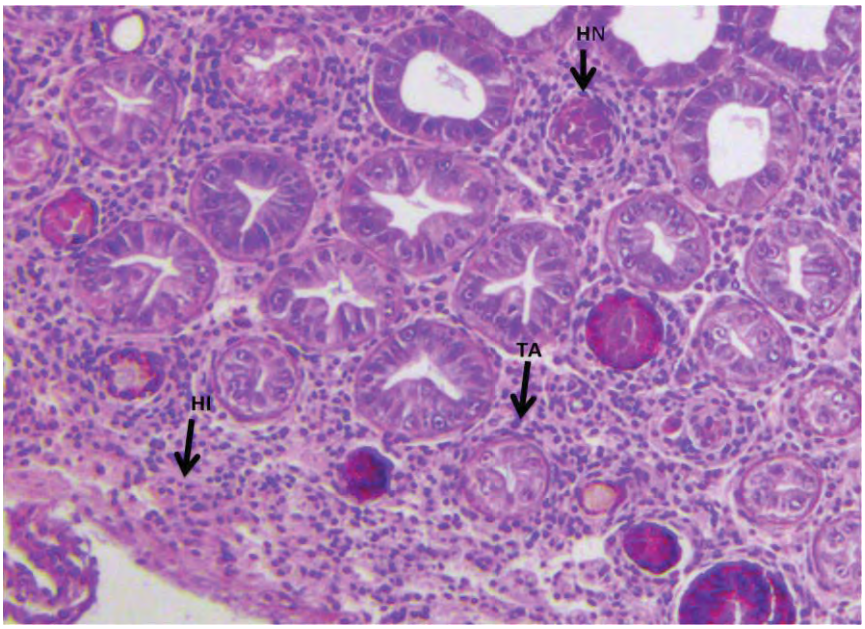

Fig. 5. Histological section of the hepatopancreas of *Penaeus vannamei* showing haemocytic infiltration (HI), multifocal haemocytic nodules (HN), tubular atrophy (TA) and melanized tubules with capsule formation .

Acute Hepatopancreatic Necrosis Disease (AHPND)

AHPND was confirmed in Mexico (first report in the Americas) in August 2013 by Dr Donald V. Lightner from the University of Arizona, during the "Sixth Meeting of the Inter-American Committee on Aquatic Animal Health", held in Yucatán, Mexico. The way by which AHPND came into the country is unknown; it has caused mass mortalities in shrimp production farms culturing *Penaeus vannamei* Boone 1931.

In April 2013, high mortalities were observed in shrimp farms of Nayarit State, Mexico during first 20 days after stocking in semi-intensive and intensive farming systems. Within a few days, mortalities also occurred in other states of the northwest region (i.e. Sinaloa and Sonora), which includes around 90 % of farmed shrimp production in Mexico.

Currently, AHPND is also present in shrimp farms located in the states of Chiapas, Colima and Baja California (Mexicali Valley), the latter two in low salinity (Morales-Covarrubias 2013; Soto-Rodríguez et al. 2015). The first approach to the diagnosis of AHPND, which was made in ponds by observation of clinical signs and wet-mount analysis, established three disease stages: an initial phase, an acute phase and a terminal phase. The initial phase involves normal hepatopancreas colour, tubular deformation (Fig. 6) and hepatopancreatic tubules that are empty at the apical region; these changes become more severe when the shrimp enters the acute phase. In this phase, the hepatopancreas has whitish to pale discolouration and presents central liquefaction when dorsally dissected. In the terminal phase, the hepatopancreas has whitish discolouration, atrophied and melanized tubules, necrosis, clusters of bacteria in the tubules (capsules), and haemocytic encapsulation, and the gut has a whitish content. Methods for confirmatory diagnosis of AHPND include histopathological study of affected shrimp tissues and the use of PCR tests (Tran et al. 2013).

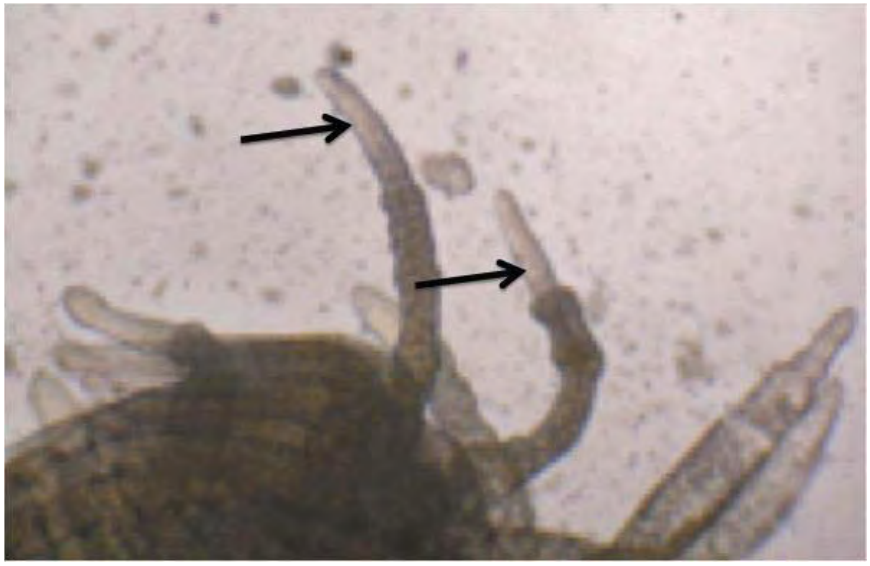

Fig. 6. Wet mount of the hepatopancreatic tubules of *Penaeus vannamei* showing tubular deformation and tubules that are empty at the apical region (arrows).

The same three phases were also observed by histopathology: an initial phase with elongated epithelial cells, non-haemocytic infiltration and bacteria on the gastric mill (Fig. 7). The acute phase had a progressive lesion from the inner (proximal) to the outer (distal) region of the hepatopancreatic tubules, including sloughing of cells into the tubules (Fig. 8); also moderate haemocytic infiltration, cell dysfunction of R, B, F and E cells in the hepatopancreatic tubules, and obvious nuclear hypertrophy in E cells.

The terminal phase presents tubular haemocytic encapsulation, severe haemocytic infiltration and bacterial clusters in the tubule lumen, haemocytic nodules with and without melanization and severe secondary bacterial infection.

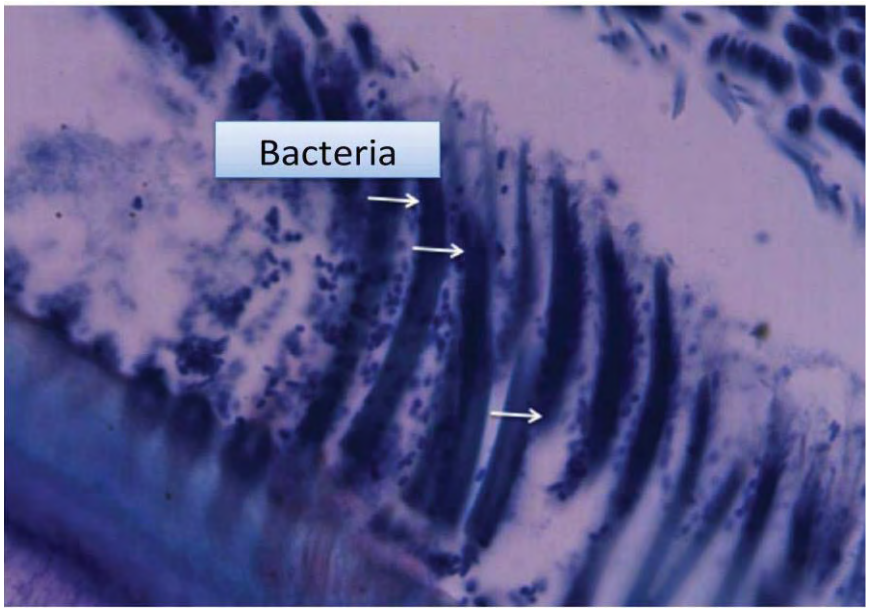

Fig. 7. Histological sections of the gastric mill of *Penaeus vannamei* showing bacterial colonies (arrows).

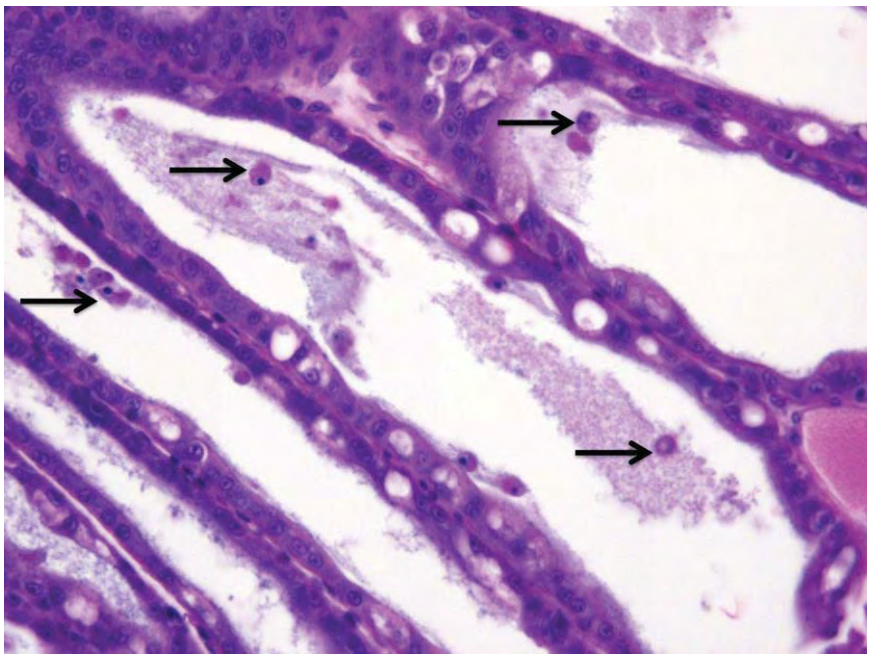

Fig. 8. Histological section of the hepatopancreas of *Penaeus vannamei* showing elongated the hepatopancreatic tubules and sloughing of cells into the tubules (arrows).

Since 2013, Mexico has been the only country in the Americas with reported scientific evidence of AHPND. However, in June 2015, it was suggested that according to PCR analysis in local and foreign laboratories, AHPND is present in populations of farmed *P. vannamei* in Honduras and Nicaragua (Undercurrent News 2015), where mass mortalities were observed in commercial semi-intensive systems. Nevertheless, this has not been confirmed by the competent authorities of these countries.

Spiroplasmosis

This bacterial disease of shrimp is produced by *Spiroplasma penaei,* which was first reported in Colombia in 2002 and has not been detected since in any shrimp farms in the world (Altamiranda et al. 2011). In 2002, spiroplasmosis produced mortalities ranging from 10 to 100 % in commercial shrimp ponds and was initially diagnosed by histopathological study of diseased shrimp, and subsequently by PCR tests (Cuéllar-Anjel et al. 2010). Affected shrimp present no external changes that suggest a bacterial disease; however, expanded chromatophores are sometimes observed. The main histological changes seen in moribund shrimp are lesions typical of systemic bacterial disease and include haemocytic infiltration, multifocal inflammatory foci in several tissues/organs, haemocytic nodules, evidence of phagocytosis, melanization and eventual fibrosis of affected areas (Nunan et al. 2005; Pantoja and Lightner 2014).

Organs and tissues that are affected by *S. penaei* include the ventral nerve cord, skeletal musculature, heart, antennal gland, lymphoid organ, fibrous connective tissue within the hepatopancreas, perigastric spongy connective tissue, gill lamellae and subcuticular epithelium. The disease progression involves the presence of necrotic cells with subsequent migration and aggregation of phagocytic cells into necrotic areas. After necrotic cells and cell-debris phagocytosis, haemocytic nodules appear that degenerate to melanized nodules and finally to a fibrous inflammatory response. For confirmatory diagnosis of *S. penaei,* a combination of histopathology and molecular techniques that include PCR and *in situ* hybridization is suggested (Nunan and Lightner 2005).

Conclusion

Based on the results obtained in this review, it was determined that the highest prevalence of bacterial disease in Latin American farmed shrimp was for septic hepatopancreatic necrosis (SHPN) caused by *V. harveyi*, *V. parahaemolyticus* and *V. alginolyticus*. This disease is probably due to stress during commercial farming and/or environmental changes in recent years. It is likely that the presence of one or more of the reported diseases should be attributed to a deficient or suppressed immune system and a damaged hepatopancreas. It has been reported by Duan et al. (2015) that an affected hepatopancreas promotes the presence, multiplication and spread of pathogens in organs and tissues of farmed shrimp.

This happens because the hepatopancreas performs several functions that include temporary nutrient storage and the synthesis, absorption, secretion and metabolism of lipids and carbohydrates. The hepatopancreas is also involved in the production of proteins required for vital functions, such as the synthesis of haemocyanin, a protein responsible for oxygen transport to all shrimp cells.

The current foci of streptococcosis infections in Guatemala and of AHPND in Mexico are probably due to the fact that these are new (emerging) shrimp diseases in the Americas. It is important to note that at present, most of the bacterial pathogens are adapted to both fresh and marine water, so from the point of pathology and disease control, it is difficult to establish a clear boundary between coastal and marine aquaculture. An inadequate farming environment (e.g. low water and soil quality) can influence shrimp health, increasing susceptibility to pathogens.

The results of this study may help shrimp farmers in implementing preventive measures that are aimed at reducing the risk of spreading bacteria between ponds and farms and to wild ecosystems, thus preventing high mortalities and their associated negative economic impacts for shrimp farms. Because diseases of aquatic organisms may have a variety of sources, it is important to conduct complete and comprehensive diagnostics procedures, covering as many factors as possible, including those related to the production system (e.g. epizootilogical mapping, climate, farm management, water quality and source of postlarvae). It is also valuable to have integrated health programmes with frequent monitoring plans and surveillance for each country, region and farm to prevent pathogen dispersion. It is urgent that the competent authorities of the countries of Latin America take action on aquatic health and establish strict border controls to protect their own productions and to prevent the entry and spread of transboundary pathogens to and within shrimp farms

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Heritability, Genetic Line and Inbreeding Effects on Resistance of Whiteleg Shrimp *Penaeus vannamei* Boone 1931 to Acute Hepatopancreatic Necrosis Disease (AHPND) in Mexico

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Abstract

The objective of this paper is to present preliminary results regarding heritability, genetic line differences and inbreeding effects for survival time in experimental challenges to acute hepatopancreatic necrosis disease (AHPND) in *Penaeus vannamei* Boone 1931. Here we present results of analyses conducted on data from a Resistance Line obtained from a merging of several Ecuadorian groups with a history of white-spot syndrome virus resistance, and a Growth Line with high genetic growth ability, obtained by selection in a Mexican hatchery. Family-identified animals from the two genetic lines and their crosses were inoculated by immersion in 2014, 2015 and 2016 using a *Vibrio parahaemolyticus* strain (M0904) AHPND+ obtained from a natural infection in Mexico. Heritabilities for survival time obtained using nested linear mixed models ranged from 9 to 18 %. Survival was greater for the Resistance Line compared to the Growth Line or the F_1 cross ($P < 0.02$). Our results point to the presence of additive genetic variation in both lines evaluated that may be exploited in breeding programmes to increase AHPND resistance.

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Additionally, our results support the idea that the Resistance Line is more resistant to AHPND than the Growth Line. Finally, comparisons between inbred and non-inbred animals suggest that the effect of inbreeding on AHPND resistance is small.

Keywords: acute hepatopancreatic necrosis disease, challenge tests, genetic resistance, heritability, *Penaeus*, shrimp

Introduction

Acute hepatopancreatic necrosis disease (AHPND) is a bacterial disease in shrimp that has resulted in substantial economic losses in shrimp farms, causing high mortality rates, mainly in juvenile shrimp (Tran et al. 2013). Since its first outbreak in the People's Republic of China in 2009, the disease has been reported in Malaysia, Viet Nam, Thailand and Mexico (Hong et al. 2016). In Mexico, it has been recognized as a cause of large atypical mortality outbreaks in *Penaeus vannamei* Boone 1931 shrimp farms since 2013 (Nunan et al. 2014; Soto-Rodríguez et al. 2015).

The development and implementation of good sanitary management practices is crucial to the control of the disease (FAO 2013; Cock et al. 2015). Disease control in aquaculture animals can also be achieved by using genetic differences for disease resistance within each species (Cock et al. 2009; Ødegård et al. 2011; Yáñez et al. 2015). For superior results these strategies may be used in conjunction, in a manner similar to that long used in plants with the concept of integrated disease control (Moss et al. 2012; Russell 2013). An additional advantage of using genetic resistance as a control method for disease in aquaculture is a reduction in the use of biologicals, drugs and chemicals with the associated advantages related to environmental sustainability.

In shrimp production, a major factor that increases the practical importance of using genetic differences for disease control is the difficulty of implementing vaccination, because it is generally assumed that shrimp do not have the capacity to acquire immunological resistance (Cock et al. 2009), although this assumption has been questioned by Witteveldt (2006) and by Johnson et al. (2008). Shrimp, as with all crustaceans, do not produce antibodies, interferon or other acquired immune mechanisms common in vertebrates (Matsunaga and Rahman 1998; Cerenius and Söderhäll 2012). To consider the inclusion of disease resistance into the breeding objective, it is necessary to measure the genetic variation of the related traits (Yáñez et al. 2014). Therefore, for genetic improvement to AHPND resistance there is a need to estimate its heritability, since this is a key element in predicting the expected response to selection and evaluating the presence of genetic variability in different shrimp populations (Falconer and Mackay 1996). In addition to genetic selection within populations, crossbreeding is another common option used in animal breeding to take advantage of genetic differences. Information about the performance of genetic lines from different origins exposed to the pathogen is necessary to measure crossbreeding effects and the possibility of producing resistance lines derived from specific crossbreeding strategies (Gjedrem and Baranski 2010).

One practical option to detect genetic differences between selection candidates or populations in aquaculture for specific diseases is to use challenge tests based on survival time and survival rate to assess resistance (Ødegård et al. 2011; Gjedrem 2015). Here we present quantitative genetic analysis results of such testing in a selection nucleus of *P. vannamei* from a large shrimp hatchery in Mazatlán, Sinaloa in the northwest coast of Mexico (Maricultura del Pacífico). In this company, a shrimp line was selected for several generations for increasing growth rate at harvest size (130 days) and for general survival in the absence of any important disease outbreak (Castillo-Juárez et al. 2015).

A fundamental motivation for the development of these experimental challenges was the interest of the Mexican shrimp industry in testing possible sources of resistance to the white-spot syndrome virus (WSSV) and AHPND outbreaks that have caused serious losses in this industry. This was also related to mounting anecdotal but compelling evidence on the existence of a higher degree of genetic resistance to WSSV in commercially available breeding shrimp from Ecuador and other sources, using both field observations made by the Mexican shrimp industry and commercial challenge tests performed at the University of Arizona. This evidence coincided with a gradual recovery in productivity of the shrimp industry in Ecuador, after following a dramatic initial reduction caused by WSSV outbreaks. Since different selection procedures have been used in each country due to their different breeding and production conditions, it is important to evaluate and compare shrimp from Ecuador and Mexico in AHPND challenge tests. In 2013, the Asociación Nacional de Productores de Larva de Camarón (National Association of Shrimp Larvae Producers) brought shrimp of Ecuadorian origin assumed to have a higher resistance to WSSV to its quarantine unit facility. Some of these animals and their crosses were used in the Maricultura del Pacífico breeding programme to yield a new genetic line designed for resistance to AHPND and WSSV (Resistance Line). In 2014, Maricultura del Pacífico began activities in its disease challenge unit facility with the scientific guidance of the Universidad Autónoma Metropolitana – Xochimilco, the Universidad Nacional Autónoma de México and the Centro de Investigación en Alimentación y Desarrollo A.C. – Unidad Mazatlán (CIAD-Mazatlán), where AHPND and WSSV challenges were performed to compare the Resistance Line with the Mexican Growth Line and their crosses.

On the other hand, it has been suggested that relatively high inbreeding levels caused by the widespread mating of highly related (full-sib) animals from single commercial lines with a “genetic lock” may have had a major role in the recent outbreaks of AHPND and WSSV in many regions of the world (Doyle 2016). We find this hypothesis unlikely, because it relies on several interconnected processes, without actual direct evidence of its occurrence. Moreover, there is a lack of direct evidence from actual measurement data on genetic resistance in animals with different inbreeding levels for the specific diseases involved. Nonetheless, as an idea that has attracted interest, it is important to provide experimental evidence to support or discard it. The objective of this paper is to present preliminary results regarding heritability, genetic line differences, and inbreeding effects for survival time in experimental challenges to AHPND performed from 2014 to 2016 on the selection nucleus population of a large breeding company in Mexico.

Here we present results on differences between "purebred shrimp", from a merging of several Ecuadorian lines with a history of WSSV resistance (Resistance Line), and a Mexican line with high genetic growth ability, selected for many generations for growth and survival in the absence of any catastrophic disease outbreak (Growth Line).

Material and Methods

Location and Population

Experimental AHPND challenges were performed from 2014 to 2016 in a facility specially designed for this purpose by Maricultura del Pacífico. All the animals were inoculated by immersion method using a *Vibrio parahaemolyticus* strain (M0904) obtained from the pure bacterial strain collection from CIAD Mazatlan´s Bacteriology Laboratory. The M0904 strain was isolated from cultured shrimp affected with AHPND in northwestern Mexico (Soto-Rodríguez et al. 2015). Collection of dead and dying shrimp was made every hour. Some of these animals were used for histopathological studies to confirm the cause of death.

Challenge conditions varied across the years (Table 1). In 2014, two 30-litre aquaria per family and three batches with different family subsets were used in order to test all the families using this data structure. Six and seven tanks containing 1 000 litres of water were used in 2015 and 2016, respectively. Each tank was seeded with animals from all the families under study. Shrimp families were identified using plastic elastomers as in Castillo-Juárez et al. (2007). These studies included a control tank where animals were not challenged against AHPND.

Table 1. Averages and (standard deviations) for variables defining conditions of AHPND challenge tests of *Penaeus vannamei* in Sinaloa, Mexico.

Year	Age (days)	Weight (g)	cfu.mL^{-1}	Duration (h)	Number of families/ individuals per line	
					Resistance	Growth
2014	55.4 (0.9)	0.5 (0.3)	1.95 x10^{5}	52	28/836	100/1 593
2015	75.8 (1.6)	2.6 (0.9)	3.16 x10^{6}	74	62/1 783	53/1 477
2016	83.1 (1.8)	1.4 (0.7)	1.09 x10^{6}	98	41/1 278	41/1 408

[1] cfu.mL^{-1}: Colony forming units.mL^{-1} at inoculation (time = 0).

Heritability

Estimates of heritability for survival time were obtained within genetic line (Resistance and Growth) and year. Linear mixed models with nested random sire/dam/progeny and fixed tank (or random aquarium in 2014) effects were applied. Sire, dam and error (progeny) variance components (V_s, V_d and V_e, respectively), were estimated with restricted maximum likelihood methodology (Ødegård et al. 2011).

Phenotypic variance was defined as: $V_p = V_s + V_d + V_e$. Heritability was estimated from sire variance component as: $4V_s/V_p$, the dam variance component as: $4V_d/V_p$ and from the sire + dam component of variance as: $2(V_s + V_d)/V_p$ (Falconer and McKay 1996).

Comparison of Resistance and Growth Lines

In 2014, the results presented here correspond to the survival pattern of the Resistance Line and F_1 Resistance Line x Growth Line cross (479 organisms from 16 families) included in the first batch, under similar contemporary conditions. In 2015 and 2016, purebred Resistance and Growth Line shrimp and several crosses were measured. The evolution of the survival of the two lines was compared using survival analysis with Kaplan-Meyer methodology (Miller 2011).

Inbreeding Effects on AHPND Resistance

Inbreeding effects on AHPND resistance were evaluated using data from 2015 and 2016, where inbred animals were produced by mating full-sibs, thus, approximate inbreeding coefficients obtained were close to 25 %, but there were also families with several inbreeding levels. Therefore, inbreeding effects were studied by regression analysis using animals with different inbreeding levels under similar conditions. We used linear statistical models to simultaneously test for crossbreeding, inbreeding and non-genetic effects on survival times. We also compared inbred and non-inbred animals within genetic lines.

Results

Heritability and Family Differences

Preliminary heritability estimates for survival time after AHPND challenge presented by genetic line and by year, derived with different variance components obtained by simple sire/dam nested within-line models (Falconer and McKay 1996) are shown in Table 2. Mean survival times per family by year, within genetic line, are shown in Fig. 1 to 6. These figures demonstrate that there is significant variation between families within genetic lines.

Table 2. Heritability estimates (%) for survival time with simple linear statistical models from sire and dam variance components by line and year.

	Genetic Line					
	Resistance			Growth		
Year	Sire component	Dam component	Sire + Dam components	Sire component	Dam component	Sire + Dam components
2014	28	1	15	0	18	9
2015	16	2	9	10	16	13
2016	22	7	15	23	12	18

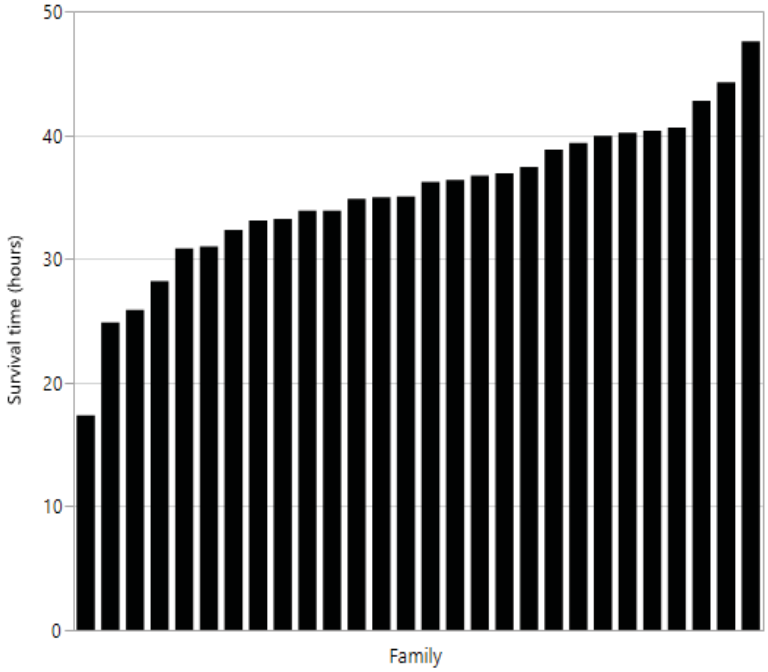

Fig. 1. Mean survival time by family for the Resistance Line in 2014 AHPND challenge test.

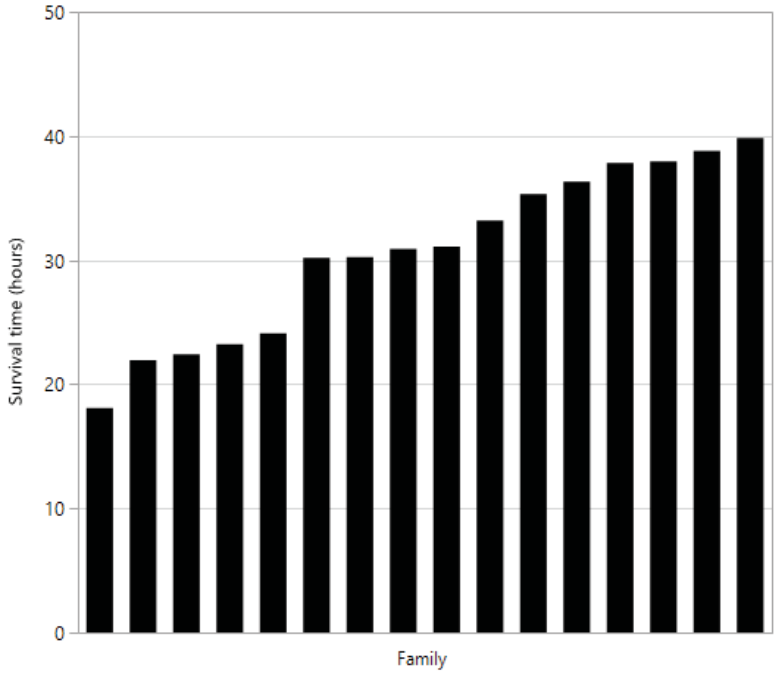

Fig. 2. Mean survival time by family for the F_1 Resistance x Growth cross in 2014 AHPND challenge test.

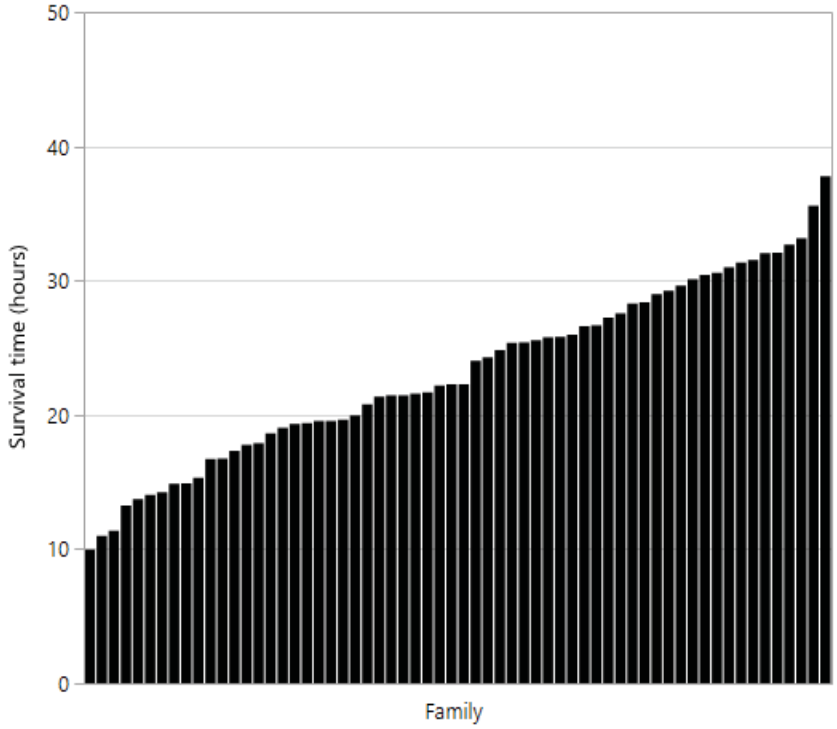

Fig. 3. Mean survival time by family for the Resistance Line in 2015 AHPND challenge test.

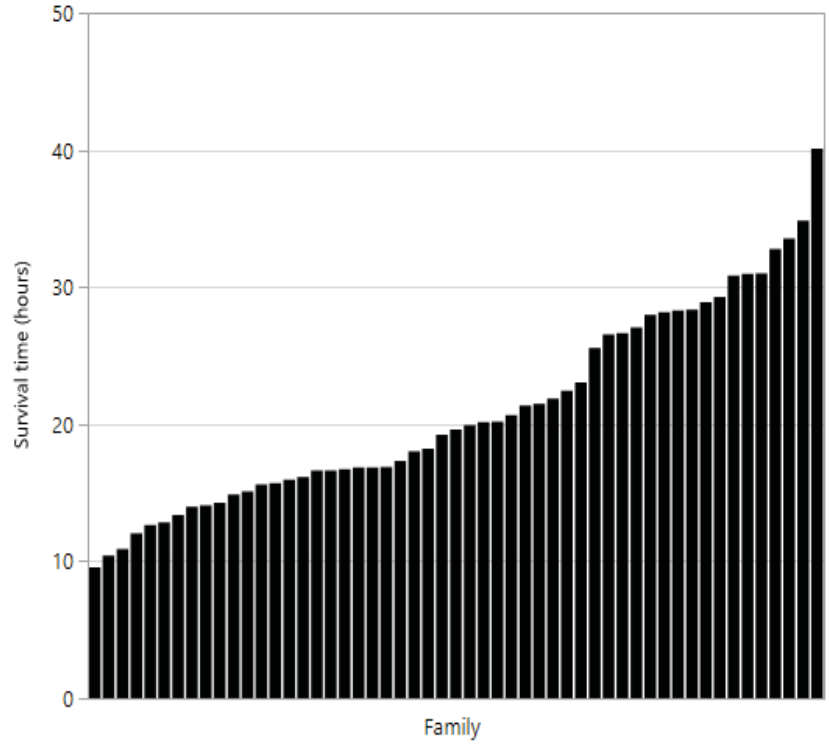

Fig. 4. Mean survival time by family for the Growth Line in 2015 AHPND challenge test.

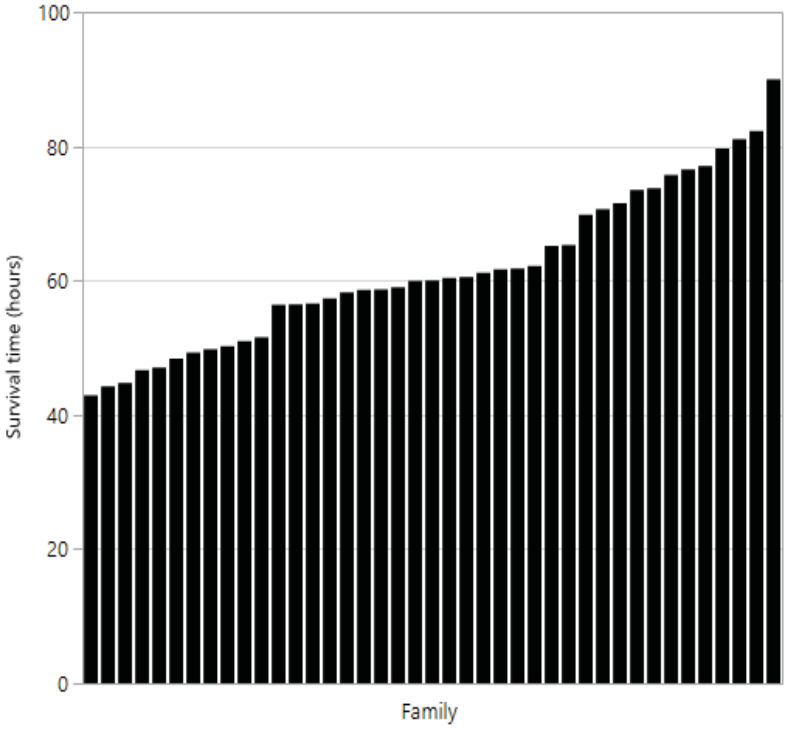

Fig. 5. Mean survival time by family for the Resistance Line in 2016 AHPND challenge test.

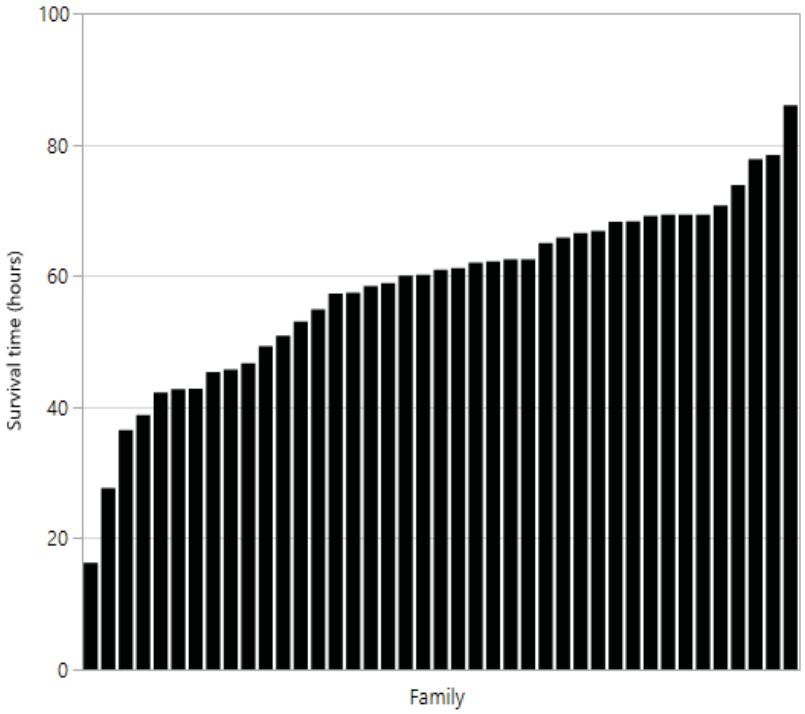

Fig. 6. Mean survival time by family for the Growth Line in 2016 AHPND challenge test.

Comparison of Purebred Genetic Lines for AHPND Resistance

Differences were observed between the genetic lines across years (2014 to 2016) regarding resistance to AHPND, measured as survival time in hours after challenge. Figures 7, 8 and 9 show the evolution of survival for the Resistance Line versus the F_1 cross (Resistance Line x Growth Line) in 2014, and versus the Growth Line in 2015 and 2016. For 2014 data, mean survival times were 35.3 h for the Resistance Line and 30.7 h for the F_1 cross. The difference between genetic groups for survival trend (right-censored) was significant ($P < 0.001$). For 2015 data, mean survival times were 23.1 h for the Resistance Line and 21.0 h for the Growth Line. The difference between genetic lines for survival trend (right-censored) was significant ($P < 0.001$). In 2016, mean survival times were 61.7 h for the Resistance Line and 58.0 h for the Growth Line. The difference between genetic lines for survival trend (right-censored) was significant ($P < 0.02$).

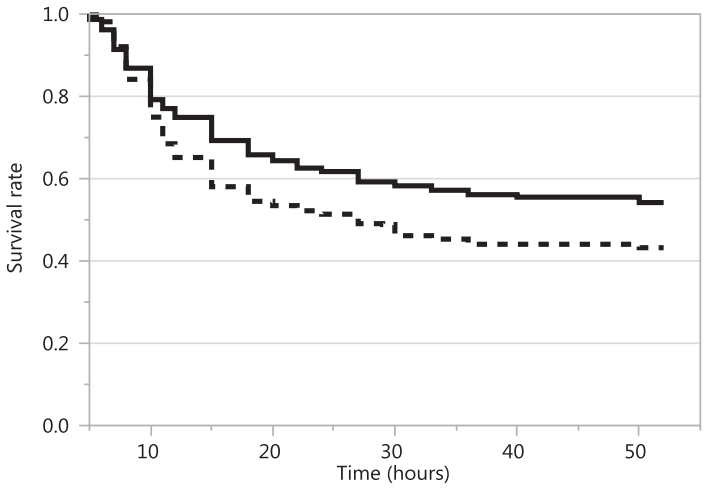

Fig. 7. Kaplan-Meyer survival plot for Resistance Line (solid line) and Resistance Line x Growth Line (F_1) cross (dotted line) in 2014 AHPND challenge test.

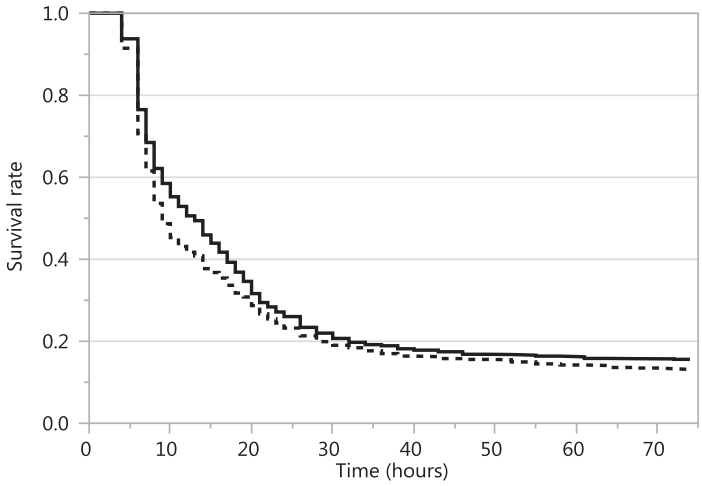

Fig. 8. Kaplan-Meyer survival plot for Resistance Line (solid line) and Growth Line (dotted line) in 2015 AHPND challenge test**.**

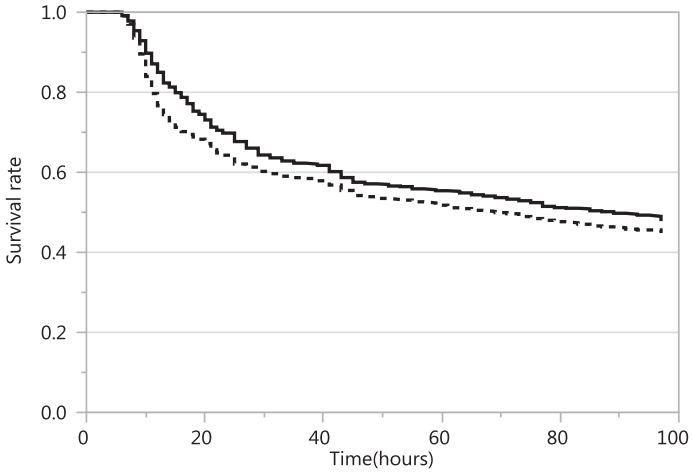

Fig. 9. Kaplan-Meyer survival plot for Resistance Line (solid line) and Growth Line (dotted line) in 2016 AHPND challenge test.

Inbreeding Effects on AHPND Resistance

The results indicate no effects of inbreeding (F) on survival times to AHPND challenges with *P*-values > 0.10 and regression coefficient estimates of survival time on F close to zero (results not shown). This was also found in testing F effects on survival rates.

Discussion

Heritability

Combined sire + dam heritability estimates were, in general, statistically greater than zero, but tended to be lower than 20 %. There are no published studies regarding the heritability of resistance to AHPND to compare with our results. Selection (if genetic variation exists) can be used in breeding programmes to yield resistant lines to specific diseases (Cock et al. 2009), although, as Moss et al. (2005) suggest, heritability estimates under disease challenge conditions are not easily translated into practical commercial conditions.

Our results revealed important differences regarding the magnitude of the sire/dam variance components between lines, possibly due to data structure and/or to actual differences in genetic parameters between lines. In any event, our challenges found additive genetic variation for AHPND resistance, which may be used in shrimp breeding programmes. Greater survival times were observed for families from the Resistance Line when compared to the Growth Line or to the Growth x Resistance cross within year. These differences were mostly observed in the left half of the distributions, which involve the families with lower survival rates. This fact is consistent with the estimation of within-line genetic differences (heritability estimates).

It is important to bear in mind that genetic parameters for disease resistance traits may change across years and environments, since they depend on the interactions between the pathogens and their hosts (co-evolution), which are in general very dynamic processes (Ebert 1998), and favourable mutations can accumulate over years and introduce genetic variation (Cock et al. 2009). Hence, heritability estimation for disease resistance traits must be performed in each breeding cycle.

Comparison of Purebred Genetic Lines for AHPND Resistance

To our knowledge, there are no published studies comparing genetic lines for resistance to AHPND challenges. Nonetheless, the differences between genetic lines in our AHPND challenges are consistent with those observed in commercial ponds in Mexico since 2013, where mortality rates have been clearly lower in the Ecuadorian-origin shrimp when AHPND and WSSV outbreaks have occurred. The higher resistance we observed in the Resistance Line is compatible with the hypothesis of a higher genetic resistance of the Ecuadorian breeding lines obtained by means of natural selection, because their breeding populations were maintained under WSSV (and probably other diseases) infection conditions for several generations. This disease challenge under natural conditions may have introduced selection pressure to these shrimp populations, leading them to develop resistance to other pathogens as well (Cock et al. 2015). Since the shrimp immune-like system is rather non-specific, the ability to succeed against one disease may also confer some protection against other diseases (Cock et al. 2009).

Inbreeding Effects on AHPND Resistance

The results obtained in this study do not support a strong association between inbreeding and disease vulnerability in shrimp populations as suggested by Doyle (2016). On the other hand, experimental and theoretical evidence of the effect of inbreeding and genetic drift in small populations on general fitness and on disease resistance in arthropods is conflicting, indicating a complex picture in the presence of natural selection that points to the risk of providing overly general conclusions (Armbruster and Reed 2005; Facon et al. 2011; García-Dorado 2012; De los Ríos-Pérez et al. 2015).

Conclusion

Our AHPND resistance challenge experiments performed from 2014 to 2016 in *P. vannamei* show that there is additive genetic variation in the Resistance Line and in the Growth Line that can be exploited in breeding programmes to increase AHPND resistance. The results presented here also support the idea that the Resistance Line formed from shrimp from Ecuador with a history of WSSV resistance is also more resistant to AHPND than the Mexican Growth Line. Finally, our experiments show that there is no effect of inbreeding on susceptibility to AHPND.

Acknowledgments

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Pathological, Genomic and Phenotypical Characterization of *Vibrio parahaemolyticus,* Causative Agent of Acute Hepatopancreatic Necrosis Disease (AHPND) in Mexico

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Abstract

Moribund whiteleg shrimp (*Penaeus vannamei*) affected by acute hepatopancreatic necrosis disease (AHPND) from farms in northwestern Mexico were sampled for pathological analysis. Bacterial isolates were molecularly identified as *Vibrio parahaemolyticus* (VP) by the presence of the *tlh* gene. The *tdh*-negative, *trh*-negative and *tlh*-positive VP strains were further characterized by enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR). The VP pure strains were used in immersion challenges with shrimp, and farmed and challenged shrimp presented the same clinical and pathological signs: lethargy, empty gut, pale and aqueous hepatopancreas and expanded chromatophores. Using fresh mount, histological analysis and bacterial density count, three stages of AHNPD (initial, acute and terminal) were identified in the affected shrimp. Pathognomonic lesions indicated severe desquamation of tubular epithelial cells of the hepatopancreas. VP had different virulence and was dose dependent. VP strains showed wide tolerance to different environment conditions of temperature, salinity and pH, and pathogenic and non-pathogenic VP strains from Mexico had similar morphological and physiological characteristics but pathogenic VP strains were most sensitive to nalidixic acid and showed resistance to penicillin. Whole genomic sequence of 22 VP strains from Asia, Mexico and South America shows the presence of similar chromosomic pathogenic mechanisms, and comparative analysis by multilocus sequence analysis (MLSA) of seven genes clearly showed that most of the isolates are independent strains.

Keywords: AHPND, antimicrobial sensitivity, genomic analysis, histopathology, shrimp, *Vibrio parahaemolyticus*, virulence

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Introduction

Production of cultured shrimp in Mexico is supported mainly from the northwest states of Sonora, Sinaloa and Nayarit. In 2013, shrimp farms from these states were affected by atypical mortalities that primarily occurred in the first days after stocking. During the first culture cycle of Nayarit State, shrimp farms presented atypical mortalities during the first 30 days of culture. Mortality events with the same characteristics as Nayarit's were subsequently observed in the states of Sinaloa and Sonora, affecting regional production and subsequently producing economic losses of over 2.5 million pesos (Julio Cabanillas, CEO of the Aquatic Animal Health Sinaloa State Committee personal communication, May 2013).

The Mexican shrimp farming industry was deeply affected, because of the large scale of the mortalities and the lack of effectivity of the antibiotics commonly used in shrimp farming (Soto-Rodriguez et al. 2006), such as enrofloxacin, oxytetracycline and florfenicol. Despite the economic losses of 2013, a slight recovery of the Mexican production of cultured shrimp was observed in 2014 and 2015. Studies conducted by our research team showed no evidence of VP_{AHPND+} colonization in the shrimp's digestive tract (unpublished data). Acute hepatopancreatic necrosis disease (AHPND) isn´t a typical vibriosis infection, rather it is an acute intoxication caused by PirA and PirB toxins delivered directly into the culture water.

Gross Signs and Histopathology of Affected Shrimp

Moribund shrimp, between 0.2 and 2.5 g, affected by AHPND from farms in northwestern Mexico revealed clinical signs that typically included slightly expanded chromatophores, lethargy and erratic swimming and were characterized by presenting macroscopic changes distinguishable in the external appearance of the digestive tract and hepatopancreas (HP) and changes to the microanatomy associated with the development of lesions in the tissue. Based on histopathological evidence and bacterial density counts for shrimp affected under natural and experimental conditions (Soto-Rodriguez et al. 2015), we identified three phases of the disease:

Initial Phase

Macroscopically, at this phase, organisms affected by AHPND showed altered HP with partial or total absence of food in the stomach and midgut. These changes can be better appreciated by dissecting the digestive tract and removing the membrane covering the HP (Fig. 1b). Microscopically, tubules of the HP presented modification and elongation of their epithelial cells (appearing like drops) towards the tubular lumen (Fig. 2a), causing the subsequent cellular desquamation. A reduction in size of the vacuoles in R and B cells was observed, which increased as the disease progressed (Fig. 2b); meanwhile other tubules were affected.

Fig. 1. Macroscopic observation of the digestive tract of *Penaeus vannamei* affected by acute hepatopancreatic necrosis disease (AHPND). (a) Healthy organism; (b) Initial phase; (c and d) Acute phase; (e) Terminal phase.

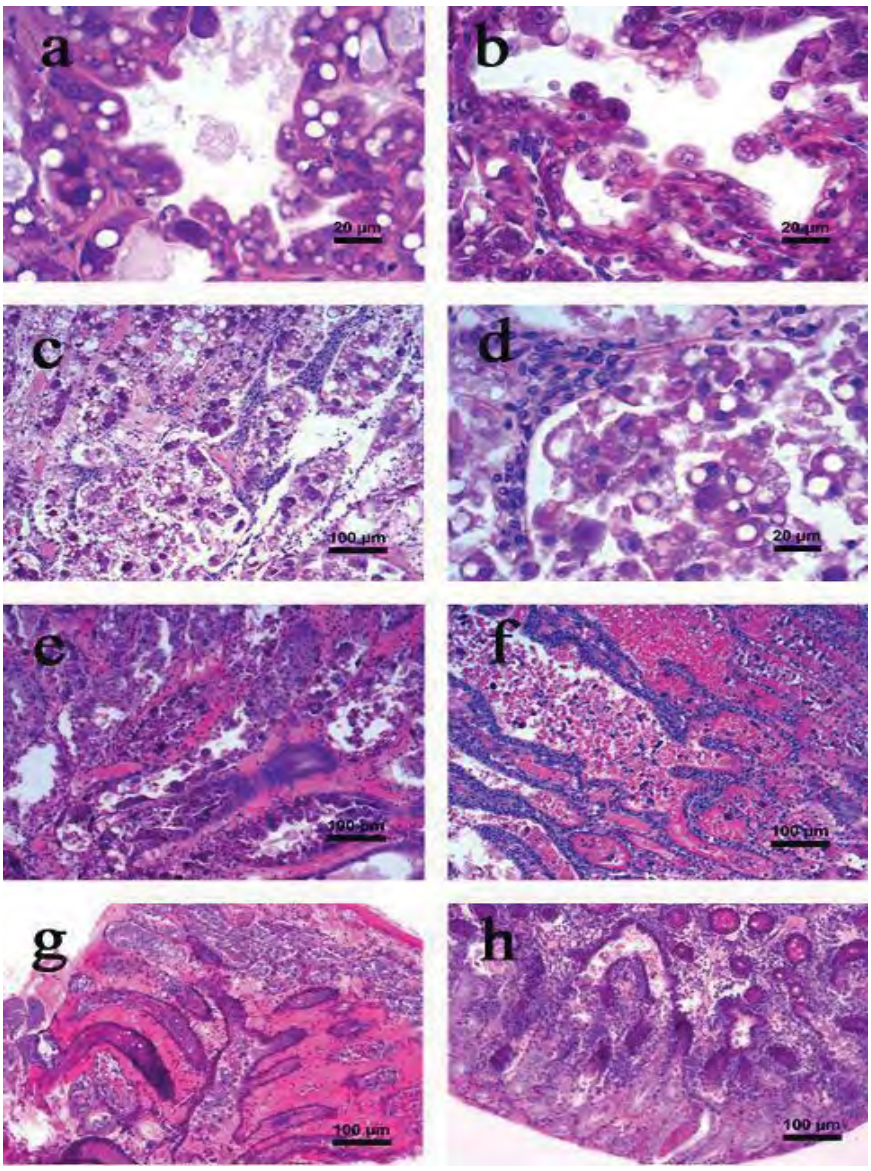

Fig. 2. Photomicrograph of the hepatopancreas of *Penaeus vannamei* with lesions associated with acute hepatopancreatic necrosis disease (AHPND). (a-b) Initial phase; (c-f) Acute phase; (g-h) Terminal phase of disease. Haematoxylin-eosin-floxin staining.

Acute Phase

Organisms affected by this phase of the disease showed anorexia, lethargy and empty digestive tract, with loss of tissue pigmentation (Fig. 1c) until the HP became whitish and atrophied (Fig. 1d). The HP tissue was friable with an aqueous consistency during the first hours post-infection, and as the disease progressed, developed a hard consistency, becoming difficult to disintegrate. Microscopically, a generalized HP disorganization was observed due to severe necrosis of the epithelium. Intertubular tissue showed haemocytic infiltration, and most of the tubules had a necrotic epithelium with a massive accumulation of dead cells into the tubular lumen (Fig. 2c and d), a pathognomonic lesion reported for AHPND (Tran et al. 2013, Soto-Rodriguez et al. 2015).

In the first hours post-infection, R and B cells may have some cytoplasmic vacuoles; however, as the disease progresses, there was a vacuole reduction (Fig. 2e) until these disappeared; meanwhile mitosis was interrupted in E cells. In this phase, there weren't bacteria in the affected tissues, as reported by Tran et al. (2013) and Soto-Rodriguez et al. (2015). Possibly at this stage, PirA and PirB toxins, responsible for AHPND (Han et al. 2015), caused the greatest damage in the epithelial cells of the HP tubules, which has not been observed in any organ or tissue of affected shrimp (Soto-Rodriguez et al. 2015). At the end of the acute phase, a large haemocytic infiltration in the intertubular tissue is observed, and the tubular epithelium is completely necrotic with loss of continuity of the epithelium or, in some instances, its absence. The tubular lumen is filled with dead cells, increasing their size due to accumulation of necrotic tissue (Fig. 2f).

Terminal Phase

Organisms at this phase showed an empty digestive tract, anorexia, lethargy, expanded chromatophores and large atrophy of the HP with whitish coloration (Fig. 1e). In addition, the HP feels fibrous when it is squashed. Histology showed black streaks, indicating focal tubular melanization, and an increase of haemocytic infiltration in the intertubular connective tissue with haemocytic capsules surrounding the affected tubules as a response to bacterial load and necrotic tissue (Fig. 2g). At this phase, the bacterial proliferation is caused by a secondary infection, possibly a vibriosis (Fig. 2h).

Identification and Characterization of Isolates From Shrimp Farms Affected by AHPND

Microbiological analyses indicated a poor presence of bacteria in the haemolymph (HL) and HP. In contrast, a high load of *Vibrio* bacteria was found in the stomach (ST). By Mann-Whitney test no significant differences were observed between bacterial densities among shrimp on thiosulfate-citrate-bile salts-sucrose agar (TCBS) and marine agar: ST (TCBS, $p = 0.109$; marine agar, $p = 0.113$) and HP (TCBS, $p = 0.131$; marine agar, $p = 0.074$) in any of the three stages of the disease. An increase in the density of *Vibrio* spp. was observed as the infection progressed from healthy shrimp to shrimp at the terminal stage, with dominance of yellow colonies (YCs) and a presence of green colonies (GCs) on TCBS that went from 20 to 38 % for the HP and from 13 to 42 % for the ST.

Field and laboratory observations have been similar to reports from Asia (Tran et al. 2013; NACA 2014) and Mexico as AHPND (Nunan et al. 2014; Soto-Rodriguez et al. 2015). Bacterial isolates were molecularly identified as *Vibrio parahaemolyticus* (VP) by the tlh gene (Bej et al. 1999). The VP strains tdh-, trh- and tlh+ were further characterized by enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) (Gomez-Gil et al. 2004); primers AP, AP1, AP2, AP3, AP4, ems2 IQ2000™ and IQ REAL™ AHPND/EMS (GeneReach, Taiwan POC) were used in the diagnostic tests for AHPND. Molecular characterization of tlh+, tdh- and trh- isolates was fingerprinted using rep-PCR, and several clones were detected. All other isolates produced different results and were considered strains; in total, 31 strains were obtained (Fig. 3). All primers and kits used as diagnostic tests have shown inconsistency to detect AHPND, except for AP4.

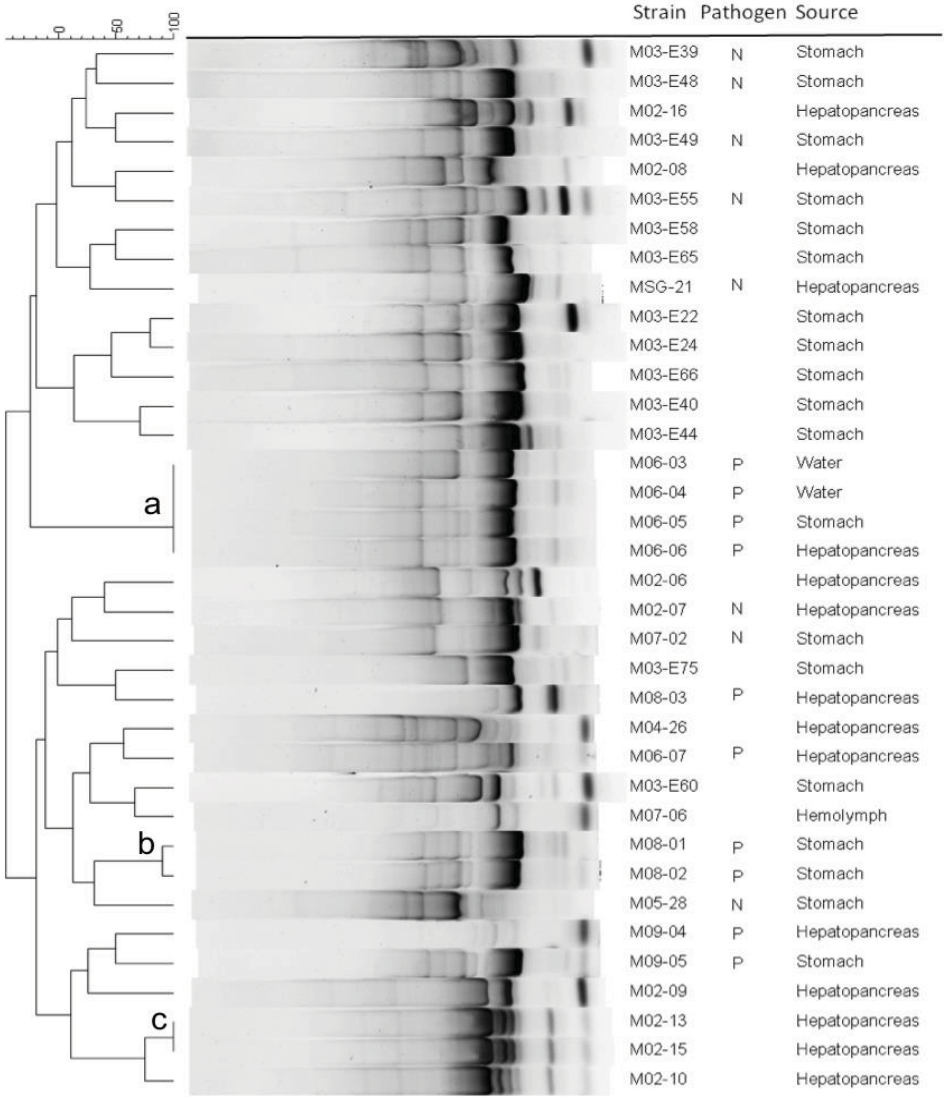

Fig. 3. ERIC-PCR fingerprinting of *Vibrio parahaemolyticus* isolated from shrimp (*Penaeus vannamei*) and water from ponds affected by acute hepatopancreatic necrosis disease (AHPND) in Mexico. N, nonpathogenic; P, pathogenic. Letters in the dendrogram denote clonal groups (95 %). Band position tolerance, 1 %; optimization, 0.2 %.

Bacterial Challenges with Pure VP Strains

In addition, experimental challenges with juvenile shrimp showed VP strains had different virulence: some of the less-virulent strains do not induce 100 % mortality, and mortality rates also rise more slowly than they do for the more virulent strains (Fig. 4).

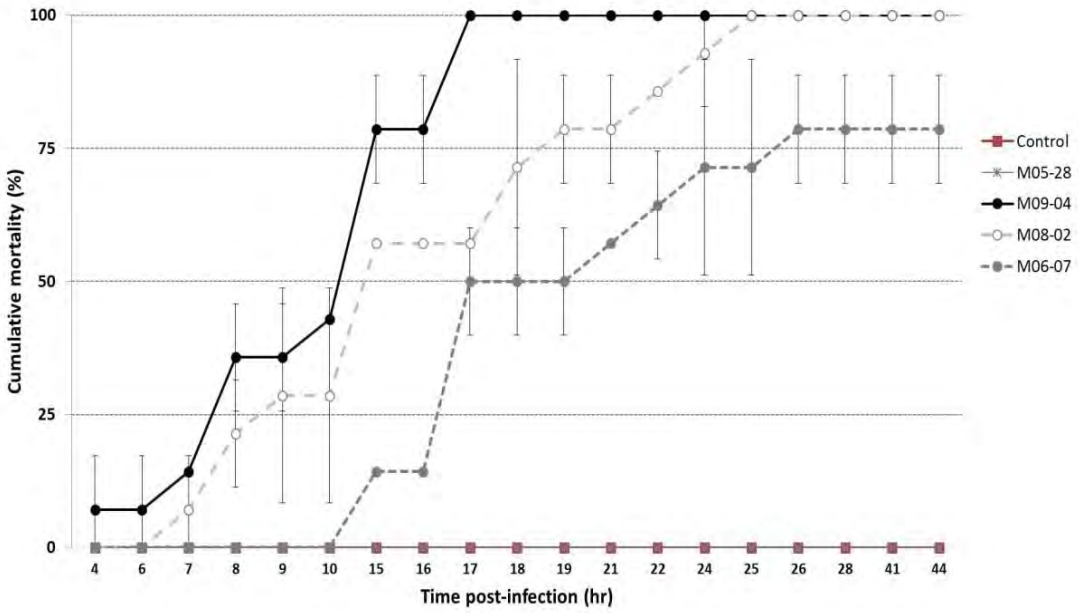

Fig. 4. Example of cumulative mortality of juvenile shrimp challenged with *Vibrio parahaemolyticus* strains. M05-28: 1.20 x 10^7, M09-04: 2.20 x 10^6, M08-02: 3.30 x 10^6, M06-07: 7.82 x 10^6 cfu.mL^{-1}. Control: tryptic soy broth+2.0 % NaCl. Bars indicate standard deviations.

The virulence of VP strains was dose dependent, where the threshold infective density was 10^4cfu.mL^{-1}; below that density, no mortality was observed. Field and experimental results showed that the VP strain that causes AHPND acts as a primary pathogen for shrimp compared with the VP strains reported to date (Soto-Rodriguez et al. 2015).

All VP strains were used in immersion challenges with shrimp, showing that farmed shrimp present the same pathological damage in the HP as shrimp coming from controlled environments. Organisms exposed to pathogenic strains (VP_{AHPND+}) systematically showed lesions associated with each disease phase previously described, but in addition, histopathological analysis of survival organisms from challenges showed lesions, considered as an additional phase of the disease (unpublished data) resulting from the evolution of the disease. In preliminary studies, survival organisms of AHPND showed filled gut and normal appearance of HP. Histology showed damage in the HP ranging from low necrotic lesions (similar to the terminal phase of AHPND) to a flattened epithelium without vacuoles and with a few elongated cells. Future studies are needed to obtain a deeper knowledge of the pathological progress of survival organisms of AHPND and to know the repair mechanisms, including the homeostatic state used by survival organisms to overcome the disease.

Phenotypic Characterization

VP_{AHPND+} and VP_{AHPND-} strains from Mexico have similar morphological and physiological characteristics. All strains are motile Gram-negative rods; non-luminescent; grow as green colonies on TCBS and as mauve, blue and beige colonies on CRHOMagar ™ Vibrio; show OF metabolism; do not produce hydrogen sulfide; and do not utilize either melibiose, L-rhamnose, sorbitol, sucrose or tryptophan deaminase. Bacterial cells of VP had an exponential growth starting at 2 hr at 30 °C; strains showed a wide tolerance to environmental conditions (Table 1), e.g. pH (5–10), temperature (20, 30, 37 and 44 °C) and salinity (from 0.5 to 10.0 % NaCl).

Table 1. Growth of *Vibrio parahaemolyticus* pathogenic (VP_{AHPND+}) and nonpathogenic (VP_{AHPND-}) strains at different temperature, salinity and pH.

Condition		**VP_{AHPND+} strains**					**VP_{AHPND-} strains**				
Temp. (°C)		**M0603**	**M0605**	**M0802**	**M0904**	**M0905**	**M0528**	**M0604**	**M0702**	**M0903**	**SMG21**
	4	-	+	-	-	-	-	-	-	-	-
	20	+	+	+	+	+	+	+	+	+	+
	30	+	+	+	+	+	+	+	+	+	+
	37	+	+	+	+	+	+	+	+	+	+
	44	+	+	+	+	-	-	-	-	-	-
NaCl (%)	0.0	-	-	-	-	-	-	-	-	-	-
	0.5	+	+	+	+	+	+	+	+	+	+
	1.0	+	+	+	+	+	+	+	+	+	+
	2.0	+	+	+	+	+	+	+	+	+	+
	3.0	+	+	+	+	+	+	+	+	+	+
	4.0	+	+	+	+	+	+	+	+	+	+
	5.0	+	+	+	+	+	+	+	+	+	+
	6.0	+	+	+	+	+	+	+	+	+	+
	7.0	+	+	+	+	+	+	+	+	-	+
	8.0	+	+	+	+	+	+	+	+	-	+
	9.0	+	+	+	+	+	-	+	-	-	+
	10.0	-	+	-	-	-	-	+	-	-	+
pH	4	-	-	-	-	-	-	-	-	-	-
	5	+	+	+	+	+	-	+	-	+	+
	6	+	+	+	+	+	+	+	+	+	+
	7	+	+	+	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+	+	+	+
	9	+	+	+	+	+	+	+	+	+	+
	10	+	+	+	+	+	+	+	+	+	+

Antimicrobial Sensitivity

Pathogenic VP strains were most sensitive to nalidixic acid and showed resistance to penicillin (Fig. 5). Some alternatives used by shrimp farmers to control AHNPD in Mexico include the use of controlled production systems, prebiotics, probiotics, biofloc technology (BFT) and the use of genetic lines more tolerant to pathogens.

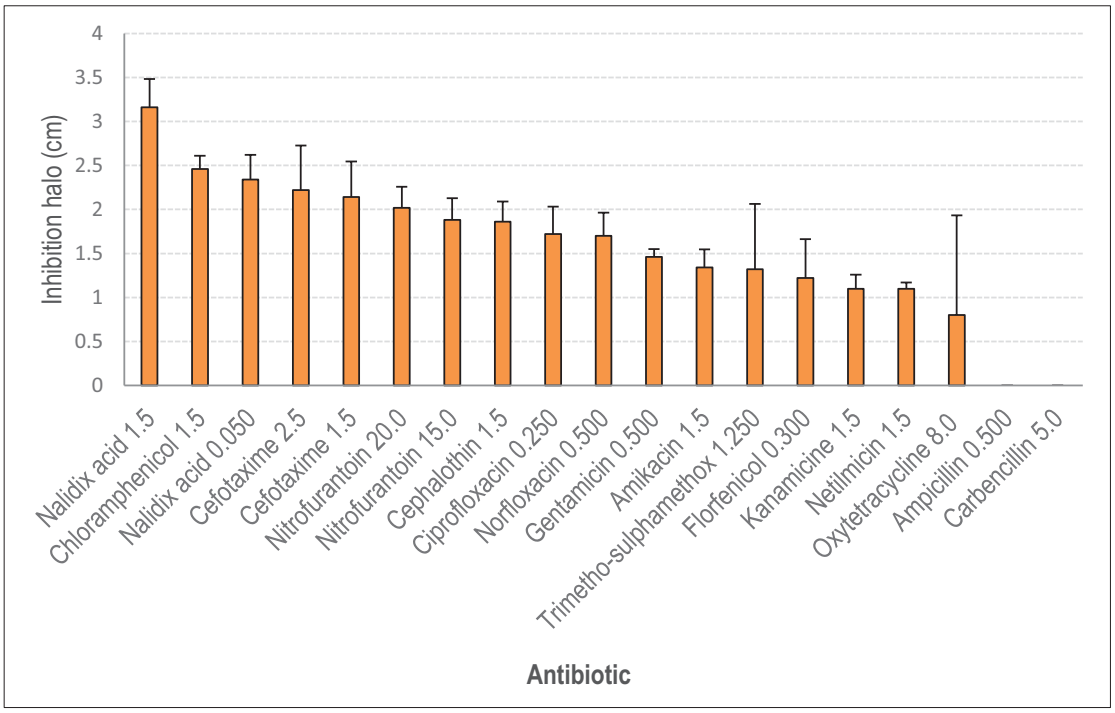

Fig. 5. Antibiotic sensitivity of pathogenic *Vibrio parahaemolyticus* (n=5) isolated from Mexico. Antibiotic concentration is in mg mL^{-1}. Bars indicate standard deviations.

Genomic Comparison

Whole genomic sequence of 22 VP_{AHPND+} and VP_{AHPND-} strains from Asia, Mexico and South America shows the presence of similar chromosomic pathogenic mechanisms. Comparative analysis by multilocus sequence analysis (MLSA) of seven genes clearly showed that most of the isolates are independent strains, although some could not be differentiated at this level and thus considered MLSA clones because they have similarity values equal or above 99.9 % (Fig. 6). By comparison of the 73.5 bp conjugative plasmid (pVp-AP) of pathogenic VP from Asia and Mexico, we observed that all VP strains have one transposon (that includes the PirA and PirB-toxin like genes), but only strains from Mexico and Viet Nam have a second transposon. Genomic characterization of pathogenic VP strains found the PirA- and PirB-like toxins as the main virulence factor in a conjugative plasmid flanked by transposases (Han et al. 2015).

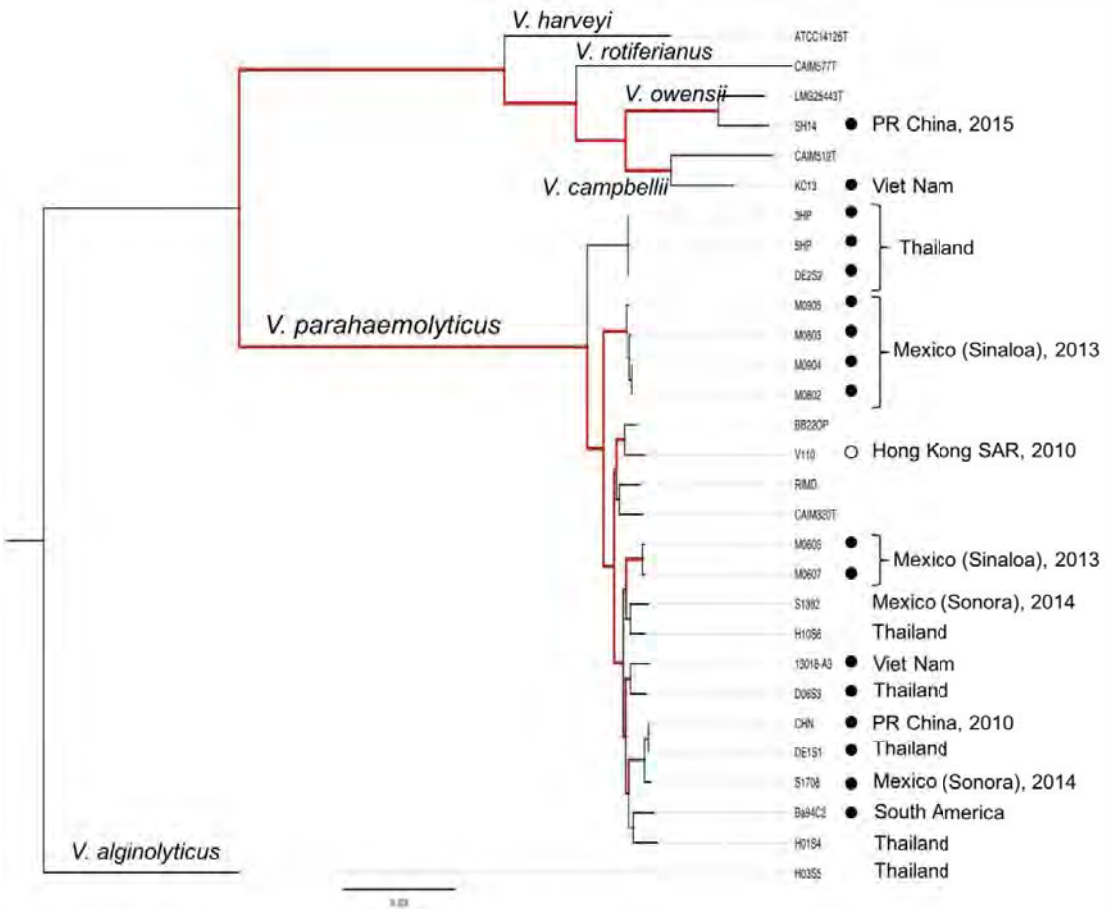

Fig. 6. Comparative analysis by multilocus sequence analysis (MLSA) of seven genes from 22 VP_{AHPND+} and VP_{AHPND-} strains from Asia, Mexico and South America.

Conclusion

The VP pure strains used in challenges with shrimp, and farmed and challenged shrimp presented the same clinical and pathological signs. AHPND had three phases (initial, acute and terminal) while challenged survival organisms showed an additional phase. VP had different virulence and was dose dependent. Our research team will do a further study on the toxin dynamics during the infectious process in P. vannamei. Pathogenic and non-pathogenic VP strains showed a wide tolerance to temperature, salinity and pH due to high metabolic diversity. This enabled them to adapt and survive in almost all marine-estuarine environments where shrimp farms are located, which implies a big risk of outbreaks and fast disease dispersion to free zones. The phenotypic profile (biochemical, morphological and physiological) was similar between pathogenic and non-pathogenic VP strains, but pathogenic VP were most sensitive to antibiotics and showed moderate resistance to oxytetracycline, one of the antibiotics most used in Mexican shrimp farms to control vibriosis.

Since 2013, some alternatives used by shrimp farmers to control AHNPD in Mexico include the use of controlled production systems, prebiotics, probiotics, biofloc technology (BFT) and the use of genetic lines more tolerant to pathogens. VP strains from Asia, Mexico and South America showed that most of the isolates are independent strains by genomic and molecular analysis, so that new pathogenic strains could be detected.

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Specific Pathogen Free (SPF), Specific Pathogen Resistant (SPR) and Specific Pathogen Tolerant (SPT) as Part of the Biosecurity Strategy for Whiteleg Shrimp (*Penaeus vannamei* Boone 1931)

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Abstract

The sanitary and genetic characterization of farmed penaeid shrimp needs to be selected based on the type of culture system and biosecurity strategy applied. This paper attempts to define the concepts specific pathogen free (SPF), specific pathogen resistant (SPR) and specific pathogen tolerant (SPT) and presents the process for development of a combined approach where white-spot syndrome virus (WSSV) SPT/SPR animals were selected to create SPF stocks for use in a low-biosecurity facility. The success of this approach was proven when they were imported and adopted as the only stocks cultured in the Kingdom of Saudi Arabia after the local species were wiped out by WSSV. Record national production has been achieved since their introduction and WSSV has been eradicated even from the wild population. It is suggested that this type of stock (WSSV SPT+SPF) could be an alternative for countries still impacted by WSSV that do not have the technical competence or the investment capacity to transform the industry to small intensive ponds where viral exclusion strategies could be implemented.

Keywords: biosecurity, disease control, *Penaeus vannamei*, specific pathogen free (SPF), specific pathogen resistant (SPT), specific pathogen tolerant (SPT), white-spot syndrome virus (WSSV)

Introduction

Diseases continue to affect the sustainability of shrimp farming, and their prevention requires the definition and implementation of a biosecurity strategy specific for each facility, culture system and sanitary zone. Biosecurity is a broad term that is often poorly understood. FAO (2003) defines biosecurity as follows:

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"Biosecurity is a strategic and integrated approach that encompasses the policy and regulatory frameworks (including instruments and activities) that analyse and manage risks in the sectors of food safety, animal life and health, and plant life and health, including associated environmental risk. Biosecurity covers the introduction of plant pests, animal pests and diseases, and zoonoses, the introduction and release of genetically modified organisms (GMOs) and their products, and the introduction and management of invasive alien species and genotypes."

In animal farming, we can synthesize that biosecurity includes all those activities necessary to prevent, control and manage the risk to animal health and life with the objective of reducing the economic impact of diseases. In other words, biosecurity is a tool for sustainability. The activities that are a fundamental part of biosecurity range from the international framework through intergovernmental agreements, to the national framework through national legislation and enforcement, to the scientific research that generates the knowledge needed to develop sound preventive measures and finally, to the implementation of such measures in animal production.

These production oriented biosecurity measures have three components: those activities related to the environment and the management of the culture conditions, those involving the pathogen and the sanitary status of the animals, and last, the characteristics of the shrimp and its genetics which is the focus of this paper.

Sanitary and genetic characterization of the shrimp

When considering the type of shrimp for culture and the biosecurity strategy to be applied, their sanitary status and genetic characteristics need to be considered.

Regarding their sanitary status, the stocks can be classified as:

- specific pathogen free (SPF), meaning that they are free from specific pathogens, but not necessarily free of all pathogens;
- pathogen free (PE), meaning that they are free of all pathogens (however, this is difficult to prove and assure);
- all pathogen exposed (APE), meaning that they have been exposed to potential pathogens, (these are, for example, the broodstock collected from ponds); or
- high health (HH), this is a commercial term and vague in terms of description of the stocks.

Regarding their genetic characteristics, the stocks can be classified as:

- susceptible, to infection and disease;
- specific pathogen resistant (SPR), meaning resistant to infection by a specific pathogen (this is a qualitative trait – they can either be infected or not); or
- specific pathogen tolerant (SPT), meaning tolerant to a specific disease, (the animal can be infected but may not develop the disease or it may develop the it to a lesser extent).

Specific Pathogen Free Stocks (SPF)

The term "specific pathogen free" has been often misused, and the proper understanding of the concept is not widespread within the shrimp farming industry. An SPF animal can be defined as one coming from a population that has tested negative for specific pathogens for at least two years (a surveillance programme must be in place), that is raised in highly biosecure facilities (i.e. with appropriate water treatment and an enclosed environment) following biosecure management measures and has been fed with biosecure feeds. Therefore, the SPF characterization refers exclusively to the history of the sanitary status of the animal, the facility and the culture conditions under which it has been raised and maintained.

Claiming SPF status requires transparency through regular auditing. An SPF animal is not necessarily more susceptible or more tolerant to pathogens, and they do not necessarily have less genetic diversity or better or worse growth performance. It only refers to its sanitary status, and this condition is not hereditary. As soon as an SPF animal is exposed to potential pathogens (i.e. by movement to a facility having a lower level of biosecurity), the SPF status is lost. Often these animals are referred to as HH.

As mentioned previously, SPF refers only to freedom from certain pathogens, not freedom from all pathogens. There is no consensus of the list of pathogens that SPF animals need to be free of. In some instances, only those pathogens listed by the World Organisation for Animal Health (OIE) are considered. It is recommended that SPF animals should be free from all known pathogens of penaeid shrimp as OIE list is not dynamic enough to include in a timely manner all relevant pathogens to shrimp culture.

The SPF approach is not a new concept, rather, it is normal practice in terrestrial animal husbandry. In fact, the idea of stocking (i.e. investing) in infected animals is difficult to understand from the veterinary point of view. In addition, the use of SPF animals provides an advantageous starting point for proper progress in breeding programmes, as sanitary variables are removed. They are also fundamental for international trade, in order to prevent the transboundary movement of pathogens and research purposes.

Industry approaches to WSSV Epizootics

Over the past several decades, outbreaks of white spot disease (WSD), caused by WSSV have caused major losses in both Asia and Latin America. In each continent, the industry took different approaches to manage the disease. While Asia chose to use SPF stocks and implement high-biosecurity measures during culture, Latin America chose to cope with the virus and to not try to exclude it out of the system.

As seen in Fig. 1, Thailand, as an example of an Asian culture system, recovered very quickly after WSSV through the use of small ponds that allow control of the culture conditions, the introduction of SPF stocks and biosecurity implementation based on viral carrier exclusion.

In fact, the use of SPF stocks changed the industry, permitting it to reach a productivity never seen before, as it allowed the development of very successful breeding programmes.

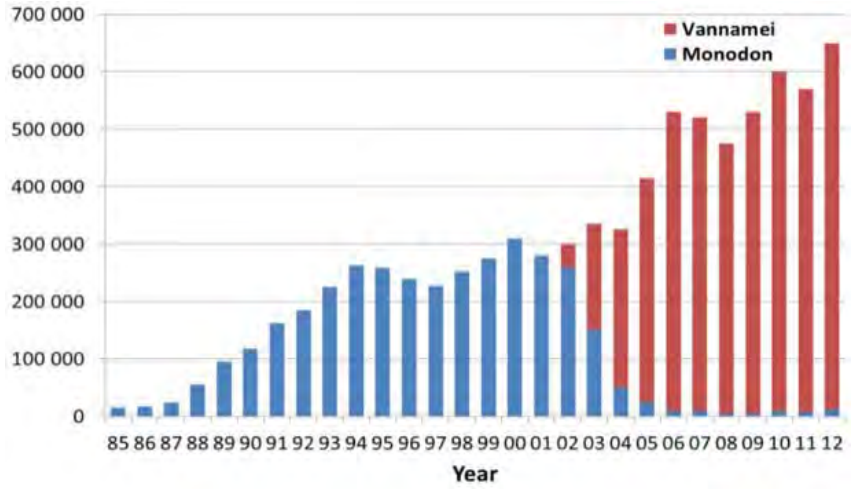

Fig. 1. Thai shrimp production (tonnes), 1985 to 2012.

The opposite approach is represented by Ecuadorian production (Fig. 2). With all the associated economic and social consequences resulting from WSD, the recovery of the industry took a longer period. However, such an approach led to the spontaneous development of shrimp tolerant to WSSV over a period of exposure to the virus, developing what we now refer to as WSSV SPT animals.

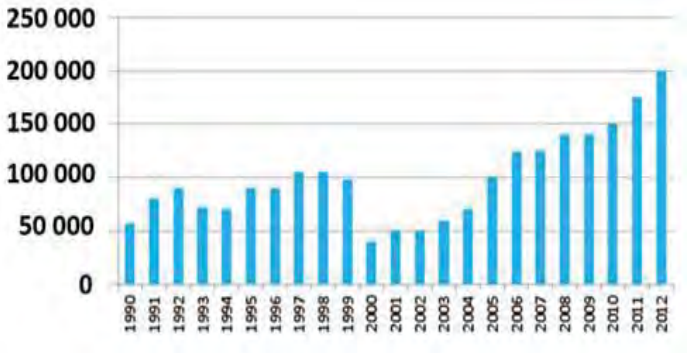

Fig. 2. Ecuadorian shrimp production (thousand tonnes), 1990 to 2012.

Analysing the reaction of both industries in retrospective, the idea of developing an SPF stock from WSSV SPT animals was conceived. From the first outbreaks of WSD in Latin America in 1999 until 2010, the degree of WSSV tolerance achieved in the population was considered acceptable following the increase in survival over the years despite of the presence of WSSV in the ponds.

The WSSV SPT+SPR animals resulting from this programme would be free of infection and therefore, there would be less chance of disease outbreaks and better productivity, while

there would be no need to implement strict biosecurity regarding WSD, with the costs associated to it.

The normal practice in Latin America is to collect adult shrimp as broodstock from the ponds. Doing so implies that most of these shrimp are likely to be infected with any pathogen present in the pond, and the impact of any disease is likely to be much more significant when there is vertical transmission rather than horizontal transmission of pathogens in the pond. Considering that acute hepatopancreatic necrosis disease (AHPND) was already affecting Asia and there was the possibility of its spread to new geographical areas, the plan was to develop SPF animals prior to the arrival of a new epizootic. Any selection for increased tolerance to new pathogens should be done on an experimental basis, rather than at an industrial scale, which had happened previously, posing an enormous cost to the farmers.

DEVELOPMENT OF WSSV SPT+SPF STOCKS

Two projects to develop SPF stocks from WSSV SPT stocks were started, one in Ecuador and a second in Nicaragua. The projects started in 2010 and lasted until 2012. The projects targeted the main pathogens of penaeid shrimp as known at that time. These included six OIE-listed pathogens and three others:

- OIE-listed pathogens (2010):
    - White-spot syndrome virus (WSSV)
    - Yellow head virus (YHV)
    - Infectious myonecrosis virus (IMNV)
    - Necrotizing hepatopancreatitis (NHP)
    - Taura syndrome virus (TSV)
    - Infectious hypodermal and haematopoietic necrosis virus (IHHNV)
- Other non-OIE listed pathogens included in the screeningg
    - *Enterocytozoon hepatopenaei* (EHP)
    - *Penaeus vannamei nodavirus* (PVNV)
    - *Streptococcus* spp.

As diagnostic tools, nested polymerase chain reaction (PCR) (IQ2000 kits) and histology were used. The company diagnostic laboratories successfully participated in the Arizona Ring Test. The projects started with a nationwide surveillance for primary pathogens and consultations with research centres and official diagnostic centres to learn about enzootic pathogens. The only primary pathogens found were WSSV, IHHNV and NHP. From then on, these were considered enzootics and selected animals would be tested individually for each of them, while they would be tested in pools of 10 animals for the rest of the pathogens (non-endemic). The first objective was to identify individuals exposed to pathogens from the ponds (APE) that were free of pathogens. The target size was between 23 and 30 g, as it was assumed that if animals could reach this size free of pathogens, then they would have specific characteristics of tolerance (SPT) or resistance (SPR) to infection. Other criteria for selection

were that the shrimp should be from ponds identified as having high productivity and that to broaden the genetic diversity, they should have as broad geographic origin as possible.

Vertical transmission of pathogen may have different pathways; it can happen within the egg (intra-ovum), on the egg surface (per-ovum) or through the contaminated faeces of the broodstock (Table 1). From a health-management perspective, we can assume that all systemic viruses have some degree of vertical transmission and therefore, infected animals are not acceptable for an SPF development project. Enteric pathogens allow certain management strategies to prevent the contamination of the nauplia. Of the enzootic pathogens, both WSSV and IHHNV have intra-ovum vertical transmission, which meant that any infected animal had to be discarded, as it could transmit the infection to offspring. As NHP is caused by a bacterium, oral treatment of the selected animals with oxytetracycline upon arrival combined with an egg washing with the same antibiotic resulted in the eradication of NHP in both projects.

Table 1. List of shrimp pathogens, their economic relevance, body distribution, type of vertical transmission and recommended broodstock strategy.

Pathogen[1]	**Economic impact**	**Distribution**	**Vertical transmission**	**Broodstock strategy**
WSSV	High (highly prevalent)	Systemic	Intra-ovum	Eliminate
TSV	High (sporadic)	Systemic	Per-ovum	Eliminate
YHV	High (sporadic)	Systemic	Per-ovum	Eliminate
IHHNV	Medium	Systemic	Intra-ovum	Eliminate
YHV/GAV	High (localized)	Systemic	Per-ovum	Eliminate
IMNV	High (localized)	Systemic	Per-ovum	Eliminate
Streptococcus	High (localized)	Systemic	Per-ovum/ Faeces/opening of mouth	Management
NHP	Medium	Digestive track	Faeces/opening of mouth	Management
BP, MBV, HPV	Low	Digestive track	Faeces/opening of mouth	Management
EHP	High	Digestive track	Faeces/opening of mouth	Management
AHPND	High	Internal & external cuticle & faeces	Surface colonization Faeces/opening of mouth	Management

[1]AHPND = acute hepatopancreatic necrosis disease, BP = *Baculovirus penaei*, EHP = *Enterocytozoon hepatopenaei,* GAV = gill-associated virus, HPV = hepatopancreatic parvo-like virus, IHHNV = infectious hypodermal and haematopoietic necrosis virus, IMNV = infectious myonecrosis virus, MBV = Monodon baculovirus, NHP = necrotising hepatopancreatitis, TSV = Taura syndrome virus, WSSV = white-spot syndrome virus.

To conduct these projects, several additional facilities were needed: quarantine 1 and 2, broodstock multiplication centres and larviculture. Water treatments were implemented, biofloc was established indoors, and all fresh feeds were tested by PCR for known pathogens. Biosecurity protocols were established and training considered crucial for the implementation of biosecurity was provided to staff.

At the time of selection of ponds from which to collect animals, a preliminary screening for enzootic pathogens (WSSV, IHHNV and NHP) of 75 animals was performed. Great variability was found between ponds. Only those ponds with low prevalence and good productivity were used for this purpose.

Animals were brought individually to the facilities where they were cold stressed (22–24 ^{o}C) in individual buckets. After 48 hr, individual testing was done for WSSV and IHHNV (in pleopods) and NHP (in faeces), and then done again for each female after ablation and spawning. In addition, each individual contributed to pool reactions (10) for TSV, IMNV, YGV/GAV, PVNV, BP and EHP. Pools of postlarvae (PL) (n=120) were again tested for WSSV and IHHNV after cold challenge. Finally, histology of the broodstock was performed for unknown pathogens.

Each animal was tested individually 2 times (males) or 3 times (females) and as a pools, at least twice. The process was repeated for three generations with consecutive negative results. Considering that international standards (OIE and European Union, EU) to declare freedom of disease require two years of testing, we could declare the populations to be SPF by 2012.

In order to add confidence to the process and with concern for the possibility of having missed latent infections, other testing procedures were used, such as using alternative tissue to the pleopod (e.g. haemolymph, gills and lymphoid organ) and different types of stressors (e.g. pH, salinity and alkalinity). In none of these trials was a primary pathogen detected from the cleaned population.

Due to its high prevalence which resulted in very high cost, the project in Nicaragua decided not to exclude IHHNV. The project in Ecuador successfully eliminated all primary pathogens, achieving SPF status. The number of PCR tests performed during the two years of development was over 64 000 in Ecuador and over 100 000 in Nicaragua. The percentage of broodstock discarded was 74 % in Ecuador and 47 % in Nicaragua.

The surveillance within the selected population continued for two years with consecutive negative results. At that time, experts from the Department of Infectious Diseases, University of Zaragoza (Spain) who had been appointed by the EU to declare zones/populations disease free, were invited to audit the programme and granted SPF status to the Ecuadorian program. This auditing procedure continues to be conducted on an annual basis.

The use of SPF populations was immediately reflected in better productivity at every step of production:

- Maturation:
    - Mortality in reception room dropped from 24 to 0.5 %.
    - Mortality after ablation dropped from 15 to 0.3 %.
    - Female mortality during production dropped from 5 to 0.1 %.
- Larviculture:
    - Days to PL12 were reduced from 20–21 to 17–18.
    - $PL.g^{-1}$ were reduced from 350 to 200.
    - Survival increased from 45–50 to 70–75 %.
    - Coefficient of variation decreased from >15 to 12 %.
- Grow out:
    - Grow out was 6 weeks shorter to reach 15 g.

In addition, these WSSV SPT+SPF stocks were cultured under intensive conditions at the same time that SPF stocks were brought in from the United States of America that were known to be susceptible to WSSV but were reported to have higher growth rate. In the intensive farm, high biosecurity was implemented; however, two of the ponds were hit by WSSV and harvesting was carried out as soon as possible. The harvested SPF susceptible stocks had a survival rate of approximately 35 %, while the survival rate of the WSSV SPT+SPF stock was approximately 70 %. These results prove the validity of using a combined approach of genetic characterization and sanitary status as a biosecurity strategy.

The proposed name for these type of SPF stocks is "Reverse SPF", as in order to develop them, the reverse process is required when compared to the development of a conventional SPF stock. Conventional SPF are originated from areas with the minimum exposure to pathogens while Reverse SPF are originated from areas where pathogens are highly prevalent and have survived these conditions.

Application of WSSV SPT+SPF Shrimp

The Ecuadorian stocks were exported to the Kingdom of Saudi Arabia, where the industry had been wiped out by WSSV. Culture systems in the Kingdom of Saudi Arabia are similar to those used in Ecuador, with big ponds (10 ha) which do not allow the implementation of viral exclusion biosecurity nor the control of culture conditions. Despite the harsh desert conditions with daily water temperature fluctuations of up to 10 °C and high salinity (often up to 55 ppt), *Penaeus vannamei* Boone 1931 performed very well, leading to national record production (Fig. 3).

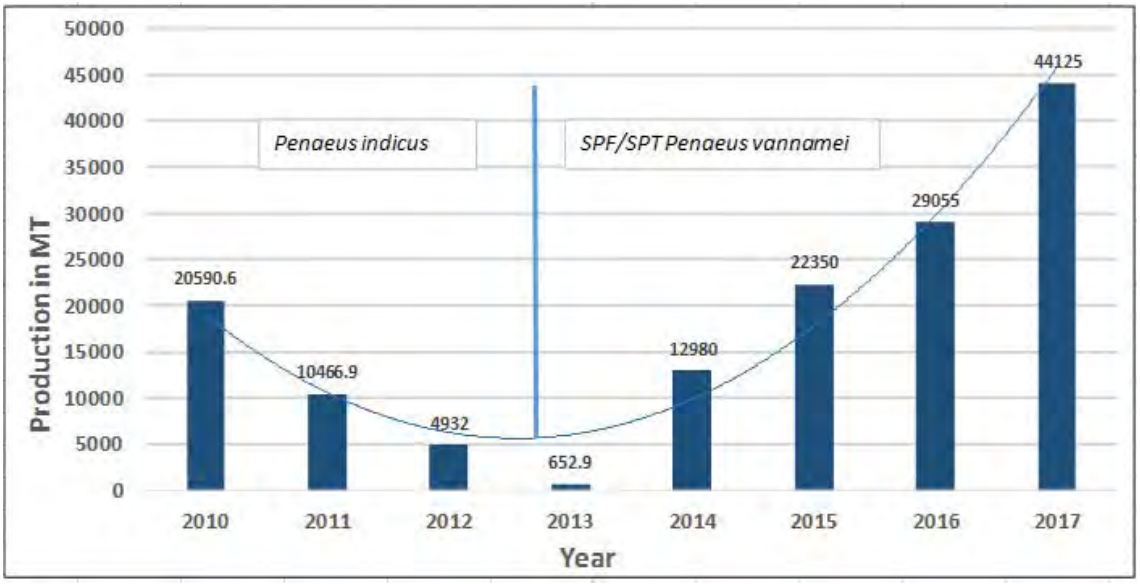

Fig. 3. Shrimp production (tonnes) in the Kingdom of Saudi Arabia, 2010 to 2016.

These are currently the only stocks cultured in the country, and no other source has been approved by the government, as other potential sources are either susceptible SPF, or WSSV SPT but not SPF. The exclusive use of these stocks all over the country is considered to be one of the keys to the success of the biosecurity strategy. In addition to the private-company surveillance programmes, the government has its own monthly surveillance programme. Since 2014, only two detections of WSSV have occurred (in 2015 and 2016), both of them localized in the south. Some mortality was observed and after-harvest survival remained at approximately 70 %. The experience of using WSSV SPT+SPF as demonstrated in the Kingdom of Saudi Arabia could be a solution for countries still impacted by WSSV that do not have the knowledge or the investment capacity to develop small, biosecurity controlled ponds. These stocks could also be used to reduce the cost of biosecurity in intensive systems; however, this would first require the improvement of growth through a breeding programme to be able to match the growth of other SPF stocks currently in the market. From this experience, it can be concluded that the use of SPF stocks is a fundamental strategy to the sustainability of shrimp farming including the extensive and semi-extensive systems with low/none biosecurity. When pathogen exclusion is not possible, SPF status needs to be combined with SPT/SPR characteristics of the stocks for the pathogens endemic in the farming region. SPF claim only defines the health status but there is a common misconception that SPF are more tolerant to diseases (misconception more widespread in Asia) or more susceptible to disease (misconception more widespread in Latin America). As a farmer, we should question stocking, in other words, investing, in animals which are infected by the time we stock.

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Environmental Factors and Acute Hepatopancreatic Necrosis Disease (AHPND) in Shrimp Ponds in Viet Nam: Practices for Reducing Risks

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Abstract

Water and sediment quality in shrimp ponds in Soc Trang, Bac Lieu and Ca Mau provinces in the Mekong River Delta, Viet Nam did not differ ($P > 0.05$) between ponds containing AHPND-infected shrimp and those with AHPND-free shrimp. However, there was evidence of wide-spread pollution in the sampling area, pond management procedures were often deficient, and general water quality conditions in some ponds were suboptimal. Farms in the study area take in water from the same canals into which they discharge effluent, favouring cross-contamination spread of shrimp diseases among farms. The risk of AHPND in shrimp ponds could be lessened by improvements in biosecurity and pond management that would result from adoption of good aquaculture practices (GAPs) described herein.

Keywords: AHPND, EMS, good aquaculture practices, pollution and shrimp farming, water quality in shrimp ponds.

Introduction

High mortality of shrimp during the first 30 days of culture for unknown reasons observed first in the People's Republic of China around 2009 was referred to as covert mortality disease and later as early mortality syndrome (EMS). The phenomenon subsequently spread to Viet Nam, Malaysia and Thailand, resulting in major losses of shrimp.

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Lightner et al. (2012) described the pathology of EMS and suggested that the malady be called acute hepatopancreatic necrosis syndrome (AHPNS). One possible cause proposed for AHPNS was a toxic agent of natural or anthropogenic origin (NACA 2012), and an environmental survey and monitoring effort was initiated in September 2012 as part of the Food and Agriculture Organization of the United Nations (FAO) programme TCP/VIE/3304 "Emergency assistance to control the spread of an unknown disease affecting shrimps in Viet Nam."[1]

The cause of AHPNS was identified in early 2013 as a bacterial infection of the hepatopancreas of shrimp with a form of *Vibrio parahaemolyticus* that contains a phage (Tran et al. 2013a,b; Oanh et al. 2013). This cause of early mortality in shrimp is now known as acute hepatopancreatic necrosis disease (AHPND). Despite elucidation of the aetiology of AHPND, findings of the environmental study are important. The study provides critical information on conditions in culture ponds and their surroundings that can be useful in modifying farm operations and pond management to lessen the risk of AHPND.

Materials and Methods

The environmental study was conducted in Soc Trang, Bac Lieu, and Ca Mau provinces in the Mekong River Delta, Viet Nam. These provinces represent the major shrimp production area of Viet Nam (Table 1), and most of the intensive shrimp culture (farms using both feeding and aeration) in Viet Nam is done in these three provinces. Although intensive farms represent only a portion of shrimp aquaculture in Viet Nam, it was thought that owners of intensive farms could provide more reliable records of management inputs and current and previous production success than could owners of extensive and semi-intensive farms.

Table 1. Shrimp production for 2010 in the three major shrimp-farming provinces of Viet Nam.

Location	Pond Area (ha)	2010 Production (tonnes)	2010 Production ($kg.ha^{-1}$)
Viet Nam	722 000	449 652	622
Three major shrimp-producing provinces:			
Soc Trang	48 000	60 830	1 267
Bac Lieu	126 000	70 462	559
Ca Mau	265 000	108 847	411

Environmental Observations and Farm Survey

Six farms were selected in each province for observations and interviews with owners. Travel among farms and provinces by car allowed observations of canal networks that supply water to both agriculture and aquaculture, agricultural activities in the vicinity of shrimp farms, population distribution and industrial development.

[1] The report can be downloaded at http://www.fao.org/docrep/018/i3422e/i3422e00.htm.

Focus was mainly on specific activities and development that represented possible sources of pollution that might negatively impact shrimp farming. At each farm, observations of farm layout, water supply, condition of infrastructure and pond management were made, and farm owners were interviewed to obtain information on items listed in Table 2.

Table 2. List of information requested from shrimp farmers during interviews.

Location of farm	**Use of feed amendments**
Farm age	Aeration rate
Number of ponds	Water exchange practice
Area of ponds	Treatment of water used for exchange or replacement
Depth of ponds	Specific chemicals used during crop, dose and application frequency: • Fertilizers and minerals • Liming materials • Disinfectants • Zeolite • Other
Source(s) of water	
Salinity range	
Pond bottom preparation: • Dry-out time • Sediment removal practice • Liming practice • Tilling practice	
Water disinfection: • In reservoir – chemicals and concentrations • In ponds before stocking – chemicals and concentrations	Probiotic use Usual appearance of water during culture Survival and success in past Observations related to onset of EMS/AHPNS Opinion about cause of EMS/AHPNS Agricultural activities in vicinity Use of agricultural pesticides to disinfect ponds in past Other sources of pollution
Species stocked	
Postlarval health	
Stocking density	
Expected production	
EMS/AHPNS status	
Feed protein content	

Environmental Sampling

The environmental sampling was conducted between 11 September and 9 October 2012 in three rounds: 22 ponds and seven canals in the first round, 21 ponds in the second round and 11 ponds in the third round. The AHPND status (infected or not infected) of shrimp in ponds was ascertained by a shrimp disease specialist from Can Tho University who made the determination based on the gross clinical signs of AHPND as described by Lightner et al. (2012). Twenty-three of the study ponds were stocked with *Penaeus monodon* Fabricius 1798 and 31 were stocked with *Penaeus vannamei* Boone 1931. Of the *P. monodon* ponds, 16 (≈70 %) contained AHPND-infected shrimp, while 13 (≈58 %) of the *P. vannamei* ponds had infected shrimp.

Water samples were dipped from the surface, placed in appropriately sized bottles and preserved (Eaton et al. 2005) as follows: at 4 °C in dark [5-day biochemical oxygen demand (BOD_5), total ammonia nitrogen (TAN), nitrite-nitrogen (NO_2^--N), and nitrate-nitrogen (NO_3-N)]; acidified to pH 2 with sulfuric acid and stored in the dark at 4 °C [total nitrogen (TN) and total phosphorus (TP)]; fixed in a BOD bottle [dissolved oxygen (DO)]; preserved with zinc acetate at pH 9 (total sulfide); particulate matter removed on Gelman GF/C glass fiber filters and frozen at -20 °C (chlorophyll *a*); treated with 1 mL.500 mL^{-1} concentrated nitric acid and stored at 4 °C (trace elements). Water samples also were collected from selected ponds, nine with AHPND-free shrimp and 13 with AHPND-infected shrimp, for pesticide and algal toxin analyses. These samples were dipped, placed in bottles and stored in the dark at 4 °C. Sediment samples also were collected from the upper 5-cm layer in these ponds for analyses for pesticides and trace elements. These samples were stored in plastic bags and held in the dark at 4 °C for later analysis. In May 2013, water samples for analysis for polychlorinated biphenyl compounds (PCBs) were collected from shrimp ponds and their water supply canal in each of the three provinces (n = 12). These samples were preserved in the same manner as pesticide samples.

Analyses of Samples

Water temperature, pH and salinity were measured *in situ* with a portable, multiprobe meter. Analyses for the common water quality variables, DO, BOD_5, TAN, NO_2^--N, NO_3^--N, TN, TP, total sulfide and chlorophyll *a,* as well as calculation of un-ionized ammonia nitrogen (NH_3-N) and un-ionized sulfide sulfur (H_2S-S), followed standard protocol (Eaton et al. 2005). The analyses were conducted at the Laboratory of Water Quality Management, College of Aquaculture and Fisheries, Can Tho University.

Trace metal, pesticide, and PCB analyses were made at the Laboratory Centre for Environmental Analysis and Technology Transfer, Institute for Agriculture and Environment, Hanoi, Viet Nam. The trace element analyses were by atomic absorption spectrophotometry using American Society for Testing and Materials methods (ASTM 2011). Pesticides and PCBs were determined by procedures recommended by the U.S. Environmental Protection Agency (http:/epa.gov/osw/hazard/testmethods/sw846/pdfs/8081b.pdf). The pesticide analyses included fenitrothion, deltamethrin and hexaconazole, which are common pesticides used in the Mekong Delta. Algal toxin analyses were made by the Laboratory of National Agro-Forestry-Fisheries Quality Assurance Department – Branch 6, Can Tho, Viet Nam. The mouse bioassay procedure was used (AOAC 2006).

The design of the survey was simple, and only basic statistical analyses – means, standard errors, Student t-test and analysis of variance with Duncan's multiple range test – were used in the data evaluation.

Results

Water Supply

Farms receive water from canals branching from estuarine reaches of rivers or the sea. In Soc Trang and Bac Lieu provinces, study farms were between 1 and 12 km from the sea, while in Ca Mau Province, distances to the sea ranged from 14 to 31 km. However, water in canals often traveled much farther than the straight-line distance from the sea to a farm. For example, a farm near Bac Lieu City was only 2 km from the sea, but it received water that flowed about 20 km in a canal before reaching the farm. Surface water in the Mekong River Delta seems almost totally interconnected – especially in the rainy season. Rice and other cereal grains are cultivated in the three provinces (http://www.gso.gov.vn/ default_en.aspx?tabid=469&idmid= 3&ItemID=13070). The areas devoted to this type of agriculture in 2010 were as follows: Soc Trang, 353 300 ha; Bac Lieu, 158 400 ha; Ca Mau, 125 800 ha. There is considerable use of insecticides and fungicides on these crops. Rice farming and other agriculture are done in freshwater areas, and water control structures greatly restrict the movement of brackishwater into freshwater canals. Nevertheless, freshwater areas drain into rivers, and pollution from freshwater areas can reach brackishwater canals that supply shrimp farms via river discharge into coastal areas. The population density of the Mekong River Delta is high; for the three provinces of this investigation, average population densities were as follows: Soc Trang, 394 people.km^{-2}; Bac Lieu, 354 people.km^{-2}; Ca Mau, 229 people.km^{-2} (http://www.gso.gov.vn/default_en.aspx?tabid=467&idmid= 3&ItemID=12941). High population density favours in a large amount of domestic and municipal wastewater, and in Viet Nam, wastewater usually is discharged untreated into canals and rivers. The minimum wet-season salinity sometimes falls to 0 ppt at a few farms; at most farms, the salinity remains above 5 ppt. Maximum, dry-season salinities of 10 to 42 ppt were reported. All farms have a wide fluctuation in salinity during a 12-month period.

Farms and their Management

Individual farms had different numbers of ponds; the smallest number was two, while the largest number was 28. Total production areas of individual farms ranged from 0.3 to 9.8 ha. Average depths of individual ponds ranged from 1.0 to 2.0 m, but most ponds averaged around 1.5 m in depth. Ponds at all farms were allowed to dry out between crops. Dry-out time was variable (7 to 60 days), but it did not exceed 15 days at the majority of farms. Sediment was removed from ponds between crops at all but two farms. At four farms practicing sediment removal, high-pressure hoses were used to wash sediment from ponds, but at others, a bulldozer was used to push sediment to pond edges. Pond bottom soil was limed between crops at all but one farm. Burnt lime, also known as quick or unslacked lime, was applied to pond bottoms at nine farms – application rates ranged from 250 to 2 000 kg.ha^{-1}. The recommended rate for disinfection of pond bottom soil with burnt lime is 4 000 to 5 000 kg.ha^{-1} (Hill et al. 2013).

At the other farms using liming material, agricultural limestone (pulverized limestone or marl) was applied at rates ranging from 500 to 2 500 kg.ha^{-1}. Agricultural limestone is not a disinfectant (Boyd and Tucker 1998). At five farms, pond bottoms were tilled with a disk harrow following liming. Water was pumped from water supply canals into farm reservoirs, into farm water supply canals, or in a few cases, directly into ponds. At farms with reservoirs, water was held in reservoirs for 4 to 8 hr for sedimentation. Chemical disinfectants were applied to water after it had been transferred to culture ponds. Calcium hypochlorite and iodine compounds were most commonly applied; other disinfectants reported to be used were: formalin, glutaraldehyde, peroxymonopersulfate, potassium permanganate and trichlorocyanuric acid. The farmers usually could not provide exact information on amounts of disinfectants applied, but the amounts of calcium hypochlorite used were much less than the recommended concentration of 10 mg.L^{-1} of active chlorine (Hill et al. 2013). We could not find references to effective doses of the other compounds, but Hill et al. (2013) indicated that the dose of iodine needed for disinfection was considerably greater than for chlorine. The two farms that did not use chemical disinfectants passed the water from reservoirs through a fine filter (mesh not specified) when transferring it to production ponds.

All farms claimed to stock postlarvae (PL) tested free of specific pathogens (SPF PL). Of course, the PL were not tested for *V. parahaemolyticus*, because this bacterium had not been implicated as the cause of AHPND in 2012. Stocking densities varied from 35 to 120 PL.m^{-2} for *P. vannamei* and from 25 to 40 PL.m^{-2} for *P. monodon*. The crude protein content of feeds differed from 37 to 45 % – usually a lower protein-content feed was used for *P. vannamei* than for *P. monodon*. Fat content of feed was 4 to 8 %. Production expected by farmers based on previous crops depended upon species and stocking density, and varied from 6 to 16 tonnes.ha^{-1} for *P. vannamei* and from 4 to 8 tonnes.ha^{-1} for *P. monodon*. Three farms reported using water exchange during grow out. However, the amount of water exchange was small – only 10 to 20 % of pond volume two or three times during the production period. Make-up water was added to replace evaporation during the dry season at all farms. Owners of farms claimed to disinfect surface water before it was used for water exchange or replacement.

Two farms used groundwater to replace evaporation loss, and this water was not treated with a disinfectant before adding it to ponds. All farms applied mechanical aeration with "long-arm" paddlewheel aerators typically used in Asian shrimp farming. The amount of aeration was increased from early in the crop until harvest, and aeration rates at the end of the production period were reported to range from 10 to 32 hp.ha^{-1}. Based on maximum, expected production values given by farmers, aeration rates ranged from 312 to 1 000 kg shrimp.hp^{-1} (average = 608 kg.hp^{-1}) for *P. vannamei* and from 250 to 466 kg shrimp.hp^{-1} (average 357 kg.hp^{-1}) for *P. monodon*. An aeration rate of 1 hp for every 400 kg of production often is recommended for shrimp ponds in Asia (Boyd 2009). At the time of the survey, ponds were in the first 30 days of culture and had low biomass of shrimp. The amount of aeration per unit weight of shrimp per horsepower was well above the minimum recommended amount. All farms applied microbial products during the grow-out period with the belief that this would improve water and sediment quality.

These microbial products consisted of cultures of live microbial cells, and some products contained up to six of the following genera: *Aspergillus*, *Bacillus*, *Lactobacillus*, *Nitrobacter*, *Nitrosomonas*, *Paracoccus*, *Rhodobacter*, *Rhodococcus* and *Saccharomyces*. Other amendments used at all farms for the purpose of improving water quality were zeolite, iodine, potassium permanganate, calcium carbonate, dolomite, benzalkonium chloride, glutaraldehyde, *Yucca* extract, potassium monopersulfate, mineral mix, enzyme preparations and chelated copper. Various combinations of these amendments were used at different farms according to the judgment of the owners.

Water and Sediment Quality

Water in the canals that supplied farms in all three provinces was of relatively good quality, but it had high concentrations of nitrogen and phosphorus and contained a relatively great abundance of phytoplankton as indicated by elevated chlorophyll *a* concentrations (Table 3). Trace metal analyses were made on samples from only two canals, and concentrations of these substances were low (Table 4). Pesticide analyses were not made for canal water.

Table 3. Average concentrations of water quality variables in canals that supplied water to shrimp ponds in the three provinces.

Variable	Soc Trang (n = 2)	Bac Lieu (n = 3)	Ca Mau (n = 2)
Water temperature (^{o}C)	27.8	29.0	32.4
pH (standard units)	8.00	8.3	8.05
Salinity (ppt)	3.0	9.3	4.0
Dissolved oxygen (mg.L^{-1})	3.70	5.91	4.34
5-Day biochemical oxygen demand (mg.L^{-1})	3.0	4.0	9.0
Total ammonia nitrogen (mg.L^{-1})	0.335	0.306	0.174
Un-ionized ammonia nitrogen (mg.L^{-1})	0.028	0.043	0.015
Nitrite nitrogen (mg.L^{-1})	0.131	0.110	0.052
Nitrate nitrogen (mg.L^{-1})	0.095	0.087	0.192
Total nitrogen (mg.L^{-1})	3.33	1.43	1.54
Total phosphorus (mg.L^{-1})	0.351	0.10	0.280
Total sulfide (mg.L^{-1})	0.052	0.059	0.126
Hydrogen sulfide (mg.L^{-1})	0.004	0.003	0.011
Chlorophyll *a* (μg.L^{-1})	78.4	26.6	14.5
Arsenic (μg.L^{-1})	1.19		6.18

Table 3 Continued.

Variable	Soc Trang (n = 2)	Bac Lieu (n = 3)	Ca Mau (n = 2)
Cadmium (μg.L^{-1})	ND[1]		0.96
Copper (μg.L^{-1})	7.0		22.6
Lead (μg.L^{-1})	0.53		50.6
Mercury (μg.L^{-1})	ND		
Zinc (μg.L^{-1})	132		554

[1]ND = not detectable.

Table 4. Water analysis data for ponds in the Mekong Delta, Viet Nam that contained shrimp either positive or negative to AHPNS**.**

	1st Sampling[1]		2nd Sampling[1]		3rd Sampling[1]	
Water quality variable	**Negative (n = 9)**	**Positive (n = 13)**	**Negative (n= 11)**	**Positive (n = 10)**	**Negative (n = 5)**	**Positive (n = 6)**
Temperature (°C)	29.07±1.10	30.06±2.00	29.15±1.54	28.96±0.69	30.0 ±2.04	28.9±1.12
pH (standard units)	8.37±0.35	8.71±0.63	8.67±0.60	8.64±0.49	8.48±0.50	8.59±0.37
Salinity (ppt)	10.1±6.4	10.1 ± 5.4	12.8±5.3	12.4±6.9	4.6 ±3.6	9.2±5.3
Dissolved O_2 (mg.L^{-1})	7.1±1.6	7.73 ± 1.6	6.7 ± 1.8	6.4 ± 1.6	6.8±0.8	6.9±1.1
BOD_5 (mg.L^{-1})	10.6±8.0	9.4±8.2	4.1±4.7	2.4±3.4	4.6±0.5	1.9±0.2
Total ammonia N (mg.L^{-1})	0.55±0.61	0.18±0.29	0.08±0.15	0.20±0.38	0.73±0.10	0.30±0.18
NH_3 (mg.L^{-1})	0.07±0.07	0.04±0.05	0.09±0.05	0.08±0.10	0.38±0.42	0.16±0.17
Nitrite N (mg.L^{-1})	0.31±0.40	0.09±0.13	0.11±0.24	0.11±0.28	0.25±0.18	0.11±0.12
Nitrate N (mg.L^{-1})	0.29±0.63	0.04±0.04	0.49±0.58	0.50±0.45	0.99 ±0.57	0.88± 0.48
Total N (mg.L^{-1})	2.15±1.32	1.62±1.29	0.86±0.75	1.07±1.43	1.82±0.19	2.53±0.15
Total P (mg.L^{-1})	0.26±0.10	0.21±0.13	0.20±0.19	0.15±0.13	0.08 ± 0.04	0.04 ±0.04
Total sulfide (mg.L^{-1})	0.05±0.03	0.07±0.05	0.04±0.06	0.02±0.03	0.004±0.006	0.001±0.001
H_2S (mg.L^{-1})	0.003±0.002	0.006±0.010	0.002±0.004	0.002±0.004	ND	ND
Chlorophyll *a* (μg.L^{-1})	33.5±43.7	31.0±31.1	23.9±22.3	29.7±45.9		
Arsenic (μg.L^{-1})	0.10±0.05	0.01±0.06				
Cadmium (μg.L^{-1})	0.20±0.16	1.22±1.51				
Copper (μg.L^{-1})	49.6±38.7	44.2±52.3				
Lead (μg.L^{-1})	7.2±6.4	4.1±3.0				
Mercury (μg.L^{-1})	2.2±1.5	2.4±1.8				
Zinc (μg.L^{-1})	313±113	282±137				

[1]There were no differences at $P = 0.05$ between ponds with AHPNS-negative or AHPNS-positive shrimp for any variables as determine by *t*-tests.

[2]ND = not detectable.

Concentrations of water quality variables in pond waters were within similar ranges in all three provinces and for the three sampling rounds, but the important point is that there were no differences as determined by t-test ($P > 0.05$) between means for water quality variables with respect to AHPND status of shrimp for any of the three rounds of sampling. The upper limits (lower limit for DO) of the normal concentration ranges of critical water quality variables and for trace metals were established (Table 5) based on information from the literature by Boyd and Tucker (1998), Boyd (2000), Prapaiwong and Boyd (2012, 2014), Chin and Chen (1987), Lin and Chen (2001), and water quality fact sheets from the Southern Regional Aquaculture Center (http://srac.tamu.edu). One or more of these limits were exceeded in some ponds with shrimp of both AHPND categories. The number of un-ionized ammonia values above normal was considerably greater in ponds with AHPND-negative shrimp than in those with AHPND-positive shrimp, and the total number of instances of values of critical variables exceeding the limits of the normal ranges was somewhat greater for the ponds with AHPND-negative shrimp as a result.

Table 5. The number of ponds in the Mekong Delta, Viet Nam that contained shrimp either negative or positive to AHPNS that were outside acceptable concentration ranges of critical water quality variables.

Variable	**Negative (n = 25)**	**Positive (n = 29)**
Salinity, < 5 ppt	3	3
pH, > 9.0	5	7
Dissolved oxygen, < 4 $mg.L^{-1}$	1	0
Un-ionized ammonia nitrogen, > 0.05 $mg.L^{-1}$	12	4
Nitrite-nitrogen, > 0.5 $mg.L^{-1}$	6	3
Hydrogen sulfide, > 0.0025 $mg.L^{-1}$	4	3
Arsenic, > 5 $\mu g.L^{-1}$	0	0
Cadmium, > 0.25 $\mu g.L^{-1}$	2	2
Copper, > 50 $\mu g.L^{-1}$	4	3
Lead, > 5 $\mu g.L^{-1}$	5	5
Zinc, > 500 $\mu g.L^{-1}$	0	2
Total number instances with values outside normal range	42	33

The only pesticides detected in pond waters were the insecticides fenitrothion and deltamethrin. These two insecticides were at detectable concentrations in more ponds with AHPND-infected shrimp than in ponds without AHPND-infected shrimp, but there were no differences between the two categories of ponds with respect to average concentrations of the two insecticides (Table 6). The highest concentration of either insecticide was 0.5 $\mu g.L^{-1}$. Algal toxins and PCBs were not at detectable concentrations in any water samples.

Table 6. Mean pesticide concentrations and standard errors (SE) along with ranges (μg.L^{-1}) for water from ponds that contained either AHPNS-negative (n = 9) or positive (n = 13) shrimp.

Pesticide	Number of ponds where detectable		Average concentration ± SE[1] (Ranges in parentheses)	
	Negative	Positive	Negative	Positive
Water				
Fenitrothion	3	5	0.006 ± 0.0065 (ND[2] – 0.140)	0.106 ± 0.0411 (ND – 0.500
Deltamethrin	2	4	0.024 ± 0.0212 (ND–0.130)	0.035 ± 0.0174 (ND – 0.170)
Sediment				
Hexaconazole	4	5	20.4 ± 1.69 (ND – 24.5)	22.1 ± 1.34 (ND–27.0)
Deltamethrin	3	5	2.6 ± 0.90 (ND – 3.74)	3.1±1.43 (ND–4.54
Fenitrothion	1	6	1.0 ± 0.01 (ND – 1.0)	2.5±1.34 (ND–9.04)

[1]There were no differences at $P = 0.05$ between ponds with AHPNS-negative or AHPNS-positive shrimp for any variables as determine by *t*-tests.
[2]ND = not detectable.

There were no differences ($P > 0.05$) for trace metal concentrations in sediment between ponds with respect to AHPNS status of shrimp (Table 7). The concentrations of trace metals in sediment were within the range of concentrations of trace metals reported in shrimp pond sediment from ponds in several countries (Boyd et al. 1994).

Table 7. Trace metal concentrations and standard errors (SE) (μg.kg^{-1}) in sediment from ponds that contained either AHPNS-negative or positive shrimp.

Variable	Negative (n = 9)	Positive (n = 13)
Arsenic	5.95±0.781	5.85±0.560
Cadmium	0.23±0.033	0.22±0.017
Copper	26.2±1.34	24.8±2.08
Lead	37.6±1.31	35.4±1.67
Mercury	0.06±0.012	0.04±0.007
Zinc	168±4.0	160±5.6

Deltamethrin and fenitrothion, as well as the fungicide hexaconazole, were detected in sediment samples (Table 6). Although each of these pesticides was detected in more ponds with AHPND-positive shrimp than in ponds with shrimp unaffected by the disease, as with water, mean concentrations of the pesticides did not differ ($P > 0.05$) with respect to AHPND status of shrimp.

Discussion

The water supply situation for shrimp farms in the Mekong River Delta obviously is not ideal. Water often travels long distances in canals, providing ample opportunities for pollution from domestic, agricultural, aquacultural, industrial and natural sources. It is particularly problematic that discharges from shrimp farms in a particular area mix with water in canals from which all shrimp farms in the area take in water. Contamination of farm influent with farm effluent facilitates transfer of shrimp diseases among farms. Disinfection of water before using it for shrimp culture ponds should be considered a critical aspect of farm management in Viet Nam. The farmers visited in Viet Nam claimed to dry-out ponds and disinfect water before stocking shrimp, but there was wide variation from farm to farm in management practices. In particular, the methods of disinfection varied, and concentrations of disinfectants used also differed among farms. Concentrations of disinfectants used at all farms appeared to be too low to effect complete disinfection.

The farmers claimed to use SPF PL; however, the PL were not checked for *Vibrio parahaemolyticus* (with phage), because the causative agent had not yet been identified at the time of the farm survey. Most farmers claimed to use feeding trays or cast net samples for obtaining information on optimal feed input. However, the amount of aeration used in *P. vannamei* ponds was sometimes less than that considered necessary to maintain nighttime dissolved oxygen concentration above 3 $mg.L^{-1}$ (Boyd 2009) – especially during the last one or two months of culture. But, AHPND apparently occurred early in the shrimp crop when aeration was adequate. All farms used microbial products during culture, and the use of these products apparently had no influence on AHPND status of shrimp in ponds. There were no apparent differences in AHPND status of shrimp with respect to pond management. This does not necessarily mean that the disease cannot be influenced by management, because some PL may have been infected with AHPND in the hatchery, and even if they were AHPND-free when delivered to farms, the concentrations of disinfectants used at farms were too low to be effective.

Ponds had relatively high concentrations of nitrogen and phosphorus, and phytoplankton was abundant in most ponds during the first month of culture, as evident from elevated chlorophyll *a* concentrations. In some ponds, pH values were high, and concentrations of salinity, dissolved oxygen, un-ionized ammonia nitrogen, nitrite-nitrogen, hydrogen sulfide, arsenic, cadmium, copper, lead and zinc were outside optimal ranges for shrimp culture. Insecticides also were detected in some ponds, but PCBs and algal toxins were not found. There were, however, no differences in concentrations and ranges of potentially harmful substances between ponds with respect to AHPND status of shrimp. The onset of AHPND possibly could have been triggered by stress related to one or more water or sediment quality variables. However, infection of shrimp by AHPND requires the presence of the disease agent, and if *V. parahaemolyticus* was not present in ponds, stress would not have led to AHPND. This is confirmed by the observation that water quality was frequently outside optimal ranges for one or more variables in all ponds regardless of AHPND status of shrimp.

It is particularly important to note that the pH did not differ between ponds of the two AHPND categories. The claim that high pH is the environmental trigger for the disease because AHPND in shrimp repeatedly regressed at pH 7 but not at higher pH (8.5 to 8.8) (Akazawa and Eguchi 2013) is likely a mote point. Shrimp ponds typically have pH between 8.0 and 9.0, and there is no practical way – at least at present – for reducing pH below 8.0 in most ponds. Of course, it may be possible to manipulate pH in the shrimp gut to lessen the risk of AHPND, but there are no data to support this hypothesis. The report of the FAO project TCP/VIE/3304 "Emergency assistance to control the spread of an unknown disease affecting shrimps in Viet Nam" recommends the use of better biosecurity and application of good aquaculture practices as a means of lessening the risk of ANPNS (ANPHD). The objectives are to reduce the abundance of *Vibro* spp. (including *V. parahaemolyticus*) as much as possible before stocking PL, to stock PL that are free of the AHPND agent and other common disease-causing agents, to prevent entry of disease organisms during culture, and to maintain good water and sediment quality during grow out to avoid stressing shrimp. Some practices that should be adopted follow:

Biosecurity

- Banning broodstock and PL that have not tested free of specific pathogens (including *V. parahaemolyticus*).
- Stocking SPF shrimp – screening should include *V. parahaemolyticus*.
- Using closed culture systems where feasible.
- Disinfecting intake water before use (see Source Water below).
- Preventing entry of wild fish and other organisms by screening inflow (see Source Water below).
- Not transferring water or shrimp from AHPND-infected ponds to other ponds.
- Disinfecting nets and other equipment used in AHPND-infected ponds with calcium hypochlorite solution (50 $mg.L^{-1}$ chlorine) or iodophor solution (200 $mg.L^{-1}$ iodine) before reuse.
- Disinfecting water in ponds where the crop is lost to disease by treatment with calcium hypochlorite (50 $mg.L^{-1}$ chlorine) before discharge.
- Using good aquaculture practices (GAPs) as described below.

Pond Bottoms

- Removing large accumulations of sediment – usually not necessary in semi-intensive culture.
- Drying pond bottoms for 2 weeks or more after harvest.
- Liming pond bottoms with burnt lime at 400 to 500 kg.1 000 m^{-2}.
- Treating bottoms with soil pH below 7.0 with agricultural limestone unless burnt lime has been applied as a soil disinfectant.
- Tilling to depths of 10 to 15 cm may be used to improve dry-out and incorporate liming materials into pond bottom soil.

Source Water

- Filtering water through 150–250 μm filter bags to remove wild fish and larger organisms that can be vectors of disease organisms. Note: this practice will not remove pathogenic bacteria.
- Holding source water in a reservoir for at least 2 weeks – for longer if possible – before filling ponds.
- Holding water in the culture ponds at farms without reservoirs for 2 weeks before stocking.
- Treating water for filling ponds with calcium hypochlorite (65 % chlorine) at 15 kg.1 000 m^{-3} before stocking PL. This can be done in the reservoir, or if there is no reservoir, treatment can be done directly in ponds.
- Applying fertilizer – preferably chemical fertilizer – to stimulate plankton growth before stocking the PL.

Postlarvae

- Using SPF PL from a reputable hatchery. *Vibrio parahaemolyticus* should be included in screening of PL.
- Acclimating the PL to pond water conditions before stocking.
- Using stocking rates reasonable for pond management strategy – especially the rate of water exchange and amount of aeration.

Feeding

- Using a feed of good quality that contains no more protein nitrogen and phosphorus than necessary.
- Storing feed in a dry place that does not have extreme temperatures.
- Using cast nets or feeding trays to assist in determining feeding rates.
- Appling feed in no greater quantities than animals will eat.
- Taking shrimp off feed if AHPND is observed. Gradually resume feeding if shrimp health improves.

Aeration

- Using about 10-hp of aeration for each 10 kg.ha^{-1} of daily feed input. The key is to avoid dissolved oxygen concentration below 3 mg.L^{-1} and preferably below 4 mg.L^{-1}.
- Positioning aerators to avoid erosion of pond bottoms and embankments.
- Using less aeration at the beginning of culture period and increasing the amount as feeding rate increases.
- Less aeration usually may be applied during the day than at night.

Fertilization during Grow out

- Using no more than 0.8 kg each of N and P_2O_5 per 1 000 m^2 per fertilizer application. Research shows that a fertilizer with an N:P_2O_5 ratio of about 1:2 or 1:3 is optimal – but there are many opinions about the best fertilization programme.

- Adjusting fertilizer application rate for plankton density. If Secchi disk visibility is less than 30 cm, delay application. In ponds with feeding, fertilization may not be necessary – especially after feeding rates reach 1 kg.1 000 m^{-2} (10 $kg.ha^{-1}$) per day.

Water Management during Grow out

- Disinfecting water in reservoirs before applying it to culture ponds to replace seepage and evaporation loss or for water exchange.
- Ceasing or reducing water exchange. At farms with large reservoirs, water may be exchanged between ponds and the reservoirs. Although water exchange often increases disease risk, its use may be necessary to lessen salinity in ponds supplied by seawater. But, no more than 5 % of pond volume per day is usually required to maintain salinity below 40–42 ppt by end of crop.

Water Quality Amendments

- Maintaining total alkalinity above 100 $mg.L^{-1}$. Liming materials may be applied as necessary, but do not make applications of burnt lime above 10 kg.1 000 m^{-2} at one time.
- Not using disinfectants (antimicrobial agents) during the culture period, because this practice is not effective, and these compounds may stress culture animals.
- Considering not applying probiotics. Although probiotics apparently are harmless, there is no evidence that they improve water quality.
- Considering not applying zeolite. Zeolite is ineffective in removing ammonia, but its use does not pose a risk to shrimp.
- Considering not applying molasses. The benefits of applying molasses for the purpose of preventing the growth of *Vibrio* spp. are unproven, but the treatment is harmless to shrimp in aerated ponds.

Water Quality Monitoring and Response

- Measuring dissolved oxygen (DO) daily in the early morning. If early morning DO concentration is below 3 $mg.L^{-1}$ regularly, more aeration is needed or the daily feed input should be lessened.
- Measuring salinity weekly. Little can be done to increase salinity, but if salinity becomes too high (over 40–42 ppt) water exchange can be used. Of course, water exchange increases disease risk.
- Measuring pH in morning and afternoon weekly. A pH above 9 in the afternoon often suggests poorly buffered water. Alkalinity should be measured, and if it is below 100 $mg.L^{-1}$, liming material should be applied. The pH in ponds varies daily – it is lowest in the morning and highest in the afternoon. It also is higher near the surface than near the bottom. Other than maintaining total alkalinity to assure buffering capacity, there is no proven way of lessening pH.

- Measuring total ammonia nitrogen concentration weekly. Values above 2 $mg.L^{-1}$ suggest excessive feed input, insufficient phytoplankton abundance to remove ammonia nitrogen effectively, or inadequate aeration.

Conclusion

Although AHPND is not caused by a toxic agent of environmental origin, environmental factors that stress shrimp will increase their susceptibility to this and other diseases. The main practices for lessening the risk of AHPND follow: use PL free of the causative agent and other specific pathogens; disinfect pond bottoms and pond water – including water added to replace evaporation and seepage; do not transfer water among ponds during culture; use management practices that assure good water quality during culture; and disinfect water in ponds in which the crop was lost to disease before releasing it into canals or other waterbodies.

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Transforming the Farm Managers into the "Family Doctors" of Their Own Ponds

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Abstract

Acute hepatopancreatic necrosis disease (*AHPND*) is caused by a virulent strain of *Vibrio parahaemolyticus* (VP_{AHPND}) not easily differentiated from other *Vibrio* spp pathogens. For the management of $VP_{AHPND,}$ an integral part of the shrimp gut and culture water ecosystems, timely responses to ecological changes in these niches is critical. For humans, companion animals, poultry and livestock, doctors or veterinarians are available to provide disease diagnosis and subsequent treatment accordingly. For shrimp, a farm manager is the best candidate to serve as the "family doctor" to prevent and/or mitigate threats from AHPND. Based on recent advances in the understanding of VP_{AHPND}, different tools have become available for AHPND management, including on-site microbiological and molecular test tools (e.g. spread plate method and insulated isothermal polymerase chain reaction (iiPCR)) for diagnosis, and ecological tools (e.g. indoor pond facilities and application of probiotics) for treatment. Working on a hand-held POCKIT™ PCR device in a format ready for on-site applications, two POCKIT™ iiPCR assays targeting different markers are available to enable identification of VP_{AHPND} in postlarvae (PL), midgut, faeces and pond water. Therefore, pond managers can be trained to use the on-site diagnostics tools and interpret test results, and to apply front-line treatments for AHPND.

Keywords: acute hepatopancreatic necrosis disease, ecological tools, ecosystem management, on-site diagnostic tools, polymerase chain reaction, probiotics, spread plate method, *Vibrio parahaemolyticus*

Introduction

Shrimp aquaculture is one of the major global agriculture industries. Emerging shrimp diseases are on the rise in recent decades largely because shrimp are often cultured at high density, exposed to environmental stress and traded globally. Efficient disease control and management practices could help minimize disease outbreaks, leading to the sustainability of the shrimp-culture industry.

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Acute hepatopancreatic necrosis disease (*AHPND*) is caused by a virulent strain of *Vibrio parahaemolyticus* (VP_{AHPND}). Without vaccines or effective treatments for shrimp diseases, the shrimp industry relies greatly on biosecurity measures to prevent the introduction of pathogens and to reduce their spread throughout the culture environment. In addition, shrimp growth and survival throughout the culture period rely heavily on good water quality, including appropriate temperature, salinity, pH, dissolved oxygen and eco-balance of microflora. A variety of *Vibrio* spp. found to cause shrimp diseases, such as *V*. *alginolyticus (*Liu et al. 2004), *V*. *harveyi, (*Karunasagar et al. 1994; Zhou et al. 2012*)* and *V*. *parahaemolyticus* (Vandenberghe et al. 1999) are an integral part of the ecosystems in the shrimp gut and in culture water. Therefore, reducing the effects of bacterial replication on the digestive system and/or reducing or eliminating the pathogenic bacteria could also help in the control of bacterial diseases.

In the pond water, bacteria can multiply rapidly and cause massive shrimp mortality once the environment becomes severely unbalanced (Vandenberghe et al. 1998; Saulnier et al. 2000; Jayasree et al. 2006). Fluctuations of intra- and interpopulational density can be monitored by quorum sensing (QS), allowing *Vibrio* spp. to initiate specific community-scale responses, including expressing and releasing virulence factors to launch an effective attack on the host. Therefore, in addition to measures that help strengthen shrimp health and the immune system, environmental management is one of the best interventions for the control of bacterial disease. As timely response to ecological changes occurring in the shrimp pond is critical, the pond manager is the best candidate to prevent potential threats from AHPND and to treat infected shrimp. Different aquaculture facilities have different individual eco-conditions, which can be quite complicated.

As the pond manager is the person who is most familiar with the shrimp production facility, he or she is therefore, the best candidate to be trained to know the "patient" and the diseases, how to use on-site diagnostics tools and interpret results, and how to treat the disease with front-line treatments. For bacterial shrimp disease management, an effective strategy will require the integration of variable information and technologies to achieve the following: i) identification of the pathogen; ii) identification of the source of the pathogen; iii) maintenance of the eco-balance among shrimp, algae and bacteria; and iv) avoiding uncontrollable issues. Like other diseases caused by *Vibrio* spp., AHPND cannot be managed 100 % effectively by a single approach.

The Pathogen and its Detection

Vibrio parahaemolyticus, a Gram-negative halophilic bacterium, can be found in shrimp and the farming environment, and also in marine environments worldwide. VP_{AHPND} can colonize the gastro-intestinal tract and produce toxin(s) that cause dysfunction and destruction of the hepatopancreas in *Penaeus vannamei* and *P. monodon* (Flegel 2012; Leaño and Mohan 2012; Lightner et al. 2012; Tang and Lightner 2014). In 2013, the VP_{AHPND} strain was found to contain a virulence-associated plasmid (pVA) (Han et al. 2015; Lee et al. 2015) encoding toxin 1 which is composed of subunits A and B with high homology to the 2 subunits of the insecticidal *Photorhabdus* insect-related binary toxin (PirA and PirB, respectively).

The VP_{AHPND} toxin 1 can cause damage to the hepatopancreas and induce AHPND-like clinical signs in diseased *P. monodon* (Lai et al. 2015; Lee et al. 2015). Most molecular detection methods developed for AHPND diagnosis target the toxin genes and/or other regions in pVA. At early stages, VP_{AHPND} in the environment (e.g. water) invades and establishes infection in the shrimp stomach; the pathogen can generally be detected by polymerase chain reaction (PCR) in the stomach but not in the midgut at this stage (Fig. 1). At the acute stage, VP_{AHPND} colonizes in the stomach and secretes significant amounts of toxin(s) which is transported into the hepatopancreas and cause the damage therein, and spreads to the midgut; VP_{AHPND} can be detected in both the stomach and midgut by PCR at this stage (Fig. 1).

Gene Transfer in the Vibrio harveyi Clade

Vibrio parahaemolyticus is closely related to other members of the *V. harveyi* clade, which consists of 11 species (Sawabe et al. 2007; Cano-Gomez et al. 2011). Different genetic transfer mechanisms, including homologous recombination, transposition, conjugation or transformation help achieve genetic material exchange between different *Vibrio* spp. The virulence genes, *PirA* and *PirB*, were found within "pathogenic islands" flanked by inverted repeats of transposase genes in the pVA plasmid (Tang and Lightner 2014; Han et al. 2015b), suggesting that the virulence genes could be transferred via genetic transfer mechanisms.

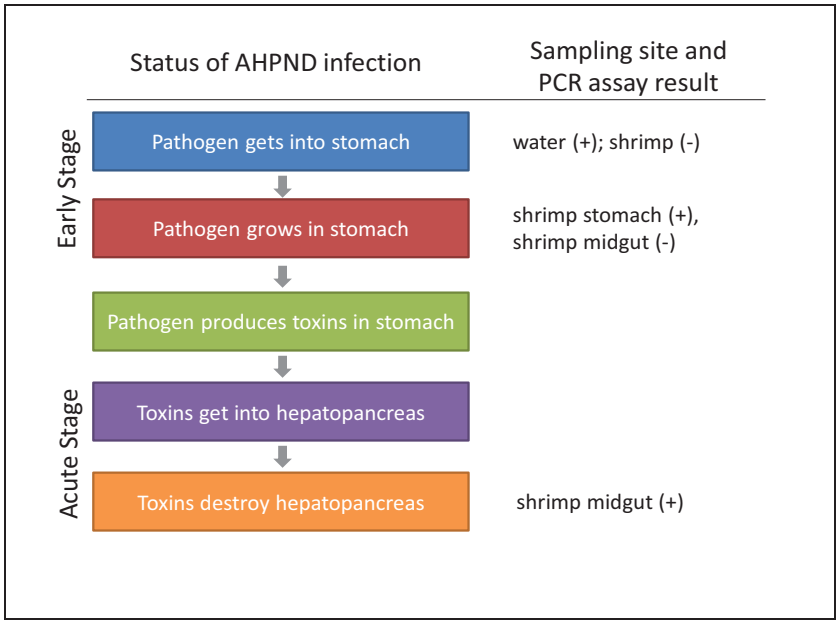


Fig. 1. Acute hepatopancreatic necrosis disease (AHPND) infection status and the corresponding polymerase chain reaction (PCR) assay results in different sampling sites. (+) = positive result, (-) = negative result.

Recent evidence indicated that the AHPND pVA plasmid can jump to other members in the *V. harveyi* clade (Busico-Salcedo and Owens 2014), and a *V. harveyi* isolate (KC13.17.5) was identified as causing AHPND in northern Viet Nam. Sequencing analysis showed that this isolate contained pVA plasmid-like sequences and the putative virulence genes (Kondo et al. 2015).

In addition, one *V. owensii* strain (SH-14) was found to cause AHPND and has a large plasmid containing the *Pir toxin* genes in a pVA-like plasmid *(*Liu et al. 2015*)*. So far, the pVA plasmid has not been found in any species outside of the *V. harveyi* clade. Since >95 % of the aetiological agents of AHPND have been identified as VP_{AHPND}, we recommend that pond managers focus on managing VP_{AHPND} for now.

Diagnostic Tools for Marine Bacterial Pathogens

Diagnosis of a disease can facilitate effective decision-making, leading to implementation of the right treatment and preventive measures. Information such as the type of pathogen (e.g. virus, bacteria, parasite or a combination of them), the severity of the disease, pathogen prevalence, pathogen titer (pathogen levels) and pathogen strain (pathogenic, partial or non-pathogenic) are all crucial for pond management. Disease diagnosis has become less challenging lately, with various diagnostics tools being available for use in a central laboratory or in the field.

Spread Plate Methods

It is a general rule in aquaculture practices that a high number of *Vibrio* spp. in the water poses a potential threat to the farmed animals (Diggles et al. 1999). It is suggested that the pathogenic *Vibrio* loads should be kept below 1 000 colony forming units (cfu).mL^{-1} in an aquaculture system (Ganesh et al. 2010). The spread plate methods can provide quantitative information on the bacterial populations. Marine agar, a non-selective medium for organisms living in high Na^+ environments, is used to obtain the total bacterial count (Fig. 2A). Thiosulfate-citrate-bile salt-sucrose (TCBS) agar, a highly selective medium for many *Vibrio* spp., can be used to estimate the numbers of *Vibrio* spp. (Fig. 2B) (Jayasinghe et al. 2006).

The combination of sucrose fermentation and bromothymol blue and thymol blue indicators in the medium leads to yellow colonies, allowing for the identification of sucrose-utilizing *Vibrio* spp. Important shrimp pathogens such as *V. parahaemolyticus* and *V. harveyi* produce greenish colonies on TCBS agar. Luminescent colony counts can help estimate the population of potentially harmful bacteria, since luminescence activity strongly indicates quorum sensing and virulence of *Vibrio* bacteria. De *Man Rogosa Sharpe* (*MRS*) agar, containing ingredients that favour growth of *Lactobacilli* and suppress competing bacteria, is often used to estimate the concentration of *Lactobacilli*.

Various microbiological indexes, I(M), have been designed to help decision-making in pond management. The optimal I(M) for each farm is different. I(M) calculation is based on the following numbers: M, total bacterial counts from marine agar plating; T, *Vibrio* spp. bacterial count from TCBS agar plating; TY, yellow colony counts from TCBS agar plating (likely avirulent *Vibrio* spp.); TG, green colony counts from TCBS agar plating (likely virulent *Vibrio* spp.). A typical I(M) example indicating good water quality is as follows: $M > 20 \times T$, $M > 10^5$, $T < 10^3$, in case $TG > TY$, $TG < 10^3$ ($TG < 5 \times 10^2$ preferred).

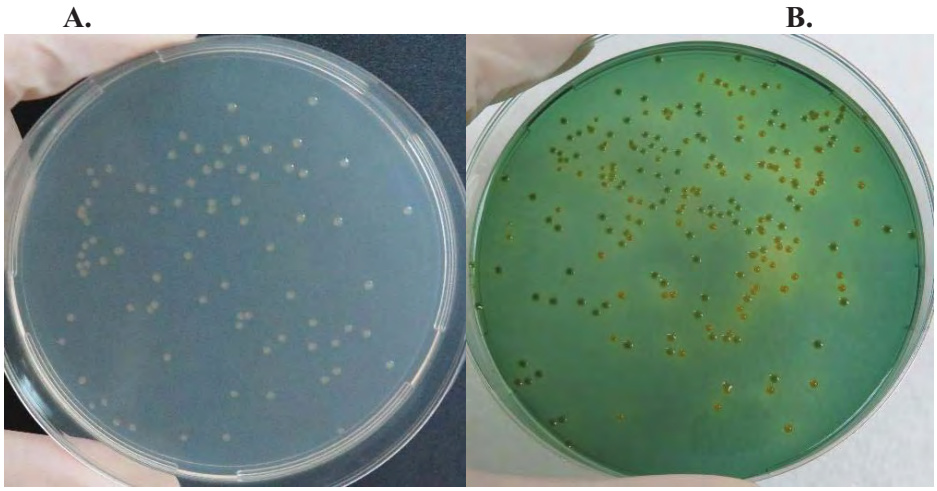


Fig. 2. Spread plate methods. Total marine bacteria and *Vibrio* spp. counts can be obtained by marine agar. (A), a non-selective medium to obtain total organisms living in high Na^+ environments, and (B) thiosulfate-citrate-bile salt-sucrose (TCBS) agar, a highly selective medium for *Vibrio* spp., where pathogenic *Vibrio* spp. such as *V. parahaemolyticus* and *V. harveyi* produce greenish colonies.

Insulated Isothermal PCR – an On-Site Molecular Detection Tool for Aquaculture Pathogens

PCR assay, with its high sensitivity and specificity, has been commonly used at various large-scale aquaculture facilities for pathogen surveillance or disease diagnosis, improving overall shrimp production in the long run. By detecting pathogens at early stages, appropriate measures can be implemented in a timely fashion to help control the spread of major shrimp pathogens, such as white-spot syndrome virus (WSSV). On-site pathogen detection tools enable farms of various scales to minimize economic losses caused by AHPND. Optimal on-site pathogen detection systems should be rapid, inexpensive, sensitive, easy to maintain and perform by anyone with minimal training, and not cross-react with shrimp DNA and any irrelevant microorganisms. The reagents should be provided in a format (such as lyophilized) that allows easy shipping and storage.

A user friendly field-deployable molecular detection method is available for on-site detection of various shrimp pathogens. The assay is based on insulated isothermal PCR (iiPCR) and works on a series of CE-IVD-marked portable devices (POCKIT™ Nucleic Acid Analyzer series, GeneReach Biotech) which can amplify target sequences, detect fluorescent signals and display simple readouts within one hour. The POCKIT™ Nucleic Acid Analyzer could run 1 to 8 samples within 55 minutes with a single programme for PCR and RT-PCR. Probe detection adds specificity and eliminates post-PCR manipulation. It could run with rechargeable battery. Dual-channel fluorescence detection allows inclusion of an internal control. User-friendly features include touch panel control and automatic data interpretation. The newly available hand-held POCKIT™ Micro Nucleic Acid Analyzer (for PCR) and POCKIT™ Micro Plus Nucleic Acid Analyzer (for both PCR and reverse transcription PCR [RT-PCR]) (GeneReach Biotech) have added flexibility to its field application and allowed shorter turnaround time. Results are obtained in around 30 min with POKIT™ Micro and 45 min with POKIT™ Micro Plus.

Both models are small (dimensions: 152 (L) x 63 (W) x 50 (H) mm), light weight (380 g), and can be charged with a micro USB Recharger (100–240V). The reliability of the iiPCR system has been shown by the fact that one WSSV iiPCR method (IQ Plus™ WSSV Kit with POCKIT™ System) was certified by the World Organisation for Animal Health (OIE) as fit for detecting WSSV in tissue of ectodermal and mesodermal origin of *P. vannamei* in 2013; that several methods on POCKIT™ have been listed in the Biodetection Technologies for First Responders: 2015 edition by Pacific Northwest National Laboratory, and that several iiPCR on POCKIT™ methods have been demonstrated to provide sensitivity and specificity equivalent to those of reference real-time PCR (qPCR) or nested PCR for the detection of a significant number of animal and human viral pathogens, including WSSV and dengue virus (Tsen et al. 2013; Tsai et al. 2014; Balasuriya et al. 2014; Wilkes et al. 2014, 2015a,b; Ambagala et al. 2015; Lung et al. 2015; Carossino et al. 2016; Chua et al. 2016; Go et al. 2016; Kuo et al. 2016; Soltan et al. 2016; Zhang et al. 2016).

Two POCKIT™ iiPCR methods targeting markers to allow identification of AHPND threat, *i.e. toxin 1* (*PirA* and *PirB*) or the AHPND pVA plasmid, are available. Postlarvae (PL), midgut, faeces and pond water are recommended for sampling. Molecular screening for the presence of the pVA plasmid can help detect a potential AHPND threat, since non-virulent strains of *V. parahaemolyticus* could be converted into virulent strains by obtaining the plasmid through different gene transfer mechanisms. The *toxin 1* gene is located in an unstable region in the pVA plasmid and could potentially be moved horizontally from one location to another location by transposases. Detection of the *toxin 1* gene in the shrimp or environment may indicate the presence of microorganisms with potential to produce the toxins that lead to disease in shrimp, alerting farmers of potential threats of AHPND.

Thus, both the pVA plasmid and *toxin 1* gene are the recommended detection targets for prevention and management of AHPND. When simultaneous performance of the two assays is not possible, it is recommended to perform the pVA plasmid test first to identify potential threat. In the case of plasmid-positive results, the *toxin 1* assay can be used to provide further evidence for the presence of the AHPND pathogen.

Both the AHPND pVA plasmid or *toxin 1* gene iiPCR assays can detect their targets sensitively and specifically. They had detection endpoints equivalent to that of qPCR assays (10^{-3}) using a serial dilution of a virulent VP_{AHPND} strain (data not shown). Both reactions did not react with 18 non-VP_{AHPND} strains from the People's Republic of China, Mexico, Thailand, Taiwan POC, the United States of America or Viet Nam, including 11 laboratory isolates (Table 1), indicating excellent target specificity. Side-by-side comparison of *toxin 1* iiPCR with qPCR using field samples (n=18) showed that eight qPCR-positive samples were all iiPCR positive and ten qPCR-negative samples were all iiPCR negative. Similarly, 11 pVA plasmid qPCR-positive samples were all pVA plasmid iiPCR positive and seven qPCR-negative samples were all iiPCR negative). These results demonstrate that both iiPCR assays have excellent agreement with qPCR to detect the AHPND virulence markers in samples.

Furthermore, easy and simple nucleic acid extraction methods are available to work with the hand-held PCR detector on site. For example, the Grind-N-Go (GeneReach Biotech) is an easy manual DNA extraction method with all reagents provided in a single cartridge; DNA extraction could be completed within five minutes, including hands-on time of about only two minutes. No device, electricity or other consumables are needed.

Table 1. Results of real-time PCR (qPCR) and insulated isothermal polymerase chain reaction (iiPCR) assays for acute hepatopancreatic necrosis disease (AHPND) showing non-detection of non-pathogenic *Vibrio parahaemolyticus* and *V. harveyi* (– = no detection).

No.	Organism	Sample Name[1]	qPCR		iiPCR	
			Plasmid	*Toxin 1*	Plasmid	*Toxin 1*
1	*V. parahaemolyticus*	BCRC 10806/ATCC 17802	–	–	–	–
2	*V. parahaemolyticus*	BCRC 12863/ATCC 17803	–	–	–	–
3	*V. parahaemolyticus*	BCRC 12864/ATCC 27519	–	–	–	–
4	*V. parahaemolyticus*	BCRC 12865/ATCC 27969	–	–	–	–
5	*V. parahaemolyticus*	BCRC 12959	–	–	–	–
6	*V. parahaemolyticus*	BCRC 12963	–	–	–	–
7	*V. parahaemolyticus*	BCRC12968	–	–	–	–
8	*V. parahaemolyticus*	BCRC 13025	–	–	–	–
9	*V. harveyi*	BCRC 12907/ATCC 14126	–	–	–	–
10	*V. harveyi*	BCRC 13812/ATCC 25919	–	–	–	–
11	*V. harveyi*	BCRC 14141/ATCC 35084	–	–	–	–

[1]BCRC = Bioresource Collection and Research Center, Hsinchu, Taiwan POC; ATCC = American Type Culture Collection, Rockville, MD, USA.

Evaluation of AHPND Status Based on Diagnostic Results

In general, bacteria are difficult to eliminate and may have long-term impacts on shrimp farms once they are introduced into a facility. Multiple strategies are available to help pond managers deal with shrimp diseases at different stages: (i) pathogen eradication/suppression, (ii) pathogen neutralization, (iii) alleviation of host clinical signs and recovery, (4) protection of shrimp from pathogens and (5) optimal ecosystem establishment and maintenance. Screening and management of potential VP_{AHPND} threats in shrimp, water, live feeds and other sources is critical. To follow VP_{AHPND} threats in the environment and shrimp tissues, PCR testing can be used to categorize AHPND infection status into five stages (i.e. safe, alert, early stage, infection and outbreak), and I(M) can be used to follow bacterial populations (Table 4). On-site detection tools for VP_{AHPND} should make the implementation of these important measures possible at shrimp culture facilities of different scales at any locations.

Particular strategies are available to help combat AHPND at different stages. At early stages, VP_{AHPND} is absent in water (negative PCR results), and bacterial population in the ecosystem can be monitored by I(M) tests. Whereas at infection stages (positive PCR results in water and stomach samples), *Vibrio* spp. in both water and shrimp can be repressed and/or killed with tools that can help manipulate the bacterial composition in the waterbody and optimize the

ecosystem for shrimp growth. Various probiotics, toxin antidotes and natural formulae (e.g. plant extracts, enzymes, minerals) are now available for different approaches to AHPND management. An example of such a protocol is "A Solution" (ScienChain Biotech, Tainan, Taiwan POC), which includes probiotics (e.g. EMS-proof, Gut-Well, Bac-Up® and Bottom-Up) and natural formulae. The following section provides some information on the use of several management strategies based on commercial products developed by ScienChain Biotech for use against AHPND.

Pond Ecosystem Management

A relatively high level of microbial heterogeneity in the culture system often helps to reduce the vulnerability of farmed animals to opportunistic colonization of bacteria (Olafsen 2001). "Bad" bacteria in the pond can compete with shrimp for food and oxygen, causing stress and disease (Moriarty 1997). *Vibrio* spp. are often the dominant bacteria found in shrimp ponds and have been the major shrimp pathogens (Lightner 1993; Chatterjee and Haldar 2012). A higher proportion of total *Vibrio* in grow-out ponds implies that microbial heterogeneity in the ponds is low. Eradication of bacterial pathogens from the pond environments has proven to be very difficult. Therefore, the best way to manage bacterial diseases of shrimp would be to facilitate microbiota balances that favour shrimp growth and prevent the propagation of pathogenic bacteria in the environment, including the digestive tracts of shrimp. Most pathogenic *Vibrio* spp. are opportunistic pathogens, and careful system management, including close monitoring for the presence of potential pathogens and for ecological conditions has been found to help curb outbreaks of vibriosis (Sung et al. 2001; Phuoc et al. 2008).

For example, the pathogenic *Vibrio* loads should be kept under control (below 1 000 $cfu.mL^{-1}$) in aquaculture systems (Ganesh et al. 2010). In intensive farming, utilization of high amounts of organic manure, inorganic fertilizer, high stocking density, feed waste, faecal matter, algal bloom and human interference should be closely monitored, as they could lead to higher loads of pathogenic *Vibrio* spp. (Lloberra et al. 1991; Moriarty 1997; Heenatigala and Fernando 2016). High salinity, alkaline pH conditions, and high levels of sulfide, ammonia and dissolved oxygen (DO) in the pond appear to favour the growth of *Vibrio* spp. The upper limit of ammonia is < 0.5 $mg.L^{-1}$ (Matias et al. 2002). Ammonium concentrations has been associated with the susceptibility of shrimp to *Vibrio* spp. (Liu and Chen 2004). According to farmers' experiences, (high DO (6–8 ppm) appears to increase the tolerance of shrimp to *Vibrio* infection. In addition, reduced water exchange in closed-water systems can help reduce the probability of pathogen introduction.

Probiotics

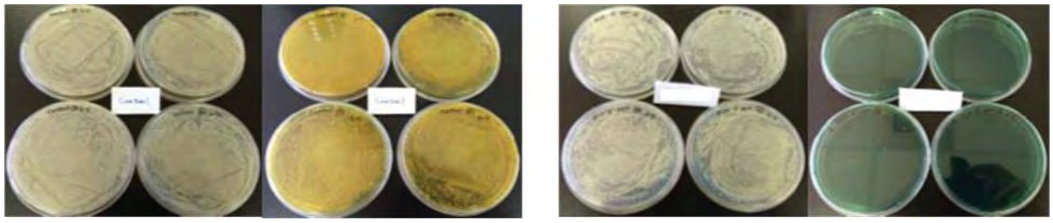
Dealing with the microflora in the ponds is difficult, as *Vibrio* spp. are always the dominant microorganisms in seawater. The most common tool for microbial control used to be antibiotics (Havenaar et al. 1992); however, with disadvantages such as alteration of microbial communities and the generation of drug-resistant bacterial strains, the use of antibiotics in water systems has been discouraged (Zanetti et al. 2001; Hansa et al. 2015). For instance, in Mexico, VP_{AHPND} strains have been found to be resistant to oxytetracycline and tetracycline (Han et al. 2015a). Different strategies such as quorum sensing disruption in the ecosystem are needed for the control of bacterial disease. Application of probiotics has been shown to improve the ecosystem in the shrimp culture facility (Pattukumar et al. 2010). Probiotics can improve ecological conditions in the culture water, remove biodebris, inhibit or compete with potential *Vibrio* pathogens for nutrients and space, colonize the gastro-intestinal tract, enhance shrimp immune systems, and provide shrimp with additional growth-promoting biocompounds. The probiotics with promising health benefit to shrimp include bacteria (e.g. *Lactobacillus* spp., *Bacillus* spp., *V. alginolyticus* and *Nitrobacter* spp.) and yeasts (e.g. *Saccharomyces cerevisiae, S. exiguous* and *Phaffia rhodozyma*) (Cruz et al. 2012). Successful strategies involving probiotics should include: (i) inclusion of organisms capable of surviving in the shrimp culture environment, (ii) inclusion of organisms producing specific anti-*Vibrio* bacteriocin to help suppress or kill *Vibrio* spp., (iii) inclusion of organisms that can be provided in a high stock concentration (e.g. 100:1 dilution) and (iv) optimization of ecological factors suitable for shrimp growth (e.g. higher DO levels). Probiotics can be applied through feed, water or a combination of feed and water in shrimp culture.

Examples of AHPND Management Strategies Using Commercially Available Ecological Tools

Reduction of Vibrio spp.

One recent example of a commercial product that was successfully used to reduce *Vibrio* spp. on brine shrimp (*Artemia* sp.) is Germ-Out, which contains natural plant extracts. The benefits of Germ-Out have been demonstrated in trials in commercial hatcheries and grow-out farms. In one case, when used at 1:10 000 x dilution (e.g. 20 mL in 200 L of seawater with 1 lb of brine shrimp eggs) at 6–10 h before hatching, Germ-Out suppressed *Vibrio* spp. only and did not affect the total marine bacterial population (including probiotics) or the hatching rates of brine shrimp (~100 %) (Fig. 3). EMS-Proof is another probiotics formula able to suppress the growth of *V. parahaemolyticus* (including VP_{AHPND}) and *V. harveyi* (fluorescent *Vibrio*). Daily feed supplementation with Germ-Out and water treatment with EMS-Proof during the grow-out phase can help to reduce the population of fluorescent *Vibrio* spp. In another trial, shrimp were fed with feed sprayed with Germ-Out (100 mL 1 000-fold diluted Germ-Out on 1 kg feed); and the pond water was also sprayed with EMS-Proof. The control group was fed with feed without Germ-Out and kept in water without EMS-Proof treatment. Both green colony and yellow colony counts on TCBS agar plate were greatly reduced with the application of EMS-Proof plus Germ-Out in two ponds (Fig. 4A). In another test, the survival rate of artificially AHPND-infected *P. vannamei* (n=30) receiving Germ-Out treatment was 77 %, greatly higher than that of the control group (0 %) (Fig. 4B).

	Marine Agar (cfu.mL^{-1})		TCBS Agar (cfu.mL^{-1})	
Germ-Out	>300	>300	1	4
	>300	>300	0	1
Control	>300	>300	>700	>700



Control		Germ-Out	
Marine Agar	TCBS Agar	Marine Agar	TCBS Agar

Fig. 3. Germ-Out treatment helped suppress *Vibrio* spp. population in *Artemia* sp. Total bacteria counts (marine agar) and *Vibrio* spp. counts (thiosulfate-citrate-bile salt-sucrose (TCBS) agar) in brine shrimp receiving Germ-Out treatment or control group receiving no treatment were determined. The numbers and plates are shown in the upper and lower panels, respectively. CFU, colony forming unit.

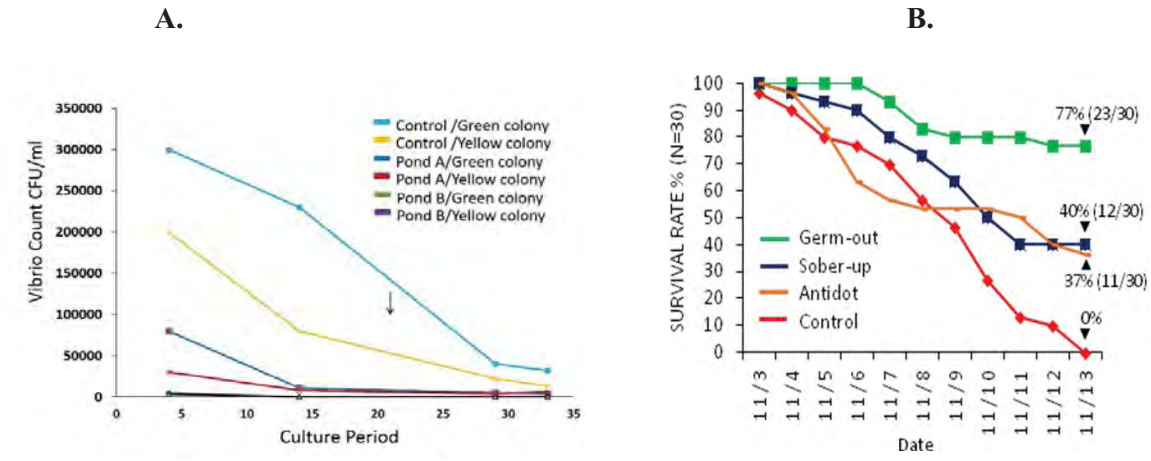


Fig. 4. (A) EMS-Proof with Germ-Out suppressed *Vibrio* spp. and increased survival rates of VP_{AHPND}-infected *Penaeus vannamei* in two ponds. EMS-Proof was activated and sprayed into the water. Germ-Out was sprayed onto shrimp feed at a 1:10 000 ratio. The control group was fed with feed without Germ-Out and the water was not treated by EMS-Proof. Green and yellow colonies on thiosulfate-citrate-bile salt-sucrose (TCBS) agar were determined. The arrow indicates the time point of control pond starting probiotics treatment. (B) Increased survival rates in artificially AHPND-infected *P. vannamei* (n=30) treated with Germ-out, Sober-up or Antidot®. The control received no treatment.

Neutralization or Removal of Toxin

The virulence of VP_{AHPND} is caused mainly by *toxin 1* (Han et al. 2015b; Sirikharin et al. 2015). Neutralization of toxin by antibody and probiotics can successfully reduce the virulence of pathogens (Sleator 2010; Paton et al. 2015). Sober-Up, a novel natural mineral mixture with

affinity for *toxin 1*, was demonstrated to absorb *toxin 1* efficiently in the shrimp stomach (Fig. 5A). This product also absorbs other algal toxins and mycotoxins. Antidot® is another formula containing secondary metabolites of *Lactobacillus* spp. and purified enzymes that can digest *toxin 1* (Fig. 5B), algal toxins and mycotoxins in the shrimp stomach. These products are best applied when positive AHPND PCR results are obtained in shrimp tissue samples. In one example, *P. vannamei* was artificially infected with VP_{AHPND} and subsequently fed with Sober-Up (final dosage 2 %; n=30) and Antidot® (final dosage 1 %) in shrimp feed. These shrimp had higher survival rates (~40 %), as compared to the control group (final dosage 0 %, n=30) (Figure 4B).

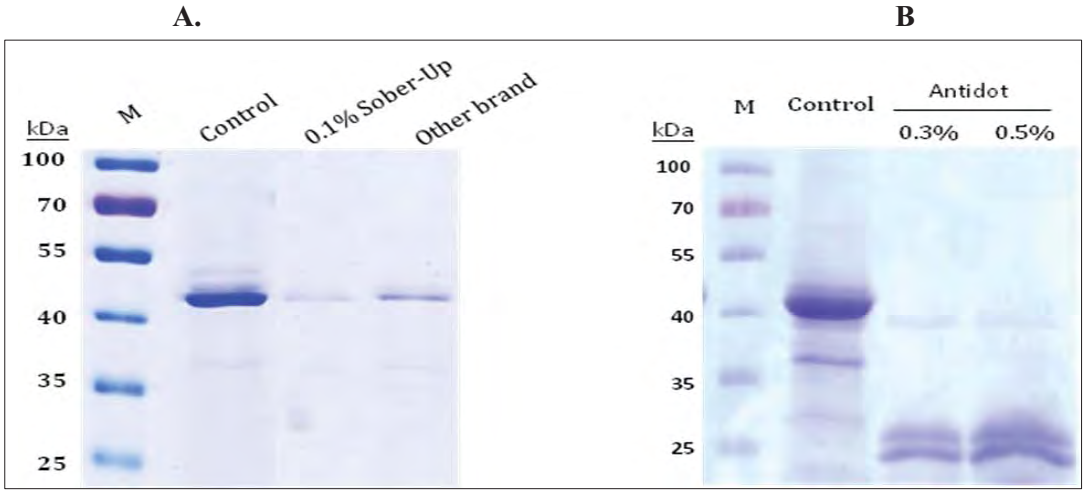


Fig. 5. *Toxin 1* was reduced by Sober-Up and Antidot®. After *toxin 1* was treated with 0.1 % Sober-Up (A), or 0.3 % or 0.5% Antidot® (B), residual *toxin 1* was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE analysis). Control, *toxin 1* protein without any treatment. (B) The same amount of *toxin 1* protein was treated with 0.3 % and 0.5 % Antidot®. The residual *toxin 1* protein was analyzed by SDS-PAGE.

Protection of the Shrimp Gastro-intestinal Tract by Probiotics

Gut-Well, a *Lactobacillus*-based probiotics with anti-*Vibrio* bioactivity, contains *Lactobacillus* spp. that can survive in a wide range of salinity and temperature. This product requires a 24 to 48-hr activation period before it is applied in feed at a ratio of 10 g per 20 kg feed in 2 liters of water for PL, or 10 g per 100 kg feed for grow-out shrimp.

Protection of Shrimp by a Combination of Tools

The complete rearing cycle of shrimp culture is a long process that includes the broodstock, hatchery production, nursery and grow-out stages. A combination of the approaches mentioned above is often required to have a successful operation. One trial based on the "A Solution" (ScienChain Biotech) including the use of probiotics (i.e. EMS-Proof, Gut-Well, Bac-Up® and Bottom-Up) and natural formulae was carried out at a nursery in Viet Nam rearing *P. vannamei*. Lower total *Vibrio* counts (TCBS plating) and more stable total marine bacterial counts (marine agar plating) were found in both PL and water treated with "A Solution", compared to the untreated group (Fig. 6).

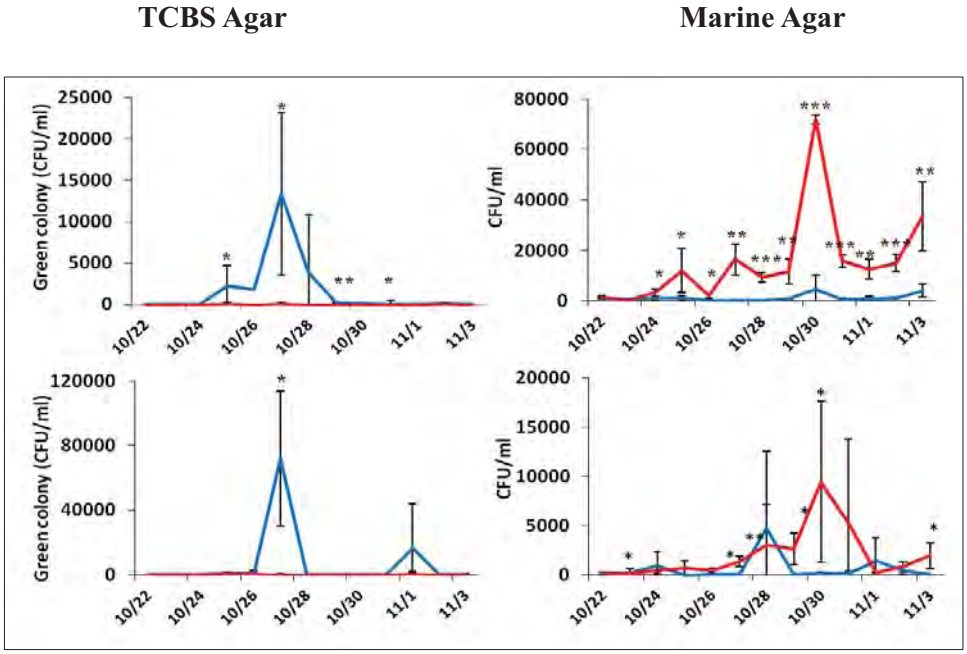
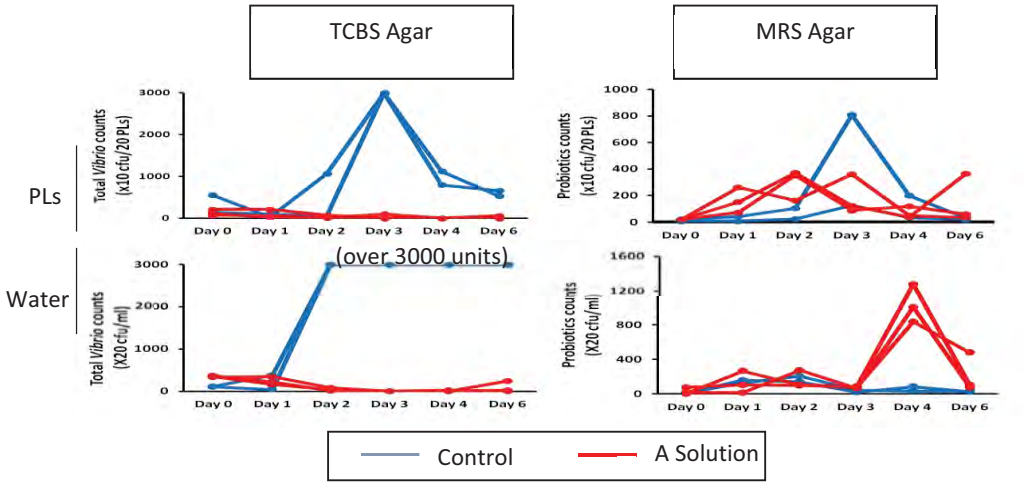


Fig. 6. "A Solution" treatment improved microbial compositions at a nursery farm in Viet Nam rearing *Penaeus vannamei*. Postlarvae (PL) and water treated with (red line) or without (blue line) "A Solution" were analyzed over a period of two weeks for their *Vibrio* counts on thiosulfate-citrate-bile salt-sucrose (TCBS) agar plate and total marine bacterial counts on marine agar plate. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

In addition, the percentages of PL with empty or disordered midguts were significantly lower in PL artificially infected with VP_{AHPND} and receiving the treatment on day 3 postinfection (data now shown). A field test with "A Solution" was also performed at a nursery farm in Panama in the summer of 2016, immediately after fluorescent *Vibrio* numeration on TCBS agar plating and results of on-site POCKIT *Vibrio harveyi* iiPCR-positive detection were obtained. A combination of *Vibrio*-controlling strategies in the "A Solution" was applied, including PCR diagnosis, *Vibrio* suppression, shrimp health maintenance and ecosystem balancing. After the "A Solution" treatment, MRS plating showed that total *Lactobacillus* spp. counts in PL and the waterbodies were elevated (Fig. 7A); and TCBS plating showed that the numbers of fluorescent *Vibrio* spp. in PL and pond water dropped significantly, compared to those found before these intervention measures (Fig. 7B).

A.



B.

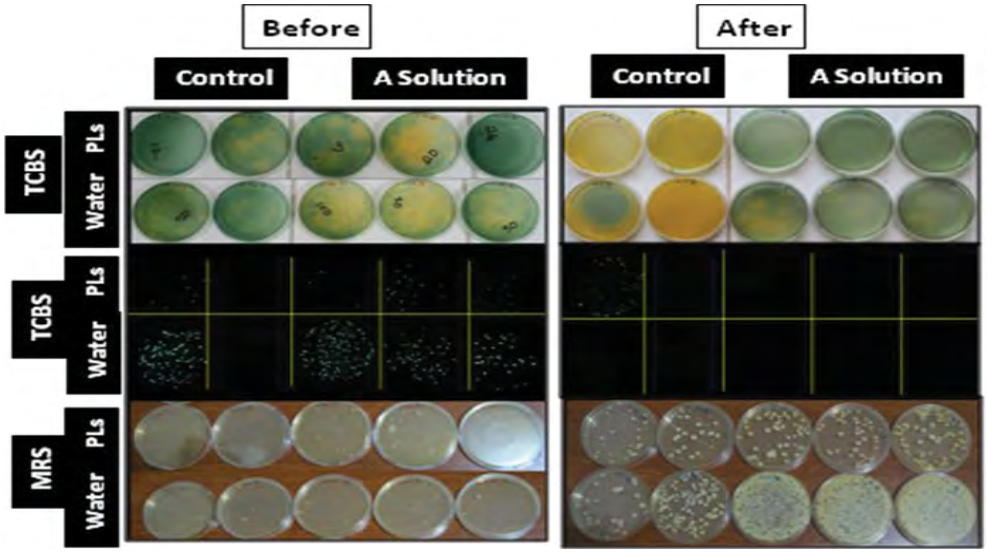


Fig. 7. "A Solution" treatment on a *V. harveyi* insulated isothermal polymerase chain reaction (iiPCR)-positive nursery farm with high fluorescent *Vibrio* counts in Panama in the summer of 2016. (A) The *Vibrio* counts on thiosulfate-citrate-bile salt-sucrose (TCBS) agar plates and probiotic counts on De *Man Rogosa Sharpe* (*MRS*) agar plates were determined in both postlarvae (PL) and water in a pond treated with "A Solution" treatment (red line) and one without treatment (blue line). (B) PL and pond water before (left panel) and after (right panel) the "A Solution" treatment were assayed for concentrations of *Vibrio* spp. on TCBS agar, luminescent *Vibrio* spp. on TCBS agar, and lactobacilli on MRS agar. The control groups received no "A Solution" treatment.

Advantages of Indoor Ponds in a Cold Climate

Basic biosecurity measures include the use of specific pathogen-free (SPF) shrimp stocks and seeds; disinfection of facilities, equipment, water and workers; coverage of culture tanks with plastic sheets to prevent pathogen introduction into hatcheries; and maintenance of optimal environmental conditions, including water, feed and stocking density in grow-out ponds throughout the culture cycle. In well-controlled environments, shrimp can be produced and supplied to the market during seasons when prices are high. Recently, a new operational concept for shrimp culture has become popular in the northern People's Republic of China.

It involves a natural biosecurity system built upon a greenhouse facility, providing a super-intensive farm with well-controlled biosecurity to keep pathogens out. In one case (Fig. 8), locally produced live shrimp from this system reached prices of US$ 5–8.kg^{-1} during the winter of 2015. However, this type of facility is extremely expensive to construct, costing about US$ 1 to 1.5 million per hectare, not including the land. In this case, stocking density was about 500–700.m^{-2} and harvest size averaged about 20 g. Eighty tonnes.ha^{-1}.crop^{-1} were harvested, resulting in 240 tonnes.ha^{-1}.yr^{-1} (valued at US$ 1.2 to 2 million per year). In winter, the temperature outside (-20 ^{o}C) can destroy the potential carriers and hosts of shrimp viruses, as well as non-viral pathogens such as VP_{AHPND} and *Enterocytozoon hepatopenaei* (*EHP*), minimizing the risk of horizontal disease transmission and providing a natural biosecurity barrier. Low water exchange also helps to minimize the risk of pathogen introduction from intake water.

Other biosecurity measures included the use of plastic sheets and bird nets on top of the facility, use of PL derived from SPF broodstock, (including freedom from the VP_{AHPND}), and well-trained pond managers who are familiar with on-site diagnostic tools and AHPND treatment options and protocols. A few AHPND outbreaks were suspected, and in these cases, the pond managers were able to make an appropriate diagnosis and apply suitable control strategies.

Fig. 8. Green houses for shrimp farming in northern People's Republic of China – a growing operation concept. The facility can provide a well-controlled, natural biosecurity barrier.

Conclusion

In shrimp culture, the "patient" is the infected pond instead of the shrimp, while the "doctor" is the pond manager. The "doctor" should understand both the disease and the "patient" very well. The "doctor" should be equipped with a comprehensive set of on-site diagnostic tools, including a hand-held PCR for molecular diagnosis and agar plating for microbiological diagnosis (TCBS plates for *Vibrio* spp., marine agar plates for total marine bacteria, and MRS plates for *Lactobacillus* spp.). The "doctor" should be provided with complete and effective treatment tools, including agents that stop or kill the pathogens, relieve clinical signs and help shrimp recover from diseases, protect them from infection and/or provide better environment for their growth.

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Polychaetes as Potential Risks for Shrimp Pathogen Transmission

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Abstract

Polychaetes comprise the benthic meiofauna of the soft-bottom intertidal zone and of shrimp ponds in coastal areas. While polychaetes provide benefits to the shrimp farming industry as (i) natural food in traditional shrimp ponds, (ii) nutrient regenerators through bioturbation and removal of organic waste in the sediment through feeding, and (iii) feed supplement to enhance maturation of shrimp brooders, the conditions present in aquaculture ponds may increase the opportunity for polychaetes to transfer pathogens to shrimp through the food chainThere is growing concern that internationally traded polychaetes, which are fed to shrimp brooders, are potential vectors for the transmission of other shrimp pathogens. The detection of the aetiological agents of two newly emerging diseases of shrimp in polychaetes, *Enterocytozoon hepatopenaei* (EHP) and *Vibrio parahaemolyticus*$_{\text{AHPND}}$ causing hepatopancreatic microsporidiosis (HPM) and acute hepatopancreatic necrosis disease (AHPND), respectively, suggests that these worms can be a host or/and passive carrier of these pathogens. This review discusses the benefits of polychaetes to shrimp farming, the risk of shrimp pathogen transmission by polychaetes at the pond, hatchery and global level, and calls for closer observation on shrimp pathogens in polychaetes used as shrimp feed.

Keywords: AHPND, EHP, pathogen transmission, risk, polychaetes, shrimp, WSSV

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Introduction

Polychaetes are ubiquitous benthic-meiofauna in shrimp ponds and the soft sediments of coastal areas. As a group, polychaetes provide ecological services for the sediment environment through bioturbation and the removal of organic wastes during feeding (Brown et al. 2011) and by being prey for animals at higher trophic levels such as shrimp, fish and birds (Hutchings 1998). Eutrophication of the pond bottom during shrimp culture results in hypoxia and entrapment of inorganic phosphorus, nitrogen and organic matter in the sediment. Polychaetes are beneficial for the pond bottom environment by recycling nutrients, making these partially available to primary producers and consumers in the pond, and by reducing the anaerobic area at the sediment-water interface through movement. Certain species of polychaete are traded at the global level as favoured baits for anglers (Arias et al. 2013, Carregosa et al. 2014) and as supplemental feed to shrimp brooders to improve spawning performance and enhance maturation (Leelatanawit et al. 2014). Polychaetes are highly adapted to a broad range of environmental conditions (Çinar 2013) and have remarkable reproductive plasticity and adaptability (Arias et al. 2013). The latter may assist polychaetes to colonize new areas (Çinar 2013) and to thrive in ponds and estuaries which are rich in organic matter.

Sediment may act as a sink to pathogens, assisting in their survival, hence becoming a pathogen reservoir or resource. Research on the link between the presence of white-spot syndrome virus (WSSV) in the sediment, benthic polychaetes and WSSV infection in shrimp (Vijayan et al. 2005; Desrina et al. 2013; Haryadi et al. 2015) sheds some light on the different facets of the role of polychaetes in shrimp farming. Burrower and detritofeeder polychaetes live in shrimp production systems such as ponds − including coastal areas receiving effluents from the ponds − where they are exposed to and potentially acquire pathogens present in the sediment. In the case of WSSV, the port of entry of the pathogen into polychaetes is most likely *per os,* and the worm in turn transfers the pathogen to shrimp after being fed upon. These findings raised interest in the possible role of polychaetes in the spread of two newly emerging shrimp diseases, acute hepatopancreatic necrosis disease (AHPND) caused by the bacterium *Vibrio parahaemolyticus* (VP_{AHPND}) and hepatopancreatic microsporidiosis (HPM) caused by the microsporidian *Enterocytozoon hepatopenaei* (EHP) (Thitamadee et al. 2016). The pathogenic form of *V. parahaemolyticus* carries a plasmid encoding two toxins, PirA and PirB, which, when expressed, are responsible for the disease in shrimp (Lee et al. 2015; Han et al. 2015). Here we not only review the benefits of polychaetes for shrimp culture, but also discuss the potential risks for further spread of pathogens based on experiences gained from studying WSSV infection in the polychaete *Dendronereis* spp., and finally suggest enhanced surveillance for shrimp pathogens in polychaetes used for shrimp feed as a starting point to mitigate or control the disease.

Overview of the Biology and Ecology of Polychaetes Relevant to Shrimp Culture

Polychaetes form a class of segmented worms in the Phylum Annelida; are highly varied in shape, size, and reproduction strategy; and occupy a variety of ecological niches (Hutchings 1998). Many polychaete species are ubiquitous macro-invertebrates in coastal habitats including rocky coastal areas and soft-bottom estuaries (Sarkar et al. 2005). They are considered opportunistic species, as these animals are the first to inhabit defaunated soft sediments with high organic matter (Kanaya 2014). The ability of polychaetes to inhabit estuaries shows their high adaptability and environmental plasticity, because estuaries and coastal areas are highly dynamic ecosystems. Naturally, polychaetes living under these conditions in estuaries have high tolerance to a broad range of salinities (8–19 ppt in water and 0–2.5 ppt in soil) (Roy and Nandi 2012), high concentrations of organic matter and to pollution. Since most shrimp ponds are built in estuarine areas, it is expected that polychaetes will be abundant in shrimp ponds and in most cases, will become the dominant benthic invertebrate species (Fujioka et al. 2007; Ngqulana et al. 2010). The ability of polychaetes to live in various ecological niches and benthic conditions has resulted in their broad geographical spread by accidental and intentional transportation. The distribution and abundance of polychaetes are affected by sediment conditions, including texture (Sarkar et al. 2005), organic content (Gowda et al. 2009), water depth, salinity, temperature (Hutchings 1998) and predation (Abu Hena et al. 2011).

Aquaculture activities produce a bulk of organic wastes that may cause the accumulation of nutrient-rich sediment and patches of hypoxial areas on the pond bottom. While shrimp avoid pond areas having low dissolved oxygen content, in contrast, polychaetes live (some even thrive) under such conditions. Polychaetes provide ecological services for the pond environment and for animal life in it through movement and transport processes, feeding activity and by being prey for animals at a higher trophic level. Errant polychaetes such as the nereids move horizontally and vertically for foraging and burrowing, causing considerable bioturbation. The mixing of sediments and pore water in the sediment-water interface during bioturbation facilitates the degradation of organic matter in a density-dependent manner (Kristensen et al. 1985; Papaspyrou et al. 2010). Polychaetes ventilate their burrows (Kristensen 1984), thereby stimulating metabolism of aerobic microbes in the sediment. This helps to restore the living area for cultured shrimp by increasing the availability of nutrients while reducing anaerobic conditions (Hutchings 1998). Since polychaete burrows can reach 30 cm depth below the sediment surface, burrower polychaetes may also help in recirculating some nutrients that may accumulate during shrimp culture. This is very relevant for traditional ponds where sediment removal is limited and spaced in time. Our observations on two burrower nereidids (*Dendronereis* spp. and *Hediste diversicolor*) is that they stay in the burrow with a vertical or horizontal orientation and that they move actively to grab their food and pull it into the burrow.

The feeding activity of polychaetes facilitates the removal of organic matter from the sediment while the nutrients are utilized for polychaete development, hence assisting in nutrient recycling in the sediment. Two species, *Nereis virens* and *N. diversicolor*, showed the ability to metabolize the nitrogenous faecal waste of clams (Batista et al. 2003a) and faecal and feed waste of halibut (Brown et al. 2011). These studies indicate that polychaetes can be a solution for aquaculture waste management, thus promoting sustainable aquaculture. Most polychaetes that live in the soft sediment are suspension and deposit feeders (Hutchings 1998), although the mouth of some worms, for instance nereidid polychaetes, is equipped with cuticular structures called jaws. Accordingly, the feeding strategy of nereidid species can change in accordance with the types of food available. For example, wild *N. diversicolor* living in estuaries mainly feed on a mucous complex containing organic matter, bacteria and fungi (Fidalgo e Costa et al. 2006), although they also feed on sediment and predate other nereids. The flexibility in feeding strategy and the ability to live under oxygen-poor conditions in organic-matter-rich sediments make polychaetes such as *N. diversicolor* (Batista et al. 2003b) and *N. virens* suitable bottom scavenger species in integrated multitrophic aquaculture (IMTA) systems (Brown et al. 2011; Van Geest et al. 2014). Large detritofeeder polychaetes such as eunicids can increase recycling of proteinaceous waste produced from aquaculture activities by enhancing enzymatic degradation (Santander-De Leon et al. 2010). Although no studies have been done on the impact of polychaetes on nutrient cycling in shrimp ponds, integrated production systems of shrimp and polychaetes in ponds should be explored as a means to raise nutrient utilization efficiencies. On the other hand, filter and detritus-feeder polychaetes are exposed to pathogens that are present in the sediment and can acquire the pathogens through feeding, as in the case of WSSV (Vijayan et al. 2005). They can be passive or active vectors of pathogens, or both, as is probably the case for WSSV. In turn, polychaetes, potentially carrying pathogens, are preyed upon by shrimp (Nunes and Parsons 2000). At present, information on the contribution of infected polychaetes to the transmission of shrimp diseases is limited, and more research is required.

Polychaetes as the Natural Feed of Shrimp in Grow-Out ponds

Three genera of polychaete have been reported as feed of shrimp in ponds and hatcheries: *Dendronereis* (Haryadi et al. 2015), *Perinereis* (Poltana et al. 2007; Meunpol et al. 2010; Leelatanawit et al. 2014) and *Marphyssa* (Vijayan et al. 2005). Members of these genera are burrowers and have a broad geographical distribution (Hutchings and Karageorgopoulos 2003; Glasby and Hutchings 2010; Ngqulana et al. 2010). *Perinereis* spp. prefer sandy sediment, whereas *Marphyssa* spp. and *Dendronereis* spp. are mostly abundant in soft and muddy sediments. The species of polychaete found in shrimp ponds may vary from one area or region to another, but also depends on the environment and the pond management. Polychaetes and other benthic invertebrates are important components of the shrimp diet in traditional shrimp ponds. Shrimp inherently rely on natural food in the pond, and polychaetes having a high protein and fatty acid content are an attractive food source. Polychaetes occur in ponds rearing giant tiger prawn (*Penaeus monodon* Fabricius 1798) all the time (Abu Hena et al. 2011).

However, the density of polychaetes tends to decrease towards the end of the shrimp-culture period (Nunes and Parsons 2000; Abu Hena et al. 2011), due to predation, as shown by gut content analysis of *P. monodon* reported by Varadharajan and Soundarapandian (2013). At low shrimp density, the predatory pressure on benthic prey is low (Balasubramanian et al. 2004), which may explain the abundance of polychaetes in traditional extensive ponds and throughout the culture time, as we observed in our own study, albeit that the dominant species may vary (Desrina 2014). Although no systematic studies have been done on the growth and weight gain of grow-out shrimp resulting from feeding on polychaetes in Indonesia, during interviews with the first author, farmers indicated that shrimp grow faster and are healthier when shrimp ponds contain lots of *Dendronereis* spp. Furthermore, in laboratory observations, shrimp show a high preference for polychaetes relative to formulated diet, and this preference might reflect the situation in ponds (Desrina 2014).

Being a benthic invertebrate, the well-being of polychaetes is also determined by pond bottom condition. The bottom of a traditionally managed pond is likely to be a selective environment for polychaetes because of the high concentration of organic matter. Many ponds are not completely dried after harvest and sediments are seldom completely removed. Nevertheless, even when the incoming water is first passed through a settlement pond, sediment accumulation in the shrimp pond will remain substantial. Accretion of organic matter-rich sediment over a 5–10 year period can result in a 30 cm thick semisolid layer of dark, highly reduced, sediment. Some polychaetes are adapted to this condition, having the ability to live in a low-oxygen niche and to feed on organic waste (Brown et al. 2011). The *Dendronereis* spp. we observed were most abundant in sediment having a soil organic carbon concentration between 5–10 % (Desrina 2014), a condition unfavourable for most other benthic organisms.

Polychaetes as Natural Feed of Shrimp Brooders

Feeding fresh feed to shrimp brooders is widely practiced in shrimp hatcheries. Because they are highly nutritious, polychaetes as a group are an important component to promote spawning performance of shrimp broodstock (Chung et al. 2011). Polychaetes contain high levels of unsaturated fatty acids such as arachidonic acid (Hoa et al. 2009; Leelatanawit et al. 2014) and the reproduction hormones progesterone (P4), 17α-hydroxyprogesterone (Meunpol et al. 2007) and prostaglandin E2 (Meunpol et al. 2010), which enhance gonad maturation of female and male shrimp brooders. Polychaetes reported to be used in hatcheries are the mud worm *Marphyssa* spp. (Vijayan et al. 2005) and the sandworm *Perinereis* spp. (Poltana et al. 2007). In addition, P. *cultrifera* has been investigated for boosting the reproductive performance of captive sole (Cardinaletti et al. 2009), indicating an increased interest in exploring the use of polychaetes not only to condition shrimp brooders but also for fish broodstock. Although polychaetes alone are nutritious enough to ensure good reproductive performance, studies on combining polychaetes and immunostimulant sodium alginates produced even better results in terms of the amount of eggs produced by spawners, total larval production and hatching rate of *P. monodon* as compared to polychaetes alone (Chung et al. 2011).

This shows that the reliance on polychaetes and other fresh feed may be reduced by using supplements that compliment the nutrition provided by the polychaetes. In our laboratory, shrimp prefer live polychaetes, although they also eat frozen ones. Freezing and thawing can result in loss of body fluid that leaks during the process and loss of odor, hence, making them less attractive to shrimp. Most companies advertise nereid polychaetes in the form of freeze-dried or frozen material. The commercial use of polychaetes as a replacement for fishmeal in the shrimp feed has been initiated.

Polychaetes as Potential Risk for Shrimp Disease Transmission

So far, only three species of polychaete have been reported to carry natural infections of shrimp pathogens: WSSV in *Marphyssa* spp. (Vijayan et al. 2005), *P. nuntia* (Supak Laoaroon et al. 2005) and *Dendronereis* spp. (Desrina et al. 2013; Haryadi et al. 2015). WSSV transmission from polychaete to shrimp was reported only for *Dendronereis* spp. and *Marphyssa* spp. However, *Dendronereis* spp. is a replicative host for WSSV (Desrina et al. 2013), while *Marphyssa* spp. appears to be only a passive vector (Vijayan et al. 2005). Recently, the DNA of two newly emerging pathogens of shrimp, EHP and VP_{AHPND}, was detected in imported polychaetes in Thailand using polymerase chain reaction (PCR). These worms were suspected as the route of entry of the AHPND agent into shrimp hatcheries in Thailand (Thitamadee et al. 2016), indicating that polychaetes may act as hosts, carriers or vectors that play a role in the spread of these pathogens at the global level. However, there is no information about the species of polychaete involved or the body parts that were positive for the AHPND aetiologcal agent. Further systematic studies are needed to verify the role of polychaetes in the epidemiology of AHPND and EHP. The niche, feeding strategies and position of polychaetes in the food chain accentuate the risk of shrimp pathogen transmission by polychaetes, although the pathway may be different for grow-out ponds and hatcheries.

There are several reasons why polychaetes pose a potential risk as vector**s,** carriers and/or hosts of shrimp pathogens in the pond environment. First, polychaetes permanently reside in burrows in the pond sediment, and hence this provides opportunities for polychaete and shrimp-pathogen encounters over an extended period of time. For generalist pathogens, continuous exposure to potential hosts is an important factor driving pathogen adaptation and broadening of the host range (Woolhouse et al. 2001), which could be the situation for WSSV and VP_{AHPND}, since both are multihost pathogens. Further, polychaetes' contact with the pathogen will become more intense during a disease outbreak when the pathogens are more abundant in the pond.

Second, the niche and feeding guild of polychaetes facilitate the acquisition of pathogens which settle in the sediment. Ponds act as sediment and organic matter traps during shrimp production. High concentrations of DNA of viral pathogens to humans, terrestrial animals (Staggemeier et al. 2015) and fish (Honjo et al. 2012) in pond sediment indicates that sediments can be a sink or reservoir for pathogens. Furthermore, sediment may provide a suitable niche for persistence of viruses, prokaryotes and parasites.

For example, WSSV retained its viability and infectivity in the sediment for 35 days (Satheesh Kumar et al. 2013), presenting opportunities for the pathogen to enter susceptible benthic inhabitants (hosts and/or vectors), such as polychaetes. Although there are as yet no reports of the presence of EHP spores in the pond sediment, the observation that faeces of shrimp suffering from white faeces syndrome (WFS) contained EHP spores (Rajendran et al. 2016; Tang et al. 2016) suggests that the spores may sink and reside in the sediment. Likewise, *V. parahaemolyticus* is ubiquitous in the sediment (Darshanee Ruwandeepika et al. 2012). For a generalist pathogen, sediment-associated pathogen transfer through the food chain increases the survival of the pathogen in the environment and its maintenance in the pond environment through transfer in the food chain. As filter feeders and detritofeeders, polychaetes may acquire pathogens present in the sediment, as reported for WSSV (Vijayan et al. 2005), although it is not known how long it took to replicate in the polychaete. Moreover, our observations with *Dendronereis* spp. indicate that this polychaete can carry a heavy infection of WSSV without showing any behavioural or gross external signs, suggesting that viral-host adaptation may exist (Haryadi et al. 2015). In the laboratory, *Dendronereis* spp. and *Hediste diversicolor* fed on formulated shrimp feed use their jaws to grab food and drag it into their burrows. However, by microscopical examination we also found sand and soil in the respective guts, suggesting that they also eat detritus. If we consider the condition in the pond, these worms can also feed on infected shrimp carcasses; thus, polychaetes may ingest pathogens directly from diseased shrimp.

Third, burrowing polychaetes can possibly avoid the chemicals used to control pathogens and pests in ponds by retracting into their burrows, allowing pathogens to survive within their host. For example, we detected WSSV with 1-step PCR from some *Dendronereis* spp. obtained from the pond bottom at up to 30 cm depth and from *Marphyssa* spp. from up to 40 cm depth, following chemical treatment to eradicate the virus after an outbreak. Oral transmission through the food chain and cohabitation are the two most important situations for shrimp pathogen transmission. Taken together, polychaetes can contribute to the epidemiology of diseases in the shrimp pond.

World shrimp production is projected to increase and as a consequence, the demands for broodstock will also increase, and thus the demand for polychaetes as an ingredient in broodstock diets. For this reason, polychaetes traded globally may pose a biosecurity risk in shrimp hatcheries. Some hatcheries raise their own polychaetes to prevent disease transmission to their facility. However, it is quite often that the polychaete production is not enough to meet the demand, forcing the company or local farm to rely on polychaetes collected from the wild. Often, polychaetes fed to brooders in hatcheries are collected from the estuary adjacent to the farm. In turn, surface waters adjacent to shrimp farms receive farming effluents, establishing a type of permanent contamination loop. Further, it is common for wild polychaete populations to have co-infection of more than one pathogen with different host exploitation and transmission strategies, or with one species of pathogen with different genotypes (Ben-Ami et al. 2011). For example, some of the *Dendronereis* spp. we examined had WSSV and haplosporidian cysts in the body cavity. This makes reliance on polychaetes captured in the wild an even more risky venture.

AHPND is caused by a strain of *V. parahaemolyticus* that has acquired at some point a specific plasmid carrying toxin genes (PirA and PirB). As the plasmid is transmissible by horizontal gene transfer and *V. parahaemolyticus* is ubiquitous in the brackishwater environment, the presence of the AHPND agent in polychaetes should not be surprising. We isolated sucrose and non-sucrose fermenting *Vibrio* from the coelomic fluid of healthy looking *Dendronereis* spp., and this suggests that this bacterium might be a normal inhabitant in the polychaetes. The ecology and biology of EHP is largely unknown, including whether shrimp is the sole host to this parasite, or whether there are secondary hosts among meiobenthic animals such as polychaetes and bivalves. It is even not clear if EHP is dependent on an animal host at all. Nevertheless, the detection of toxin-containing plasmid DNA in polychaetes (Thitamadee et al. 2016) strongly suggests that the pathogenic agent or parts of it have been in close contact with polychaetes.

Findings from a previous study on WSSV in *Dendronereis* spp. (Desrina et al. 2013) showed that the polychaete *Dendronereis* spp. with WSSV was widely distributed in Indonesia and that this polychaete can harbour the virus without notable signs of disease, such as white spots under the epidermis or sluggishness. Also, the occurrence of WSSV in *Dendronereis* spp. correlated positively to the WSSV infection in shrimp (Desrina 2014). We may infer, somewhat tentatively, that a similar situation may be applicable to AHPND and EHP, considering the ubiquity of *V. parahaemolyticus* in shrimp culture operations and the plasticity of the plasmid and the nature of Microsporidia. The occurrence and growth characteristics of the disease agents of AHPND and HPM in polychaetes need further investigation, and as do the species and source of the polychaete(s). Most importantly, transmission studies to show that the disease is indeed transmitted from polychaetes to healthy shrimp are needed. This information is important to determine the strategy and method of control. For example, if polychaetes are only a mechanical vector, then depuration for 48 hr until the gut is cleansed may be applicable.

Having said all this, there are potential vectors of pathogens in the pond environment other than polychaetes, such as crabs and crayfish. However, they are not permanent residents in ponds and can move to neighboring shrimp ponds, increasing the risk of horizontal transmission.

Conclusion

Our current knowledge on the involvement of polychaetes in shrimp pathogen transmission is limited by: (i) the few studies that have been conducted, (ii) the scant knowledge of the life history of the pathogen (especially in the case of AHPND and HPM), (iii) the absence of knowledge on the defense responses of polychaetes important for shrimp farming, (iv) the biology and ecology of polychaetes in ponds, and (v) the distribution of pathogens in polychaete host tissue(s). When reporting pathogen occurrence, it is advisable to identify the polychaetes to the lowest taxonomic level possible, because polychaetes form a large class of annelid worms and species susceptibility to a given shrimp pathogen may thus vary.

However, the mere presence of the pathogen DNA in the polychaete does not prove that: (i) the whole pathogen is alive, it might just be an inert residue, (ii) the pathogen develops in the polychaete, or (iii) that the pathogen will be transferred to the shrimp and cause disease. Experiments need to be carried out to investigate these issues. It may be concluded that the ecological niche in pond settings and the feeding habits of polychaetes allow these animals to acquire shrimp pathogens and transmit them to shrimp upon feeding. However, this cycle may start with an unnatural abundance of the pathogen in the environment (e.g. due to incomplete, inappropriate or inaccurate pond cleaning), resulting in the accumulation of the pathogen in the polychaete, or that the pathogen is undergoing (epi)genetic changes adapting to the polychaetes. Further studies on the epidemiology of shrimp pathogens and the role of polychaetes and pond management strategies in influencing this multifaceted interaction are needed. Since HPM and AHPND are caused by otherwise normal inhabitants of the pond, control measures may include sound shrimp health management (e.g. better management practices, BMPs), crop rotation to break the pathogen cycle and lowering stocking density. Excluding polychaetes altogether from the pond environment might be a way forward to lower the risk of shrimp pathogen transmission by polychaetes, but is unrealistic.

Nevertheless, the way forward is the rigorous screening of polychaetes used as feed for shrimp for the presence of pathogens, more specifically WSSV, VP_{AHPND} and EHP, as is currently being done for shrimp. Specific nested PCR tests are available and in place to detect these pathogens, for WSSV since 1995 and for VP_{AHPND} (Flegel and Lo 2014; Sirikharin et al. 2015) and EHP (Tangprasittipap et al. 2013) since 2013 and 2014, respectively. Even differential PCRs are available to differentiate pathogenic and benign strains of *V. parahaemolyticus* (Sirikharin et al. 2015). It is also important to check the resident polychaetes in shrimp ponds for the presence of these pathogens, in particular for VP_{AHPND}, as this bacterium can also multiply outside a host. Early detection and monitoring are the first steps in mitigation or control of pathogens such as WSSV, APHND and EHP in shrimp ponds.

In summary, polychaetes have been "under the radar" for quite some time as vectors of shrimp pathogens and are often not part of biosecurity regimens and regulatory frameworks. However, recently there is increased interest in polychaetes, not in the least because important pathogens such as WSSV, APHND and EHP are found in and possibly transmitted by these organisms. The lack of fundamental insight into the biology of polychaetes, their behaviour and vectorial competence in ponds, as well as the lack of hygiene in polychaete-producing farms requires the increased attention of scientists, practitioners and regulators in filling in this void. Hopefully this review is an incentive and encouragement for such a venture.

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Aquaculture Biosecurity Challenges in the Light of the Ballast Water Management Convention

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Abstract

Shipping plays a crucial role in supporting global trade, including the transport of products from the aquaculture industry. However, ships may also unintentionally transport invasive species and pathogens in their ballast water which pose biosecurity risks for aquaculture. The Ballast Water Management Convention was developed to manage the biosecurity risks posed by ballast water and has entered into force in September 2017. The management measures and technologies arising from the convention provide some solutions and opportunities for the aquaculture industry. Among these is the potential transfer of treatment technologies between shipping and aquaculture in order to deal with bio-invasion and biosecurity. However, there are residual weaknesses in the regulatory regimes for ballast water management which may reveal a continuous risk from shipping to the aquaculture industry. Gaps include knowledge and management of other aquatic bacteria or viruses that could cause outbreaks in the aquaculture industry and threaten food security and human health. Solutions include focused risk assessments for aquaculture and regional collaboration.

Keywords: compliance, invasive species, pathogens, regulation, shipping, water treatment

Introduction

Shipping is one of the key stakeholders of global trade and plays a crucial role in transporting more than 90 % of all international goods across the globe (Wan et al. 2016). Altogether, there are more than 50 000 merchant ships sailing the world's oceans, and this represents a global tonnage of 600 million tonnes (Globallast 2016).

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Naturally, this global trade also includes the trade of fish meals, animal feeds and aquaculture products. Nearly 40 % of fish output (wild caught and farmed) is traded internationally, making seafood one of the most extensively traded commodities in the world. It is considered that exports of fish products from developing countries represent a larger proportion of total exports compared to that of tropical beverages, nuts, spices, cotton and sugar combined (Asche and Khatun 2006). In this respect, the aquaculture industry is dependent on the capacity of shipping to transport these goods and products, and at reasonable prices. There is however, another linkage between these two industries, and that is the biosecurity risks posed by the global movement of ships which may unintentionally transport pathogens with the potential to affect aquaculture. There is a need to understand and evaluate how these two industries are inherently connected. In this paper, we offer an overview of the existing regulatory regimes developed by the member states participating in the global objectives of the International Maritime Organization (IMO) as well as the United States of America's Coast Guard to decrease the risks of transfer of pathogens. We focus on the management tools and frameworks dealing with the risks associated with the transport of ballast water and eventually reflect on the potential transfer of technologies between shipping and aquaculture in order to deal with bio-invasion and biosecurity.

History of Bio-Invasions Associated with Shipping

Invasive species are viewed as a major threat to aquatic ecosystems and have been reported to affect global economies and societies (Carlton 2002; Occhipinti-Ambrogi and Savini 2003). In the United States of America alone, the impact of aquatic invasive species is estimated to range between millions and billions of dollars annually (Lovell et al. 2006). The shipping industry has been identified as a major source of transport of exogenous species across ecosystems, with about a third of the introductions due to fouling on ships' hulls and another third due to ballast water exchanges (Gollasch 2006, 2007; Galil et al. 2014). Aquaculture as a whole represents the other major source of invasions, and approaches to diminish these risks have been proposed (Leung and Dudgeon 2008). The impacts of shipping on the occurrence of biological invasions was recognized over 100 years ago, when the first suggested introduction of a non-indigenous marine species, the diatom *Odontella sinensis*, known from the tropical and subtropical coasts of the Indo-Pacific, was reported in European waters where it produced dense plankton blooms in the North Sea and more recently in the Baltic Sea (Olenina et al. 2009). Unlikely to have been carried by ocean currents from such distant seas, Ostenfeld (1908) suggested that this species was introduced by shipping as part of the biofouling community on a vessel’s hull or discharged with the water or sediments contained in ships’ ballast tanks. Later, other phytoplankton species such as toxic dinoflagellates were also demonstrated to be transported via ballast water (Hallegraeff and Bolch 1992). This is also the case for species of zooplankton, for which *Acartia tonsa*, for example, was first described in Europe in 1927 and for which haplotypes were found in the Baltic Sea that were 100 % similar to specimens from Rhodes Island, United States of America (Rémy 1927; Drillet et al. 2008).

Fortunately, not all species can survive the transfer through ecosystems in tanks or on ship hulls; and those surviving the transfer may not become invasive. In order to be successful at invading a new area, an exogenous species must first be pumped into ballast water tanks or colonialize the hull of a ship; it must survive transportation to a new location where it would be either discharged or would release offspring; then it must be able to colonize the new ecosystem and establish itself to the point that it becomes considered invasive (Carlton 1985; Smith et al. 1999). There is a broad principle that estimates that only 10 % of all potential invasive species make it to the next step of this succession (Williamson et al. 1986; Williamson and Fitter 1996; Boudouresque and Verlaque 2002; Coutts et al. 2010).

However, aquaculture pathogens are particularly of concern because they usually remain unnoticed until disease outbreaks are recognized, by which point they have already affected a multibillion dollar industry (Minchin 2007; FAO 2016). Furthermore, there is clear evidence of the role of ships in spreading protozoans, bacteria and viruses to different world regions in ballast water and sediments, as well as in biofilms on ballast tank surfaces (Drake et al. 2007) and ship hulls (Sylvester et al. 2011). As most aquaculture activities are usually in the vicinity of ports and quite often create nutrient-rich sediments, there is a possibility of transfer of pathogens from ballast water to aquaculture facilities. For example, the human pathogen *Vibrio cholerae* was released by ships' ballast waters in Mobile Bay, United States of America in 1994 and led to the poisoning of oysters, which were subsequently taken off the market for a period of time, leading to significant economic losses (McCarthy and Khambaty 1994). Other famous cases include the spread of the parasite *Bonamia ostreae* or bonamiosis along the south coast of Britain, potentially via barges fouled with infected native oysters (*Ostrea edulis*) (Howard 1994). Vertical transmission of certain molluscan diseases such as *Perkinsis* spp. have also been observed as a result of the fouling of ships' hulls by contaminated commercial molluscs (Gollasch 2002). Contamination via biofouling communities has also been held responsible for the spread of amoebic gill disease (*Neoparamoeba pemaquidensis*) in cultured Atlantic salmon (Tan et al. 2002).

Ballast Water as a Vector of Concern

As previously mentioned, global trade is heavily dependent on the import or export of raw materials such as wood products, grains, coal, iron and other minerals. These commodities are transported across the oceans in specialized bulk carrier ships in one direction, taking on ballast water for their return voyages when not carrying goods. Although ship owners try to transfer goods during voyages from and to different ports to avoid travelling empty, there is often a need to balance cargoes in weight. Other ship types such as container vessels may adapt their ballasting regime to the amount and type of cargo in each port visited through a route across the globe. Ballast water is used in ships as a means to stabilize the ship when no or limited cargo is present (Fig. 1). It is an important aspect of the routine activities on-board and ensures that the ship and the crew are safe. Because of this vital role, ships will to continue to use ballast water for many more decades, if not forever.

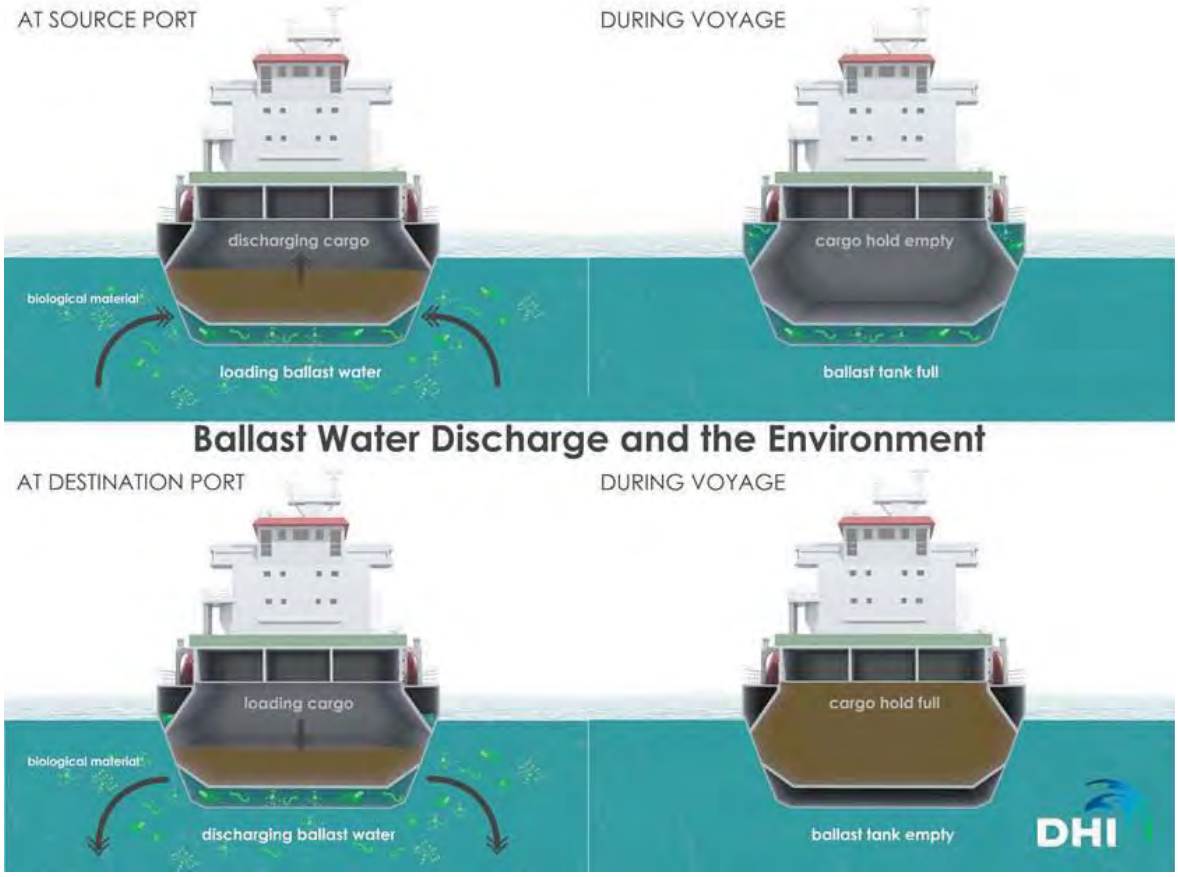

Fig. 1. Cross-section of ships showing ballast tanks and ballast water cycle. (Adapted from GloBallast 2016).

Because of the consequences of bio-invasions generated by the exchange of ballast water across ecosystems, this topic has received quite a bit of attention in the last decades, and much research on organisms transferred through ballast water or its sediments has been carried out (Carlton and Geller 1993; Eno et al. 1997; Ruiz et al. 1997; Briski et al. 2011; Seebens et al. 2013). Ballast water is estimated to be responsible for the transfer of between 7 000 and 10 000 different species of marine microbes, plants and animals globally, each day (Carlton 1999).

The annual amount of ballast water transported is large; estimated to be between 3.5 billion tonnes (Endresen et al. 2004) and 10 billion tonnes (Gollasch 1998). Large ships such as bulk carriers may pump 10 000 to 20 000 m^3 of water per hour (GloBallast 2016). Relating this to a traditional pond size in the Asian shrimp industry, this would equate to 1–3 shrimp ponds per hour. The International Maritime Organization (IMO), with its headquarters in London, has come up with a list of the ten most unwanted marine species carried by ballast water (Table 1).

Table 1. The International Maritime Organization's top-ten most unwanted species. Note that this list still omits viruses and bacteria, which may be more problematic for aquaculture.

Organism(s)	Species
Cholera	*Vibrio cholerae* (various strains)
Cladoceran water flea	*Cercopagis pengoi*
Mitten crab	*Eriocheir sinensis*
Toxic algae	Red/brown/green tides of various species
Round goby	*Neogobius melanostomus*
North American comb jelly	*Mnemiopsis leidyi*
North Pacific seastar	*Asterias amurensis*
Zebra mussel	*Dreissena polymorpha*
Asian kelp	*Undaria pinnatifida*
European green crab	*Carcinus maenas*

Ballast Water Management

To address the issue of biological invasions through shipping, the IMO has worked towards the development of a regulatory regime which provides measures to protect the environment from bio-invasions. This includes the *Guidelines for the control and management of ships' biofouling to minimize the transfer of invasive aquatic species* (Biofouling Guidelines, resolution MEPC.207 (62)), which are intended to provide a globally consistent approach to the management of biofouling.

In 1991, the Marine Environment Protection Committee (MEPC) of the IMO adopted the *International guidelines for preventing the introduction of unwanted aquatic organisms and pathogens from ships' ballast water and sediment discharges* through the resolution MEPC.50 (31). A few years later in 1997, the developments and discussion generated from these first guidelines supported the IMO-MEPC in adopting the *Guidelines for the control and management of ships' ballast water to minimize the transfer of harmful aquatic organisms and pathogen* through the resolution A 868(20). Eventually, the International Convention for the Control and Management of Ships' Ballast Water and Sediments was adopted in 2004 through the resolution MEPC.253 (67). This last resolution is also referred to as the Ballast Water Management Convention, or BWMC. As a convention and not a guideline, this last is legally binding. The convention was to enter into force exactly one year after at least 30 countries representing 35 % of the world merchant shipping tonnage have ratified it (Article 18). In order to prepare for the convention to enter into force, there has been a large amount of work carried out by IMO (Fig. 2). To support the preparation of stakeholders to the entry into force of the convention, the GEF-UNDP-IMO GloBallast Partnerships Programme was developed (Globallast 2016). This programme was initiated in late 2007 and was intended to be finished in 2012, but has been extended until the spring of 2017.

Following the ratification of the convention by Finland in September 2016, the BWMC has entered into force in September 2017. The world merchant fleet is now bonded to the convention. This entry into force will ensure that a good part of the bio-invasion risks associated with ballast water exchanges will be managed and reduced.

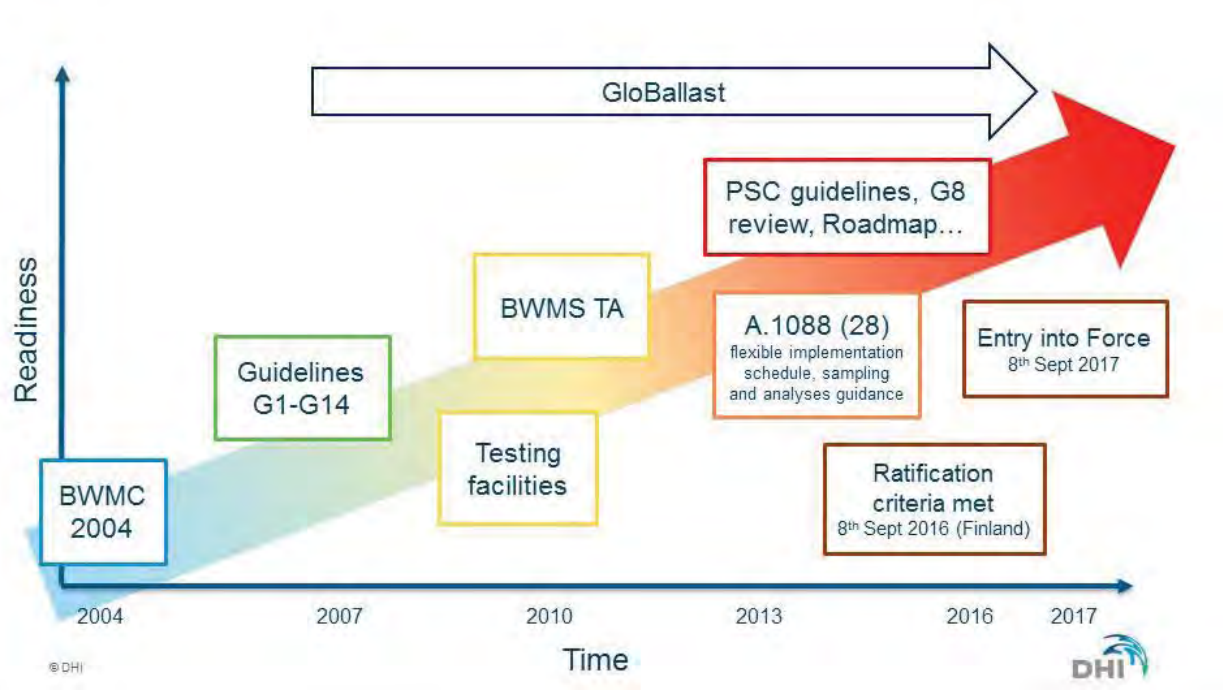

Fig. 2. Developments associated with the preparation of the entry into force of the Ballast Water Management Convention. (Adapted from Drillet (2016)). BWMC = Ballast Water Management Convention, BWMS TA = Ballast Water Management System Type Approval, PSC = Port State Control.

In short, from September 2017 to 2024, more and more ships will have to comply with the convention in that they will have to ensure that every single ship will have a ship-specific Ballast Water Management Plan (BWMP) describing how the ship is managing its ballast water and its sediments, an International Ballast Water Management Certificate (delivered by a flag state, eventually through a recognized organization), and a Ballast Water Record Book, where every single ballasting or de-ballasting event will have to be reported. The BWMP will in most cases include the treatment of ballast water using a Ballast Water Management System (BWMS) which must receive a Type Approval by an administration (see following paragraphs). However, ship(s) on short-sea voyage(s) between specified ports or locations across international borders may be granted an exemption from applying ballast water management systems under the convention (regulation A-4), if it is decided that the risk of transfer of invasive species is acceptable. A risk assessment should be carried out and Guideline G7 details the recommended process for this. The regulations allow an exemption to be granted for multiple ships and voyages between specified ports and locations, thereby supporting a regional approach to exemption through the identification of a "Same Risk Area" or SRA (Stuer-Lauridsen et al 2018).

Draft guidelines for the risk assessment of a SRA were proposed (Saunders et al. 2016 and references therein) and were accepted by the MEPC in 2017. Nevertheless, while short-sea shipping may have a possibility to be exempted of using BWMS, it is expected that most ships will have to fit or retro-fit a system type approved to treat water.

The Type Approval of Ballast Water Management Systems

In line with the convention, all equipment on-board a ship is type-approved and proven to work according to a strict set of specifications; BWMS therefore have also to be tested. There are to date 59 type-approved systems under the IMO umbrella, and the guidelines used for carrying out these evaluations are referred to as the G8 and G9 guidelines. Globally, there are at least 19 organizations involved in testing BWMS, and these are represented by the NGO Global TestNet (Global TestNet, 2018).

This network was initially supported through the work of The GEF-UNDP-IMO GloBallast Partnerships Programme and became independent from this support when signing the Busan Memorandum of Understanding (MoU) in 2013. The Global TestNet aims to increase levels of standardization, transparency and openness in testing BWMS. Typically, the BWMS are tested using volumes of 250 m^3 of water through the use of pumps; the water is stored in tanks before testing the capacity of the BWMS to ensure a valid discharge through a stringent biological evaluation by an independent laboratory (Fig. 3).

Fig. 3. Top left: the DHI ballast water technology and innovation centre in Singapore, bottom left: cultures of standard test organisms (*Tetraselmis* sp.); right: inside a 250 m^3 retention tank used for mimicking ballast water transported during a ship's voyage.

Similar to the BWMC of the IMO, the United States of America has implemented its own regulation to deal with the risk management of biological invasions through ballast water. This is commonly referred as the United States Coast Guard (USCG) regulations, and they became effective in June 2012 (U.S. Coast Guard. Standards for Living Organisms in Ships' Ballast Water Discharged in U.S. Waters. 33 CFR Part 151 and 46 CFR Part 162). Being a national regulation, this applies only to ships discharging ballast water in United States waters. The discharge standard of both the USCG and the IMO regulations is similar, and a ship may discharge water containing less than the following number of organisms:

a) 10 viable organisms per m^3 >50 μm;
b) 10 viable organisms per mL between 10 and 50 μm
c) one cfu of *Vibrio cholerae* per 100 mL or one cfu per 1 g (wet weight) zooplankton;
d) 250 cfu of *Escherichia coli* per 100 mL; and
e) 100 cfu of intestinal enterococci per 100 mL.

There are some differences between the two regulations, for example, in terms of the definition of "viability": the USCG regulation considers that an organism discharged in its territorial waters should be dead, whereas the IMO considers that non-viable organisms should not be taken into account in the discharge assessment, because of their incapacity to reproduce. Other differences between the IMO and USCG regulations exist in the guidelines and protocols describing the testing procedures for granting a type-approval to a BWMS. To date, the USCG has only approved six systems and more applications are being processed. Yet, this this is seen as a bottleneck by the shipping industry, who must fit systems onboard ships as soon as possible. The G8 guidelines which are used as a basis for the testing of BWMS under the IMO umbrella initially presented limitations because they were developed before any BWMS was ever tested. Some of these limitations have been raised to the IMO-MEPC, as well as in peer-reviewed papers (Miller et al. 2011; Drillet et al. 2013). In light of these issues, the MEPC has re-opened the G8 guidelines for review and a new version with a set of more stringent testing obligations was submitted to MEPC, and approved in October 2016. The revised testing Guidelines G8 are now mandatory (as a code) and this ensures that no new type-approval will be given to systems tested under the old G8 guidelines after 2018; and all systems installed on ships after 2020 will be required to be tested under the revised G8 guidelines (the Code). This revision by IMO ensures that the convention will become better at reaching its objectives of decreasing the rates of bio-invasions.

Remaining Weaknesses, Biosecurity Challenges and Gaps

Although the BWMC provides an important tool for managing the environmental risks from ships' ballast water, some stakeholders still consider that the 27 years taken to achieve this has been too long, that too few states representing the highest tonnage have signed up to the Convention (Wan et al. 2016) and that gaps remain in the protection measures (Drillet et al. 2016).

The BWMC is an international agreement and therefore only regulates ships exchanging ballast water across international borders, not wholly within domestic waters. The regulation applying to ships travelling solely in a single country's water are specific to that particular country. The convention therefore creates a scenario where exchange of ballast water between distant ports of a single country may be unregulated (if not regulated at the national level), while the discharge of ballast water between ports in neighbouring countries (for example across a strait) is subject to the regulations set out by the BWMC despite the expected higher ecosystem similarity at the local scale.

For example, in the Southeast Asian context, a ship ballasting on the west coast of Thailand in the Andaman Sea and travelling to and deballasting in a Thai port in the Gulf of Thailand (ca. 1 500 nautical miles away) would not require an application under the BWMC, while a ship sailing from Singapore to the island of Pulau Batam (Indonesia), less than 10 nautical miles away, would have to comply with the convention. This is both biologically and administratively unsound (Stuer-Lauridsen and Overgaard 2014; Saunders et al. 2016). Currently the only way to resolve such issues is voluntarily through regional sea approaches and working groups such as the Helsinki Commission (HELCOM) for the Baltic Sea, OSPAR Commission for the North-East Atlantic sea region and the Regional Marine Pollution Emergency Response Centre (REMPEC) for the Mediterranean Sea.

Furthermore, while the BWMC will reduce the risks of transfer of organisms larger than 10 µm and of bacteria that are harmful to humans, including *Vibrio cholerae*, *Escherichia coli* and *Enterococcus* spp., the convention does not mention any other aquatic bacteria or viruses that could cause epizootics in the US$ 160 billion aquaculture industry and threaten food security and human health (Drillet et al. 2016). Therefore, it has been proposed that risk assessments should be carried out and eventually flagged to United Nations (UN) interagencies such as UN-Oceans or GESAMP (Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection) to circumvent the potential residual risks which may have been omitted in the BWMC (Drillet et al. 2016).

Opportunities: Common Treatment Methodologies

While the gaps outlined above are being addressed, there is also an opportunity for the aquaculture industry, partly due to the efforts of the shipping industry, to advance the technologies available for water treatment. The BWMC has permitted the development of a range of technologies which have been tested in independent test facilities in a robust and controlled manner (see above-mentioned regulations and guidelines). Some of the technologies developed for the shipping industry are therefore applicable to aquaculture as well. For a ships' ballast water, the technologies can be either port-based or ship-based, with the latter being easier to implement because it is more flexible and ensures that systems are able to function during worldwide operations and capable of treating very dirty/murky waters (Tsolaki and Diamadopoulos 2010). Ballast water treatment methods can be categorized as physical separation, mechanical or chemical methods (Tsolaki and Diamadopoulos 2010).

Filtration, either by screen or hydro-cyclone filters, is effective against sediment particles and a wide range of organisms. Hydro-cyclones require less pump pressure than screen filters and allow separation of sediments and other suspended solids to approximately 20 μm. Particles or organisms smaller than this require additional treatment methods.

Thus, filtration or separation treatment is generally run in combination with additional treatment methodologies such as ultraviolet radiation, heat treatment, electromagnetic pulse applications, oxidizing and non-oxidizing biocides, and deoxygenation (see review by Tsolaki and Diamandopoulos 2010). These treatment approaches have also been tested and used in aquaculture (Otte and Rosenthal 1979; Summerfelt 2003). Therefore, lessons learned in the development of systems for the shipping industry could be readily transferred to the aquaculture sector in order to maintain the required high levels of biosecurity in farms (FAO 2010).

Conclusion

Ballast water is a vital aspect of maritime safety, as it ensures the stability and safety of the ship and therefore protects its crew. The USCG and IMO regulations stand as a cornerstone of the upcoming achievements from the shipping industry. These regulatory regimes, developed to support the management of the risks inherently associated with the use of ballast water, will help to reduce the rate and number of exogenous species transferred across ecosystems. Nevertheless, the understanding of the risks generated by the exchange of ballast water for the aquaculture industry is generally low. Limitations in the testing of BWMS have been identified and reveal potential impacts on human health risk management (Cohen and Dobbs 2015). This may also be true for understudied pathogens in ballast water tanks and their potential impacts on aquaculture, even after the installation of BWMS in all ships worldwide (see recent work by Ng et al. 2015; Kim et al. 2015; and Kim et al. 2016).

There is a gap in our knowledge and a residual weakness in the regulatory regimes for ballast water management which may reveal a continuous risk from shipping to the aquaculture industry (Drillet et al. 2016). Until this is evaluated, there are dispositions in the BWMC about the measures which port states are to take in the event of a bloom of potentially harmful organisms occurring in their waters and where a ship may ballast water from (regulation C-2). In such cases, port states are required to inform the ships and eventually propose measures to decrease the risk of ballasting water containing such organisms in ballast water tanks.

The aquaculture stakeholders may benefit from this disposition by ensuring that countries monitoring the water quality in their port test for pathogens potentially affecting aquaculture, such as those reported by the World Organisation for Animal Health (OIE, 2017). Impact assessments of the risk for aquaculture and marine spatial planning may be used as proper tools to ensure improved biosecurity (Drillet et al. 2014).

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New Paradigm for Controlling EMS/APHNS in Intensive *P. vannamei* Boone 1931 Culture Ponds

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Abstract

Since 2014, a gradual paradigm shift has been taking place in Thailand, where the farmers have changed the dynamics of their ponds to maximize diversity to control *Vibrio* and prevent APHND/EMS outbreaks. The objective is a rather simple one… to keep the pond bottoms clean of sediments and sludge. To achieve this, farm modules can use a combination of recirculation and flow-through water exchange to maintain a low-risk, sustainable culture system. There are four major components that the "new" farm design incorporates into the strategy to maintain clean pond bottoms: reduction of grow-out pond size, increased reservoir to grow-out pond ratio, increased aeration/energy capacity, and construction of a "shrimp toilet" at the center of the pond. The most significant shift in strategy is the reservoir to grow-out ratio. Traditional farms that were once 20 % reservoirs and 80 % production were changed to 60 % reservoir and 40 % grow-out capacity. Transitioning a traditional farm into an intensive, controlled and sustainable "shrimp toilet" culture system may be the best solution to overcome APHND, Vibriosis and viral diseases.

Keywords: EMS, AHPND, disease control, intensive pond culture, *Penaeus vannamei,* shrimp toilets

Introduction

The Chinese word for "crisis" is "Wei-Chi", translated as a combination of "danger" and "change", and would be an accurate description of the state affairs of the global shrimp industry today. Although the cause of these fungal, bacterial or *Vibrio*-based outbreaks has been blamed on climate change (e.g. drought, typhoons, El Niño, earthquakes) and incrementing coastal pollution, the real cause remains a mystery.

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The spread and permanence of new pathogens, (early mortality syndrome (EMS), acute hepatopancreatic necrosis disease (APHND) and *Enterocytozoon hepatopenaei* (EHP) specifically) worldwide together with unpredictable climate conditions presents a new challenge to the global shrimp industry.In most shrimp-farming countries, it is becoming more evident and alarming that traditional shrimp farming may actually never recover, given the nature of these bacterial and fungal pathogens.

Bacteria are in constant competition for pond nutrients, favoring the dominance of specific strains over others. Under suboptimal pond conditions of low biodiversity, which are characteristic of most shrimp-pond environments, *Vibrio* bacteria can double every 10–20 minutes, which could change the dynamics of the pond environment quickly. Given the presence of the APHND plasmid, this rapid proliferation of potentially pathogenic *Vibrio* could elevate quorum sensing to toxin-producing levels. Or as some researchers have postulated, *Vibrio* proliferation can serve as a stimulus to trigger viral diseases such as white-spot syndrome virus (WSSV).

Because of the evolving and ubiquitous nature of the *Vibrio* + plasmid combination throughout Southeast Asia, shrimp farming countries such as the People's Republic of China, Viet Nam, Thailand and Malaysia have yet to recover fully from the EMS/ APHND epizootic. In addition, overall shrimp production from Indonesia and India also seems to have peaked out in 2015 and is on the decline in 2016. Table 1 shows the tendencies of the major shrimp-farming countries worldwide (according to the authors' own observations for 2016). The only exception is in Thailand, where a slight improvement of 10 to 20 % over 2015 is expected.

Table 1. Trends in shrimp production for major shrimp-producing countries.

Country	Trending 2016	Target Market (majority)	Cause[1]
Mexico	↓	Domestic	EMS, WSSV
Brazil	↓	Domestic	WSSV
P.R.China	↓	Domestic	EMS, EHP
Indonesia	↓	Export	EHP
India	↓	Export	EHP
Thailand	↑	Export	System & genetics
Viet Nam	↓	Export	EMS, EHP
Ecuador	↓	Export	*Vibrio* in hatcheries

[1]EMS = early mortality syndrome, WSSV = white-spot syndrome virus, EHP = *Enterocytozoon hepatopenaei.*

Management Strategy: Control *VIBRIO* => Control AHPND => Control WSSV

Over the past two to three years, a gradual paradigm shift has been taking place in Thailand, where the farmers have changed the dynamics of their ponds to maximize diversity to control *Vibrio* and prevent APHND/EMS outbreaks. The objective is a rather simple one… to keep the pond bottoms clean of sediments and sludge. Farm modules with layouts such as that shown in Fig. 1 can use a combination of recirculation and flow-through water exchange to maintain a low-risk, sustainable culture system.

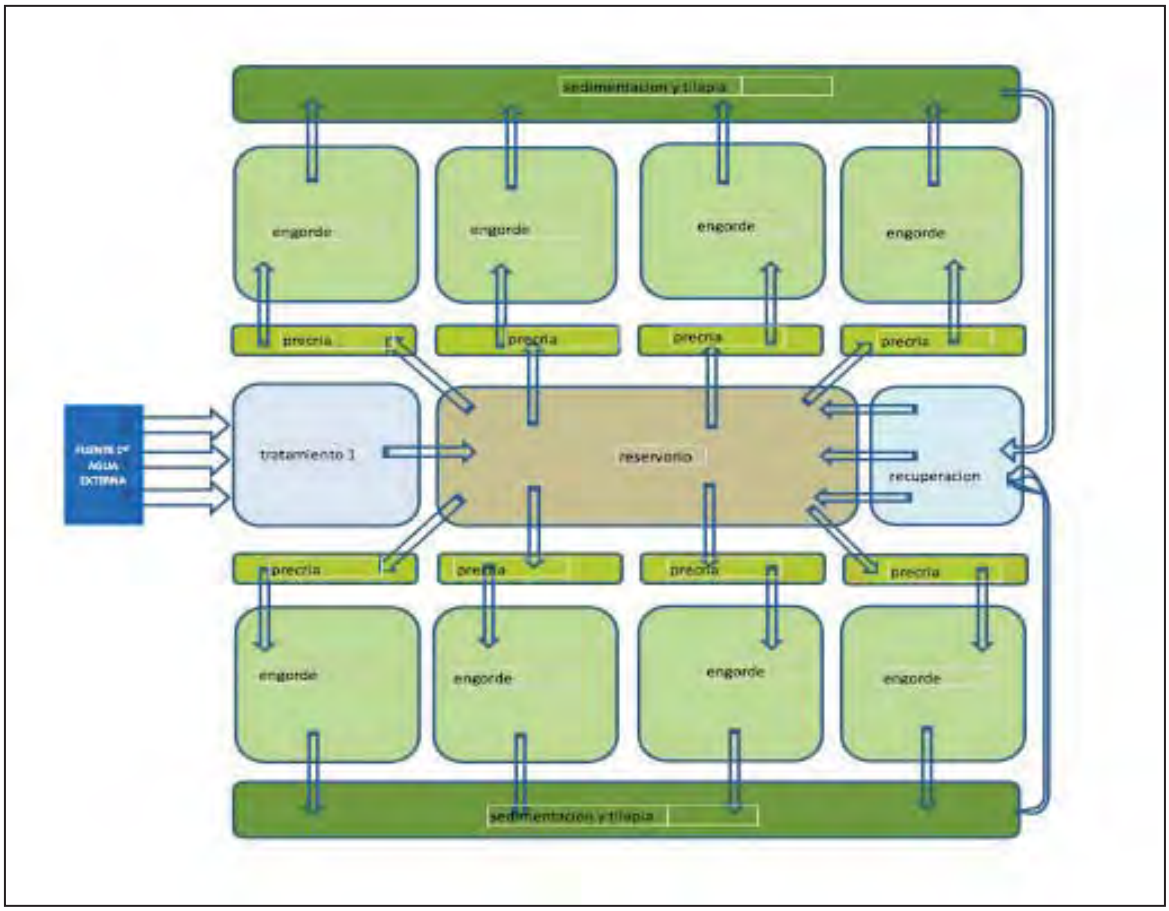

Fig. 1. Farm model for a low-risk, sustainable culture system.

However, this task is easier said than done, as the inputs that go into an intensive pond are very costly and are site dependent. Farms that have made the investment and renovation process have demonstrated that with good water quality and clean pond bottoms, very high harvest yields can be achieved. Albeit a very sizable investment, transitioning a traditional farm into a high-energy, high-exchange, and controlled culture system may be the best solution to overcome APHND, Vibrosis and viral diseases of shrimp. Table 2 summarizes the key differences between the traditional Thai shrimp ponds and the new pond design and inputs. The success of this new system is not due to any one or two criteria, but is due to a combination of many inputs.

Table 2. Key differences between traditional Thai shrimp ponds and the new pond design and inputs.

Pond Description	Traditional	New
Size (area)	1+ ha	1 000–4 000 m^2
Shape	Rectangular	Square
Depth	1.0–1.5 m	1.5–2.5 m
Bottom	Earthen	Lined (HDPE)[1]
Aeration	20–40 $hp.ha^{-1}$	55 to 75 $hp.ha^{-1}$
Discharge location	Side gate	Center drain
Water exchange	<50% over cycle	1 000%+ over cycle
Polyculture (reservoirs)	none	Tilapia
Feeding frequency	4–5 times, daytime	300+ times/12–24 hr
Feeding amount ($kg.m^{-2}crop^{-1}$)	1–2 $kg.m^{-2}$ (before EMS)	3–4 $kg.m^{-2}$

[1] HDPE = high-density polyethylene.

There are four major components that the "new" farm design incorporates into the strategy to maintain clean pond bottoms. These include:

- Reduction of grow-out pond size
- Increased reservoir to grow-out pond ratio
- Increased aeration/energy capacity
- Construction of a "shrimp toilet" at the center of the pond

The diagram given in Fig. 2 shows the difference in configuration of the ponds for a "traditional" farm design prior to APHNS/EMS and the "new" post-APHNS/EMS layout.

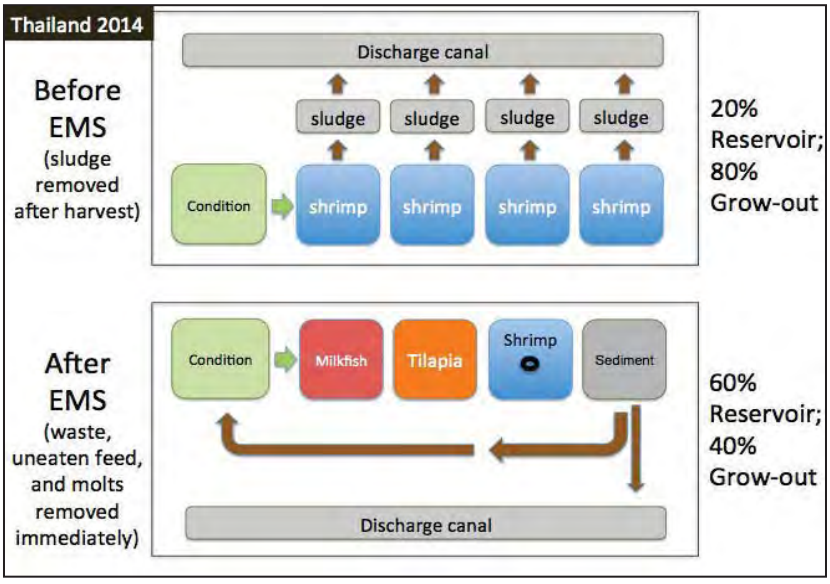

Fig. 2. Comparison of pond configuration between a "traditional" farm design prior to AHPNS/EMS and the new post-APHNS/EMS layout.

Grow-Out Pond Dimension, Reservoir Capacity, Aeration and Shrimp Toilet

There is a direct efficiency correlation between the pond size, area and depth of the "shrimp toilet"; energy/water movement and water flow volumes to remove the accumulated solids from the pond effectively.

Pond Dimension

The dimension of the lined grow-out pond must be as close to square (or round) as possible. Reducing pond size from an average of 0.8 ha (8 000 m^2) down to 0.2 to 0.3 ha improves oxygenation and more importantly, water movement efficiency to push settled organic matter towards the center of the pond. To compensate for the reduced surface area, water column depths of up to 3 m are developed to increase stocking densities to 300–500 animals.m^{-2}. In addition to reducing pond size, investment in upgrading the power grid and capacity was possibly the highest renovation expense in transforming a traditional farm to an intensive farm. Figure 3 shows a photograph of a post-APHNS/EMS shrimp pond in Thailand.

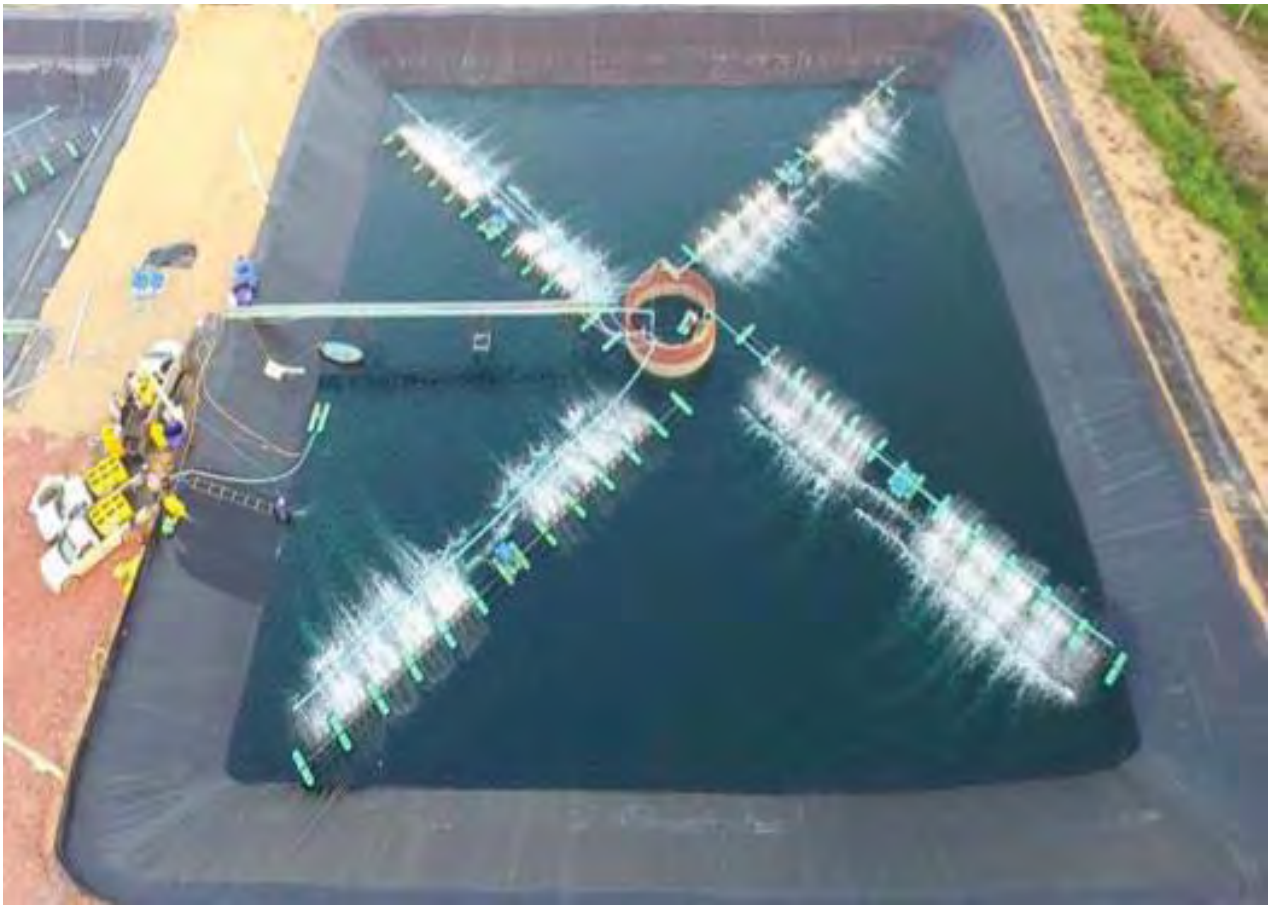

Fig. 3. Photograph of a 50 x 50 x 2.5 m deep post-AHPNS/EMS shrimp pond in Thailand.

Reservoir Capacity

The most significant shift in strategy is the reservoir to grow-out ratio. Traditional farms that were once 20 % reservoirs and 80 % production were changed to 60 % reservoirs and 40 % grow-out capacity. Psychologically, this change alone was probably the most difficult for the farmers to accept, given that less area would be dedicated to shrimp production. However, the loss in overall production from fewer production ponds has been more than compensated by higher and more reliable yields. Reservoir capacity is largely dependent on the availability of good quality water for the "new" farm. Coastal beachfront farm locations with unlimited ocean or well-point water could pump prefiltered water directly into the grow-out ponds, such as along the central coast of Viet Nam. However, farms located in estuaries where several farms share the same water source often sacrifice production ponds to become sedimentation and water storage ponds.

Daily water exchange during the course of the grow-out cycle begins at 2–5 % over the first two months and increases to up to 10–15 % over the last month of grow out. Total water exchange for a given pond could amount to over 1 000 % exchanged over the entire grow-out cycle. This water budget is 5 to 10 times higher than water exchange rates in traditional semi-biofloc ponds. Figure 4 illustrates a flow-through or recirculating aquaculture system (RAS) for enhanced biosecurity and water storage.

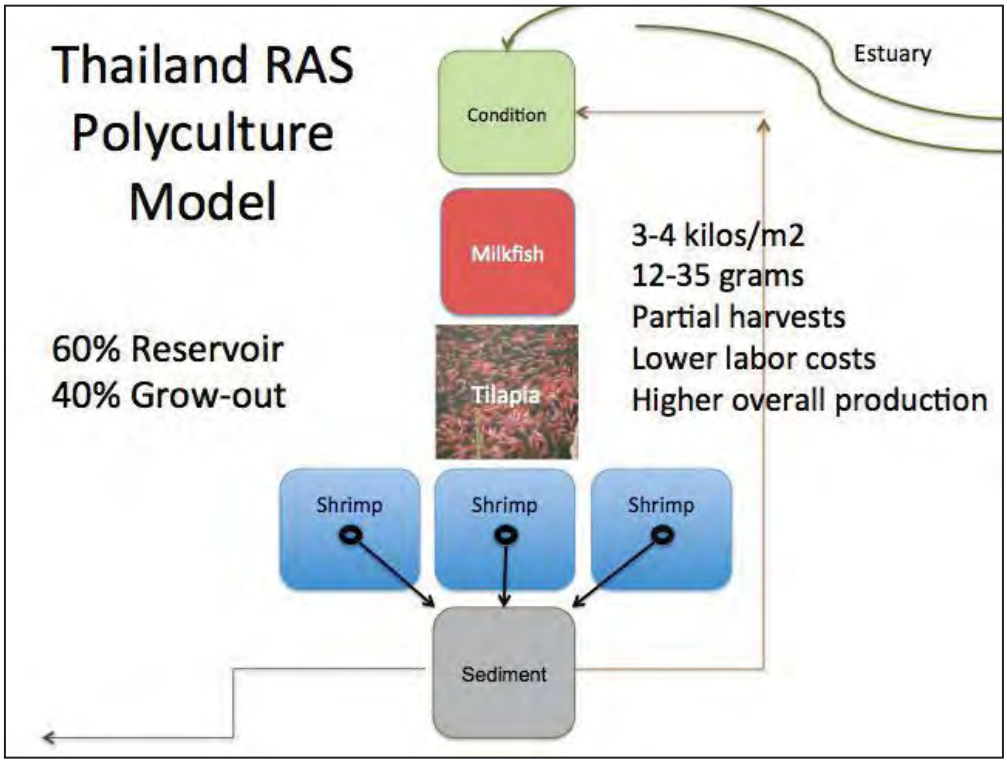

Fig. 4. Diagram illustrating a flow-through or recirculating aquaculture system (RAS) for enhanced biosecurity and water storage.

Farm modules such as in the layout shown in Fig. 1 can use a combination of recirculation and flow-through water exchange to maintain a low-risk, sustainable culture system.

Shrimp Toilet

Inclusion of the "shrimp toilet" has vastly improved the efficiency of concentrating and removing all sediments (faeces, uneaten feed, moults, algae and biofloc) from the pond bottom. Because the bottom of the cone or "shrimp toilet" is up to two meters below the bottom of the pond and discharge canal, submersible or floating pumps (2 hp) are used to pump out the sediments continuously. This relatively inexpensive excavation includes a center pit and a smooth lined cover using plastic sheets or HDPE. A photograph of a shrimp toilet is given in Fig. 5.

Fig. 5. Photograph of a "shrimp toilet".

The surface area of the "shrimp toilet" should be 5–7 % of the total area of the pond. Thus, a 4 000 m^2 pond would require a shrimp toilet measuring 16 m in diameter. The slope within the center depression should be 25–30 degrees to facilitate the solids to fall into the center pit. The "shrimp toilet" should be lined to create a smooth surface (Fig. 6).

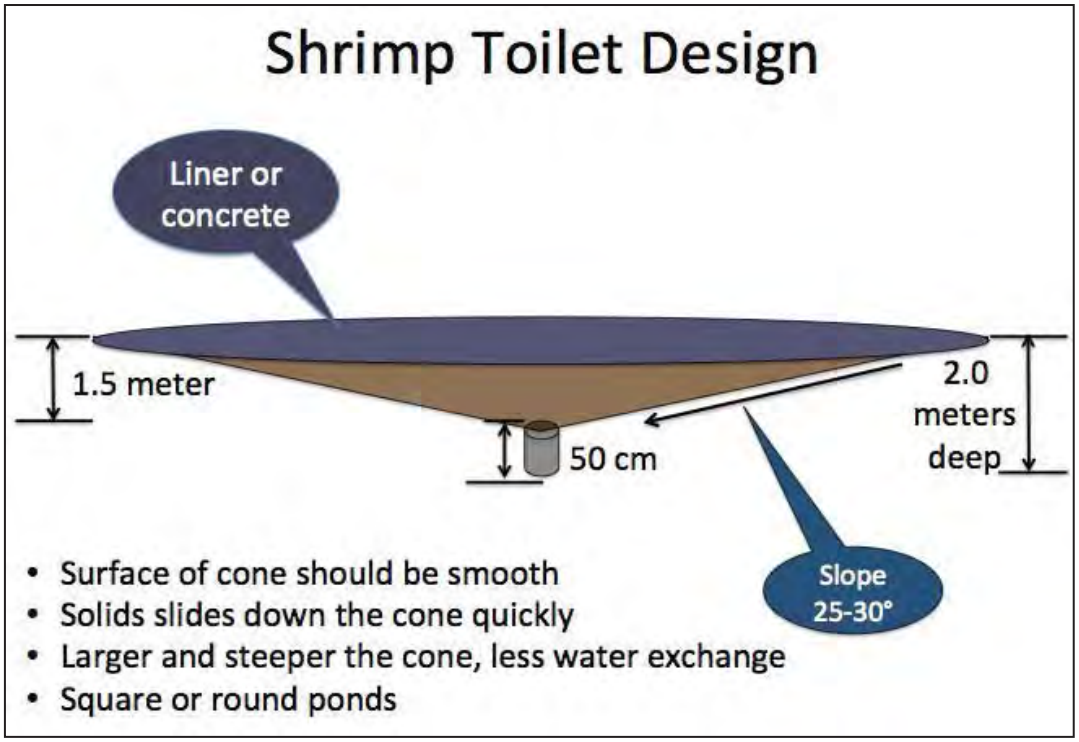

Fig. 6. Design of a "shrimp toilet".

Water Movement and Aeration

Creating a current strong enough to push settled organic matter to the “shrimp toilet” requires an energy budget of 70 to 100 hp of energy per ha, depending on the surface area and depth of the pond. Contrary to popular belief, the aerators or paddlewheels need to be operating day and night, regardless of dissolved oxygen levels. Continuous water exchange to remove accumulated sediments is a 24/7 operation. To ensure water quality, water going into the grow-out pond first goes through a series of sedimentation ponds, fish reservoirs, conditioning reservoirs and finally, a 200 μm filter (Fig. 7). This multistep water treatment process continues to evolve as some farms seed their conditioning reservoirs with macro-algae to further strip excess nutrients from the incoming water.

Fig. 7. Photograph of a shrimp pond in Thailand showing pond water exiting the "shrimp toilet" and incoming water passing through a 200 μm filter before returning to the same pond.

Polyculture

In areas of medium to low-salinity estuaries, many farmers stock their reservoir ponds with tilapia (*Oreochromis* sp.) and sometimes, milkfish *Chanos chanos* (Forsskål 1775), if available. The anti*Vibrio* "treatment" from the mucus membrane of the tilapia has been documented to lower the risk of a disease outbreak. A standing fish biomass density of around 1–2 $kg.m^{-2}$ of tilapia in the reservoir is recommended. The tilapia or milkfish are mostly underfed, as the fish graze on the excess biofloc and organic matter. Figure 8 shows the role of tilapia as a biomanipulator in shrimp ponds, while Table 3 presents the criteria for fish biomass in a water-conditioning reservoir.

Table 3. Criteria for fish biomass in a water-conditioning reservoir.

Criteria	Reservoir
Stocking size	50–70 g
Stocking density	10 $fish.m^{-2}$
Stocking biomass	500 $g.m^{-2}$
Harvest size	400–500 g per fish
Harvest biomass	5 $kg.m^{-2}$
Aeration	Yes; 2–3 per reservoir
Feeding	Yes

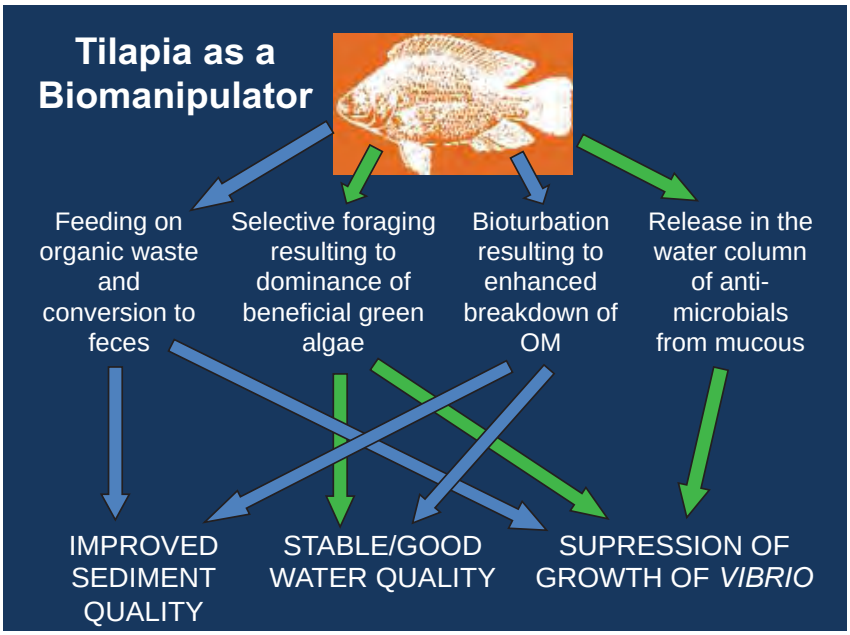

Fig. 8. The role of tilapia as a biomanipulator in shrimp ponds.

Shading the Pond

A technique that was made popular in Brazil and now adopted in other countries is to shade the nursery, grow-out ponds and reservoirs with black or green netting to reduce phytoplankton blooms and maintain slightly lower water temperatures. As high water temperature and blue-green (Cyanophytes) algal blooms have been associated with APHND, partial or complete blocking of direct sunlight helps to stabilize culture conditions, enabling beneficial bacteria to dominate the pond. A viable option to covering the ponds with shade cloth or orchid net is to apply commercial pond dye to darken and shade the pond water directly (see Fig. 9).

Fig. 9. Blue-green algal bloom, Shading ponds in Vietnam and Philippines, use of pond dyes to shade pond water.

Cost of Production

Table 4 shows the cost to produce one kg of 17–33 g shrimp in Thailand using the new "shrimp toilet" intensive culture method. Although the cost to produce 3–4 kg.m^{-2} of large shrimp may be high, as long as a profit margin of at least 30 % can be realized, the farmer will continue to farm. The most important benefit of this new "shrimp toilet" technique is that shrimp yields per crop are much more predictable and the risk of crop loss is much lower.

Table 4. Cost to produce one kilogram of 17–33 g shrimp in Thailand using the new "shrimp toilet" intensive culture method.

Cost Breakdown	Thai Baht (THB)	USD
Feed	65	$1.86
Electricity	40	$1.14
Probiotics, treatments	20	$0.57
Seedstock (juveniles)	15	$0.43
Misc.	5	$0.14
Total	145	$4.14

Harvest Size	Weight (g)	% of Harvest	THB	USD
60 kg^{-1}	17	25%	150	$4.29
40 kg^{-1}	25	25%	180	$5.14
30 kg^{-1}	33	50%	220	6.29
Average			192.5	$5.50

Production Cost per Kilogram	Farm Gate Value	Net Profit	% Profit
$4.14	$5.50	$1.36	33%

Production numbers using specific pathogen free (SPF) "fast-growing" stocks in Asia range between 3 and 8 kg.m^{-2}. Target production for a typical "shrimp toilet" farm is 3–4 kg.m^{-2} per crop, times three crops per year, or close to 100 tons per year.

Importance of the Best Genetics for the Culture System

Having the most ideal culture system that can effectively control pathogenic *Vibrio* and other diseases is only half the battle. The other 50 % is having the right genetics to optimize the culture system (or the culture system to optimize the genetics). Given that a tolerant but slow-growing specific pathogen resistant (SPR) strain of *Penaeus vannamei* Boone 1931 is no longer an essential requirement to produce shrimp successfully in an intensive "shrimp toilet" farm, it makes sense to stock fast-growing, SPF origin shrimp to maximize output. Table 5 ranks four different sources of breeding stocks according to four different performance traits at stocking densities above 100 animals per m^2. In the new "shrimp toilet" grow-out model, stocks of *P. vannamei* with the fastest growth rates would be the origin of choice.

Table 5. Ranking for four different sources of broodstock according to four different performance trials at stocking density above 100 animals.m^{-2}. Scores are 1, 2 or 3 stars, with 3 being the highest.

Trait	Ecuador (SPR)	Mexico (SPR)	Brazil (SPF)	Hawaii (SPF)
Survival	***	*	*	*
Growth/week	1.0 g	1.0 g	1.0	2.0 g
High density	*	*	*	***
Uniformity	*	*	*	***

Conclusion

As new shrimp diseases continue to spread to other shrimp-farming countries worldwide, farmers who adopt new technologies will thrive in these challenging times. Transitioning a traditional farm into an intensive, controlled, and sustainable “shrimp toilet” culture system may be the best solution to overcome APHND, Vibrosis and viral diseases.

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Surveillance and Animal Health Monitoring – Early Detection of Disease

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Abstract

Disease in aquaculture systems is the outcome of three major components, namely, the health of the animals being cultured, the condition of the culture environment and the presence of the pathogen. The early detection of signs of disease or poor health is crucial to taking measures to minimize the economic impact of disease. Routine animal health monitoring allows the generation of information necessary for immediate decision-taking. A well-established surveillance programme is key to achieving these results and it should focus on the primary enzootic pathogens in the key stages of production, as well as in the wild. It should also include exotic pathogens, as these could be introduced to the culture system through various means, including water currents, the importation of aquatic animals from infected countries or via ballast water. This paper describes the current surveillance programme for shrimp diseases being implemented in Kingdom of Saudi Arabia by the National Aquaculture Group (NAQUA), which has around 4 500 ha of culture surface. The criteria for identifying the morphological changes that indicate deviation from optimal health, its possible causes, and the mitigation measures are discussed. As the productivity of an aquaculture system is directly related to the health of the stocks, close monitoring and optimization of animal health is a key tool for profitable farming.

Keywords: aquatic animal health, disease surveillance, early disease detection, Kingdom of Saudi Arabia, shrimp diseases

Introduction

The rapid growth and development of the aquaculture business sector and international trade of aquatic animals and their products has increased the emergence of epizootics and the spread of new diseases.

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Diseases are an integral part of livestock production and are often an expression of the complex interactions between the host, the pathogen and the environment. The spread of diseases and the associated socio-economic concerns remain as one of the most relevant challenges to the industry. Prevention of diseases in aquaculture is required, as cures are hard to achieve and intervention after the onset of a disease is often difficult and costly. Well-established biosecurity practices that are strictly applied are fundamental to ensure minimal economic losses due to disease outbreaks and therefore the sustainability of the aquaculture industry. Although disease outbreaks cannot be completely averted even in the best managed ponds, early detection of disease is a key aspect of effective biosecurity.

Continuous surveillance and animal health monitoring are the major tools used to enhance biosecurity and mitigate the impact of diseases. This article describes the ongoing surveillance programme of the shrimp production at the National Aquaculture Group (NAQUA), Kingdom of Saudi Arabia, which uses approximately 4 500 ha of culture surface. This programme includes assessment criteria for morphological changes (both macroscopic and microscopic) that indicate deviation from optimal health, their possible causes, and mitigation measures applied to decrease their adverse effects and restore shrimp health. It is emphasized that productivity is always directly related to the health of the cultured animals and therefore, close monitoring and optimization of animal health are key for profitable farming.

The Surveillance Programme

The key objective of the surveillance programme is the early detection of primary enzootic pathogens, as well as exotic pathogens which are emerging or pose high risk. The health status of the shrimp population in the Kingdom of Saudi Arabia is exceptionally good. To date, the only viruses that have been detected are white-spot syndrome virus (WSSV), *Baculovirus penaei* (BP) and Taura syndrome virus (TSV). Of these, TSV was last detected in 2010, with infections limited to the southern part of the country. Other serious shrimp pathogens remain exotic to the Kingdom of Saudi Arabia (Table 1)**.**

It is important to take into account that surveillance programmes have to be designed based on the sanitary status of the zone, country or region. In addition to the commercial production, the NAQUA surveillance programme also covers the *Penaeus vannamei* Boone 1931 Specific Pathogen Free (SPF) Programme for all the pathogens of penaeid shrimp listed by the World Organisation for Animal Health (OIE) (i.e. WSSV, AHPND, IMNV, IHHNV, YHV, TSV and NHP) and other known important shrimp pathogens (EHP, CMNV, HPV, MBV and BP). As discussed below, the frequency of sampling is based on the degree of economic impact of the diseases, the type of culture system (indoor or outdoor) and the environmental conditions.

Table 1. Sanitary status of the Kingdom of Saudi Arabia with regard to major shrimp pathogens.

Enzootic pathogens	Exotic pathogens
White-spot syndrome virus (WSSV)	Infectious hypodermal and haematopoietic necrosis virus (IHHNV)
Taura syndrome virus (TSV)	Monodon baculovirus (MBV)
Baculovirus penaei (BP)	Necrotizing hepatopancreatitis (NHP)
	Hepatopancreatic parvolike virus (HPV)
	Acute hepatopancreatic necrosis disease (AHPND)
	Enterocytozoon hepatopenaei (EHP)
	Infectious myonecrosis virus (IMNV)
	Yellow head virus (YHV)
	Covert mortality nodavirus (CMNV)

Surveillance of Broodstock and Hatchery Operations

SPF monitoring programme

A major portion of the surveillance programme is focused on broodstock testing, in both the breeding and commercial broodstock development programme. To remove the IHHNV inserts from the stock, after each spawning, all brooders used for the SPF programme are tested individually for IHHNV by polymerase chain reaction (PCR) methods, followed by histopathology. The progenies from each set of brooders are reared separately to avoid mixing of populations. Later, a cold challenge at 20 °C for 48 hr will be given to the juveniles to rule out latent infection by certain pathogens, since pathogens like WSSV multiply faster and express into disease at low temperatures. All the major enzootic and exotic shrimp pathogens (see Table 1) also will be tested at 2 % prevalence by PCR or at 10 % prevalence by histology for all other serious pathogens (see Table 2) before stocking into nurseries as part of SPF status monitoring and validation.

Table 2. Specific pathogen free (SPF) monitoring programme.

Process	Target sample	Target pathogen	Percentage of stocks tested	Diagnostic method
Broodstock SPF programme	Broodstock after spawning	IHHNV	100%	PCR
		All	100%	Histology
	Juveniles (cold challenge)	WSSV, IHHNV, BP, NHP, MBV, HPV,AHPND, EHP, TSV, IMNV, YHV, CMNV	Once per batch (2% prevalence)	PCR
		All	Once per batch (10% prevalence)	Histology

Surveillance of commercial broodstock

Broodstock for commercial seed production are selected individually for quality before they are moved to maturation. Any mortality during maturation will be tested for WSSV by PCR and all animals with clinical signs will be analyzed by histology. One of the important sources of potential pathogen entry into the system is through live or fresh feeds. Hence *Artemia* biomass used as maturation feed is cooked to make sure pathogens are eradicated prior to feeding. All batches of commercial as well as breeding programme broodstock are checked fortnightly by animal health monitoring, examination of wet mounts and histology.

Table 3. Surveillance of broodstocks.

Process	Target sample	Target pathogen	Frequency	Diagnostic method
Broodstock production	Routine	All	Fortnightly	Animal health monitoring, wet mount, histology
	Clinical signs	All	N/A	Histology
Broodstock for maturation	100%	All	Individual selection	Animal health monitoring (morphological)
Broodstock at maturation	Standard mortalities	WSSV	100% mortalities	PCR
	Clinical signs	All	N/A	Histology

Surveillance of hatchery

Bacterial infections are one of the causes of mass mortalities at early stages. So *Artemia* and algae that are used to feed larvae must be free of green colonies on thiosulfate-citrate-bile salts-sucrose (TCBS) agar. PCR analysis for WSSV, IHHNV, AHPND and EHP is also done for *Artemia* because these pathogens can be introduced to the system by contaminated *Artemia*. All the larval tanks are checked on a daily basis by animal health monitoring and wet mount as shown in Table 4.

Table 4. Surveillance of hatchery.

Process	Target sample	Target pathogen	Frequency	Diagnostic method
Larval production	Routine	All	Daily	Animal health monitoring & wet mount
	Artemia	WSSV, IHHNV, AHPND, EHP	Every batch	PCR
	Algae and *Artemia*	Vibrio (green colonies – TCBS)	Weekly	Microbiology

Surveillance of nurseries and grow-out ponds

Considering that shrimp will lose their SPF status once they are exposed to the external environment of the nurseries, samples from each nursery are also cold challenged at 20 °C for 48 h prior PCR testing for WSSV and then transferred to grow-out ponds to make sure that they are free of WSSV. Samples from all nurseries and grow-out ponds also undergo animal health monitoring and wet-mount examination, as described in Table 5. During winter, due to the risk of WSSV, the nurseries and ponds are monitored fortnightly by animal health monitoring and examination of wet mounts. During the summer, the procedure is performed once a month.

Table 5. Surveillance of nursery and grow out.

Process	Culture period	Target sample	Target pathogen	Frequency	Diagnostic method
Nursery and grow-out ponds	Summer	Cold challenge (only nursery)	WSSV	Before transfer to grow out ponds	PCR
		Routine	All	Monthly	Animal health monitoring & wet mount
		Clinical signs	WSSV	N/A[1]	Rapid field test/PCR
			All	N/A	Animal health monitoring & wet mount
			AHPND	Based on hepatopancreas wet-mount results	PCR
			All	N/A	Histology
	Winter	Routine (only nursery)	All	Fortnightly	Animal health monitoring & wet mount
		Clinical signs	WSSV	N/A	Rapid field test/PCR
			All	N/A	Animal health monitoring & wet mount
			AHPND	Based on hepatopancreas wet-mount results	PCR
			All	N/A	Histology

[1]N/A = not applicable.

Surveillance of wild animals

Wild crustaceans (e.g. crabs, shrimp, zooplankton) can be carriers of shrimp pathogens. These are collected and tested for WSSV and in the case of wild shrimp, also for TSV. For this purpose, samples from various locations (including feeder canals) and from local fishermen who are engaged in fishing in the same area are collected. The information thus generated is useful in making decisions regarding stocking season, type of stocking, stocking density and even emergency harvest. The details of testing are given in Table 6.

Table 6. Surveillance of wild animals.

Location	Target sample	Target pathogen	Frequency	Diagnostic method
Main feeder canals, subfeeder canals & intakes	Crabs, shrimp & zooplankton	WSSV (crabs, shrimp & zooplankton); TSV (shrimp)	Monthly (summer) Fortnightly (winter)	PCR
Local fishing boats (wild catch)	Shrimp	WSSV, TSV, AHPND, EHP	Monthly	PCR
	Crabs	WSSV		
	Filter feeders	TSV, AHPND		

Surveillance of seafood markets

Because of the transboundary nature of many pathogens (many diseases are spread globally through the importation of infected animals), and the fact that the Kingdom of Saudi Arabia is importing seafood from other countries having much lower sanitary status, samples are collected from local fish markets and analyzed for both enzootic and exotic pathogens. The details of testing are given in Table 7.

Table 7. Surveillance of seafood markets.

Location	Target sample	Target pathogen	Frequency	Diagnostic method
Local fish markets	Shrimp from different countries	WSSV, TSV, IHHNV, NHP, AHPND, EHP, MBV, HPV	Monthly	PCR

Animal Health Monitoring Programme

The Animal Health Monitoring Programme helps to determine if there is any building up of problems that could eventually lead to a disease outbreak (whether it is caused by primary or secondary pathogens). A primary pathogen is an agent that can cause disease by itself (such as viruses), while a secondary or opportunistic pathogen requires a primary cause to initiate the onset of disease (e.g. a primary pathogen or a change in culture conditions). An effective animal health monitoring programme takes into account first-level diagnosis, real-time physicochemical parameters of water and soil, and animal performance data obtained from the field. The complete information will help in obtaining a proper diagnosis. In order not to lose the opportunity for early detection of disease, it is important to prioritize tanks, nurseries or ponds with abnormalities or poor performance.

Importance of Correct Sampling

The observation of animals is time consuming. It should be taken into account that the number of animals that will be sampled is very small when compared to the population size; and that the information generated from these few animals will be used to determine the health status of the whole population. Hence, it is crucial to conduct targeted sampling.

Weak animals can be obtained from the pond sides (especially during an outbreak of white-spot disease (WSD), see Fig. 1b), from check trays (Fig. 1a) or from outlet screens after a thorough flushing (Fig. 1c, d). Assumed prevalences of 2 % and 5 % are most commonly used for surveillance of presumed exotic pathogens with a 95 % confidence limit. More details of prevalence calculation are given in the Table 8.

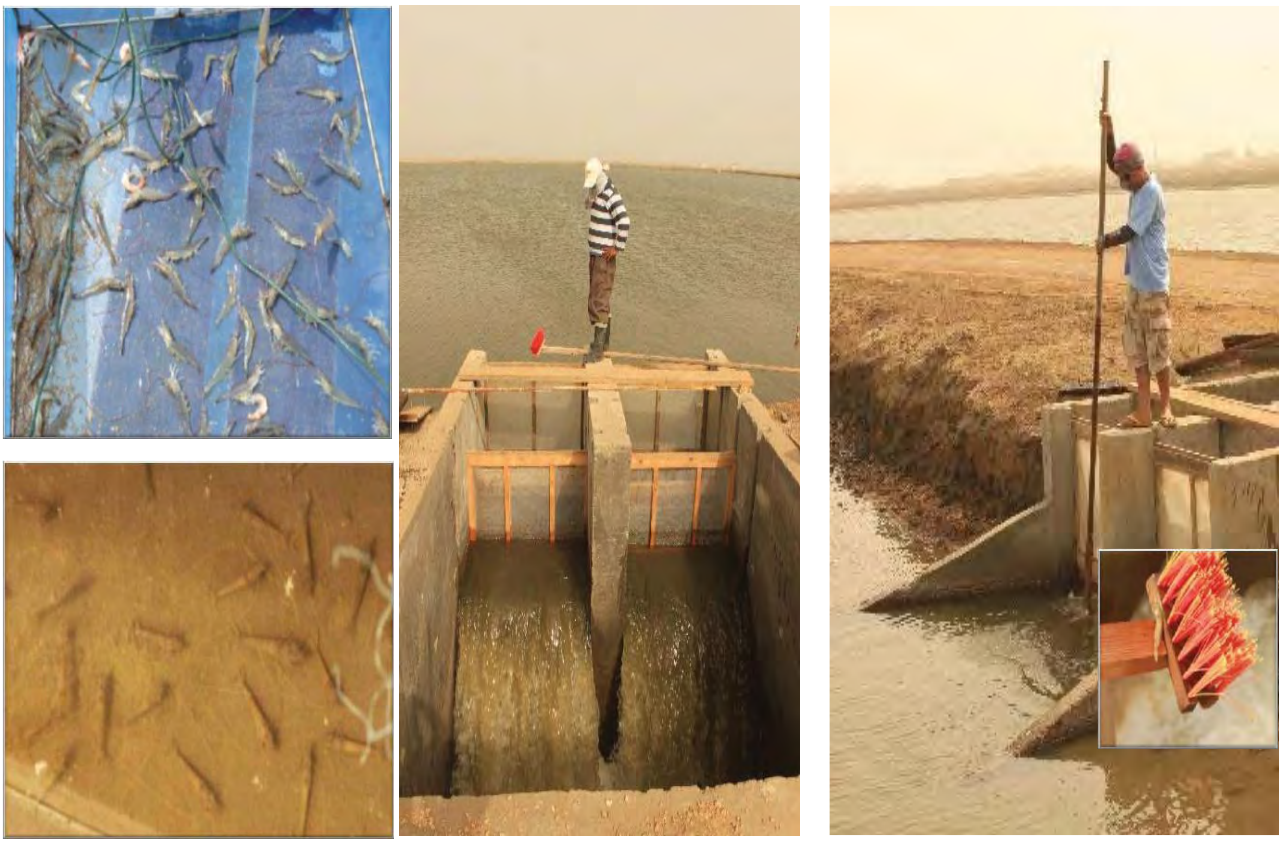

Fig. 1. (a) Weak animals in check tray; (b) weak animals near pond side; (c, d) collection of weak animals from outlet screen after thorough flushing.

Observation of Culture Conditions

Some observations of culture conditions that could indicate the health status of the pond population are given below.

Table 8. Estimation of sample size needed to detect different levels of prevalence in a population (modified from Amos 1985).

Population size	Prevalence (%)						
	0.5%	1%	2%	3%	4%	5%	10%
50	46	46	46	37	37	29	20
100	93	93	76	61	50	43	23
250	192	156	110	75	62	49	25
500	314	223	127	88	67	54	26
1 000	448	256	136	92	69	55	27
2 500	512	279	142	95	71	56	27
5 000	562	288	145	96	71	57	27
10 000	579	292	146	96	72	29	27
100 000	594	296	147	97	72	57	27
1000 000	596	297	147	97	72	57	27
>1 000 000	600	300	150	100	75	60	30

Bird activity

Birds are the best indicators of abnormalities in a pond, and will be the first sign a farmer can notice when entering a farm. Birds are attracted to the animals that move to the pond surface, either due to a disease outbreak, mortalities or even dissolved oxygen depletion. Weak animals gathered at the pond peripheries also attract birds. Figure 2a-c shows bird activity in culture ponds.

Fig. 2. (a-c): Bird activity in culture ponds due to oxygen depletion.

Water discolouration

One of the basic and important aspects of successful shrimp farming is managing water transparency and productivity in a stable manner. A sudden increase or decrease in algal or microbial population can result in drastic changes in water quality. Excess feed will provide nutrients for algal and microbial communities to develop exponentially. This is particularly common in the second phase of production. If the phytoplankton population is not managed properly, it could cause "die-off" and eventually lead to high transparency and an anaerobic pond bottom. This dead and decaying organic matter could provide nutrients for pathogenic bacteria. The acceptable range of transparency for semi-intensive ponds is 45–60 cm on Secchi disk. Figure 3a-d shows water transparency and discolouration in shrimp ponds.

Fig. 3. (a) Normal pond water; (b) *Dunaliella salina* bloom; (c) filamentous algae; (d) dinoflagellate bloom.

Presence of extraneous populations

Crabs and fish must be excluded from shrimp culture ponds, as they can introduce primary pathogens like WSSV, increase the chance of spread of muscle microsporidians, increase food conversion ratio (FCR) and even prey on postlarvae (PL). Carnivorous fish (Fig. 4a,b) play a significant role in reducing the survival of PL when they are stocked into a pond. The results of a small trial that was conducted to assess the impact of fish in the ponds at the time of stocking is given in Table 9. A similar trial was conducted with juveniles; however, no significant difference in survival was observed.

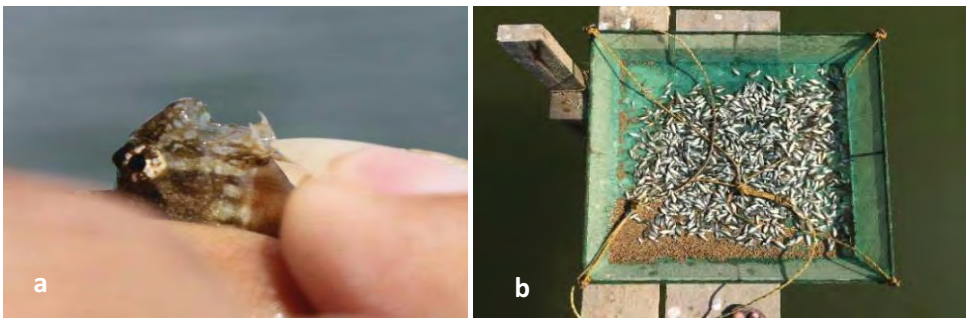

Fig. 4. (a) Fish used for the trial; (b) fish found in check tray.

Table 9. Results of a trial conducted to determine the effect of carnivorous fish on the survival of *Penaeus vannamei* during the early stages (trial duration – 24 hr).

	Stocking	Survival
Happa 1	200 postlarvae + 5 fish with feeding	24%
Happa 2	200 postlarvae + 5 fish without feeding	10%
Happa 3	200 postlarvae + no fish with feeding	93%

Morphological and physiological observations

Behaviour

Behaviour of the animals is the first observation you are should make when conducting shrimp health monitoring. Animals with clinical signs of disease and weak animals at the shore are a clear indication of abnormal behaviour.

Body colouration

Discolouration of the carapace and pleopods also often indicates the occurrence of disease or stress. Reddish discolouration can be a sign of WSD. Discolouration of the shrimp body is shown in Fig. 5a-c.

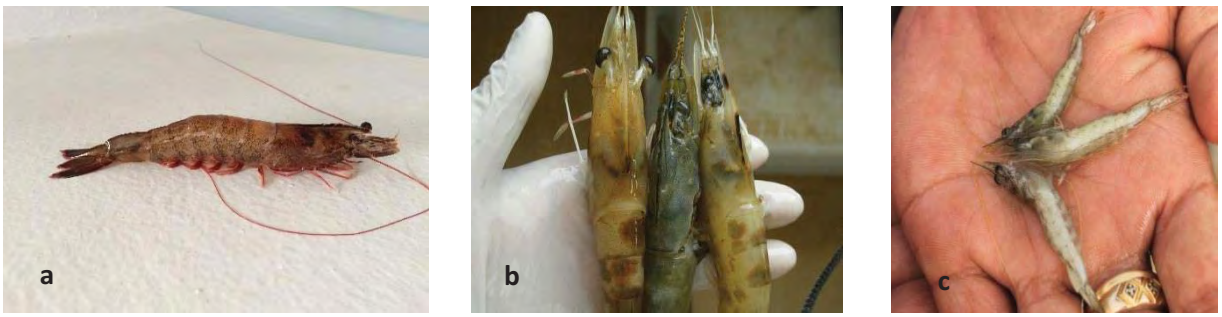

Fig. 5. Discolouration of the shrimp body. (a) reddish discolouration; (b) whitish discolouration; (c) yellowish discoloration.

Presence of loose shell

Loose shell is another crucial indication of abnormality. It may be a clinical sign of enteric bacterial infection. Inflammation of the hepatopancreatic tubules results in malfunctioning and eventually leads to impairment of nutrient absorption and lipid storage. This will result in weight loss and subsequently gap formation between the shell and muscle. It is important to remember that loose shell can also be due to malnourishment. Loose shell caused by enteric bacterial infections is shown in 6a,b.

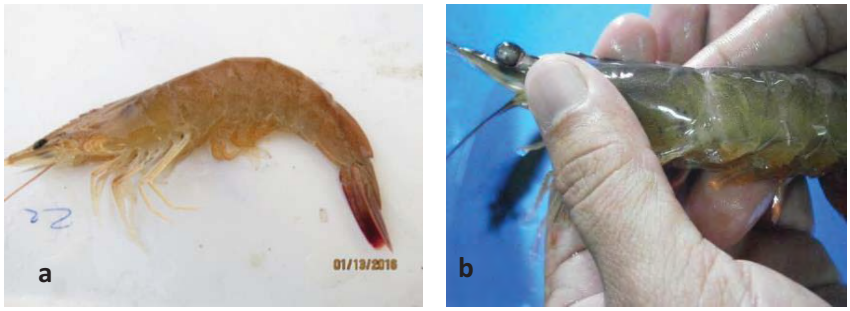

Fig. 6. (a, b) Loose shell due to enteric bacterial infection.

White spots on carapace

Even though the presence of white spots on the shell has no diagnostic value, as they are not pathognomonic, it can raise an alarm to increase vigilance for WSSV in the pond (Fig. 7a,b). In the Kingdom of Saudi Arabia, WSSV is the major enzootic disease affecting the shrimp industry. However, the presence of white spots on the shell could be non-specific.

Fig. 7. (a,b) White spots on the carapace of shrimp.

Gill colour

It is important to check gill colour frequently; if they are becoming brown (Fig. 8a,b) or black (Fig. 8c) it will be either a sign of poor bottom condition, algal bloom or even bacterial infection. These can be easily distinguished by placing the affected animals in an aquarium or bucket with clear water with sufficient oxygen.

If the animals are able to clear the discolouration by themselves, it can be concluded that pond bottom deterioration, heavy algal bloom or algal die off are possible causes. However, if the animals do not clear the colour, it means that the dark gill colouration is caused by melanization and thus could be related to bacterial infection. In both cases, a thorough water exchange can help to restore the normal condition of the shrimp.

Fig. 8. (a,b): Brown gills; (c): black gills

Tail muscle colouration

Tail discolouration can occur for various reasons; for example, when the animals are infected with muscle Microsporidia such as *Ameson* sp. the tail becomes whitish and opaque (Fig. 9a); this disease is known as cotton shrimp disease. In some cases, there will be reddish discolouration of the abdominal segments (Fig. 9b,c). This could be due to IMNV or to a systemic bacterial infection. Whitish muscle can also be caused by muscle cramp.

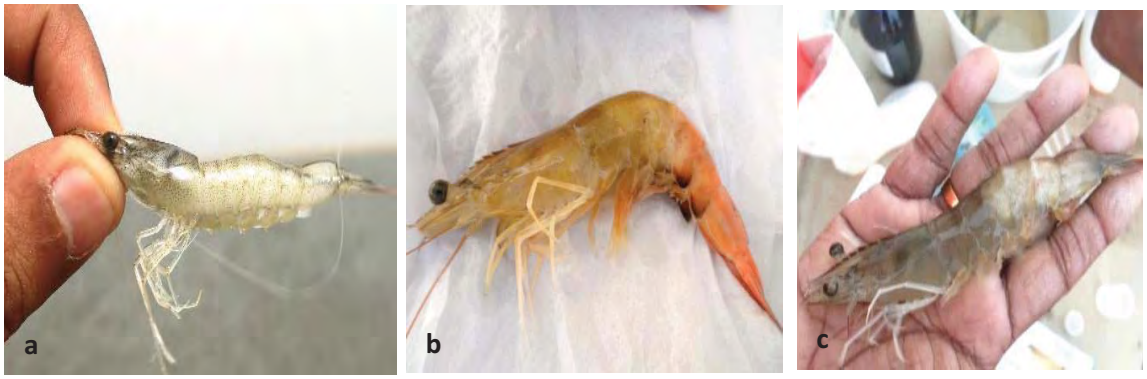

Fig. 9. (a): Muscle microsporidian infection; (b,c): systemic bacterial infection.

Cuticular melanization

Cuticular melanization is another striking character occurring mostly due to bacterial infection if the bacterial flora of the culture water is dominated by pathogenic bacteria (e.g. *Vibrio parahaemolyticus*). A thorough water exchange will help to replace the bacterial flora and remove moults, as the chitin component of shells acts as a substrate for *V. parahaemolyticus.* Melanization caused by infection by *V. parahaemolyticus* in the cuticle of *P. vannamei* can be seen in Fig. 10a-c.

Fig. 10. (a-c): Cuticular melanization/erosion caused by *Vibrio parahaemolyticus*.

Uropod reddishness/tail rot

Reddish and melanized uropods (Fig. 11a,b) are often the result of poor pond bottom quality and bacterial infections. This condition is common in poorly managed broodstock ponds and also in recirculated aquaculture systems (RAS).

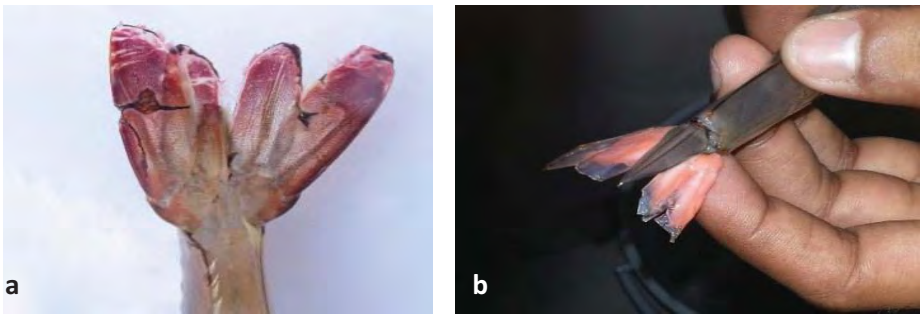

Fig. 11. (a) Uropod reddishness; (b) necrosis (tail rot).

Haemolymph clotting test

In shrimp, haemolymph will normally clot within 1–1.5 min after extraction. However, if the animals have viral infections (like WSSV) or bacterial infections (like vibriosis), the clotting time will be extended. Care should be taken to conduct this analysis immediately after sampling, because stress can also cause extended clotting time. Extraction of haemolymph and the haemolymph clotting test are shown in Fig. 12a,b.

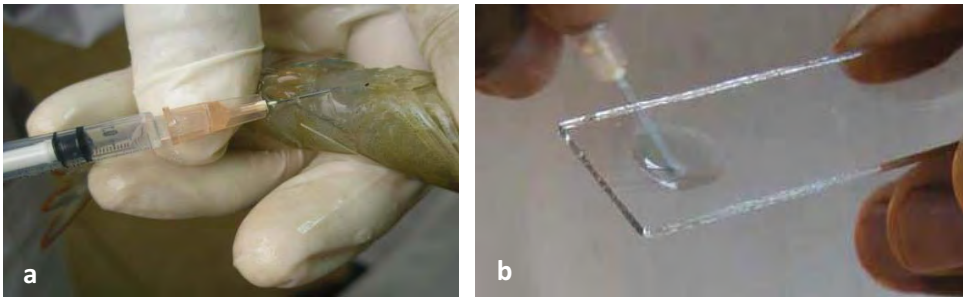

Fig. 12. (a): Extraction of haemolymph; (b): performing haemolymph clotting test.

Microscopic Observations or Wet Mount

Gill wet mount

Gill wet mount is done to determine the presence of parasites, debris or melanization in the gills. These results will give an idea about the pond conditions and also the health status of the animals. Gill melanization is one of the clinical signs of systemic vibriosis; in some cases it can also be caused by toxicity. Photomicrographs of gill wet mount showing gill melanization (Fig. 13a) and various infections (Fig. 13b,c) are given below.

Fig. 13. (a) Gill melanization, probably due to bacterial infection; (b) filamentous bacteria (*Leucothrix mucor*) infection in gills; (c) *Zoothamnium* sp. infection in gills.

Hepatopancreatic tubular constrictions

It is always important to observe changes in the tubules of the hepatopancreas, as this organ is exposed to water and feed quality. Hepatopancreatic tubular constrictions are the first sign of toxic effects due to acute hepatopancreatic necrosis disease (AHPND), vibrios, blue-green algal toxins, etc. Usually, if the percentage of hepatopancreatic tubular constrictions is high, it is not advisable to stock the larvae, as this can have a significant impact on production and also on health. Fig. 14a,b shows constrictions in the hepatopancreatic tubules of *P. vannamei* collected from grow-out ponds.

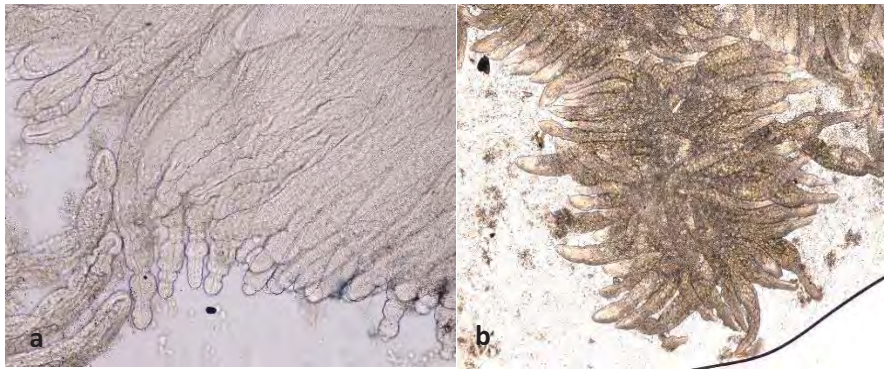

Fig. 14. (a,b) Hepatopancreatic tubular constrictions.

Hepatopancreatic tubular melanization

Tubular melanization of the hepatopancreas could be due to bacterial infection or the result of chronic toxicity. In some cases, if hepatopancreatic tubular constriction is not managed properly, melanization can occur, resulting in the malfunctioning of the hepatopancreatic tubules and therefore, poor absorption. Animals with more hepatopancreatic tubular melanization will also have loose shells. Figure 15a-c presents photomicrographs of melanized hepatopancreatic tubules of animals with enteric bacterial infection.

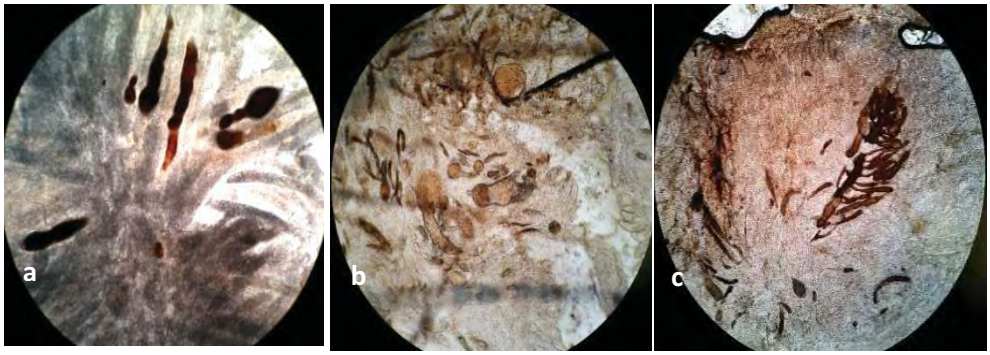

Fig. 15. (a,b,c) Hepatopancreatic tubular melanization in *Penaeus vannamei*.

Lipid vacuolization in the hepatopancreas

Observations of lipid storage levels in the hepatopancreas may help in optimization of feeding. It is determined by checking for the presence of lipid vacuoles in the hepatopancreas (Fig. 16a-c).

- Correct feeding: more 80 % of the animals with medium to low lipids 1 h before and more than 80 % with high lipids after 1 h of feeding
- Under-feeding: less than 80 % of animals with high lipids after 1 h of feeding
- Over-feeding : more than 60 % of animals with high lipids before 1 h of feeding

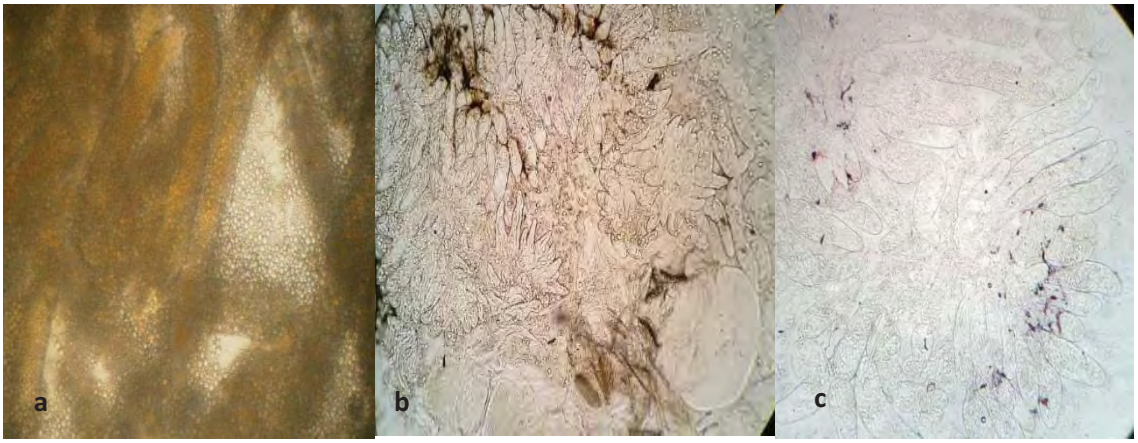

Fig. 16. (a): High level of lipids storage in hepatopancreas; (b, c) low level of lipids.

Gut content analysis

Wet-mount examination of gut contents is done to detect the presence of diseases like *Baculovirus penaei* (BP) (Fig. 17a), monodon baculovirus (MBV) (Fig. 17b) and intestinal gregarines (Fig. 17c). All these pathogens can affect the growth of cultured shrimp.

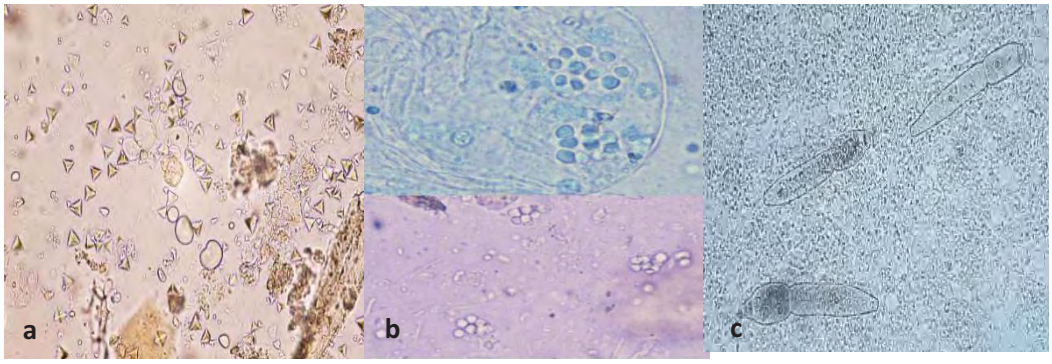

Fig. 17. (a) *Baculovirus penaei* (BP); (b): Monodon baculovirus (MBV); (c) gregarine in midgut (courtesy Dr D.V. Lightner).

Factors Influencing Frequency of Monitoring

Health monitoring frequency should be flexible to adapt to the season and sanitary status of the biosecurity zone. It has to be increased when there is a disease detection or outbreak at the farm, at neighbouring farms or any other farm within the same biosecurity zone. During winter and after a heavy rain, the farms and nurseries need to be monitored more critically, as low temperature can trigger diseases like WSSV. The objective of performing an animal health monitoring and surveillance programme is to achieve early detection of disease, thus minimizing the economic losses. If the clinical signs or field observations are ignored, that can cause failure and will be very expensive.

Reference

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Effect of Biofloc on the Survival of Whiteleg Shrimp, *Penaeus vannamei* Boone 1931, When Challenged with a Pathogenic Strain of *Vibrio parahaemolyticus*, the Causative Agent of Acute Hepatopancreatic Necrosis Disease (AHPND)

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Abstract

To understand better the effect of different water conditions on the survival of whiteleg shrimp, *Penaeus vannamei*, when challenged with a pathogenic strain of *Vibrio parahaemolyticus* (VP_{AHPND}), a series of challenge trials with biofloc were conducted. Challenges investigated the effect of holding individual shrimp for short periods (5–10 days) in either biofloc or in clear water prior to exposure to VP_{AHPND} in either biofloc or in filtered biofloc. Shrimp reared and challenged in unfiltered biofloc had the lowest mortality rates (0 and 6.7 %; $P < 0.05$), followed by those held in clear water for 10 days and challenged in clear water (33.3 and 20 %; $P < 0.05$). Shrimp reared in unfiltered biofloc and but challenged in clear water had the highest rates of mortality (80 and 60 %). A second validation trial, included the use of filtered biofloc. Shrimp reared and challenged in unfiltered biofloc had the lowest rate of mortality (13.3 %), followed by those reared in biofloc but challenged in 2 μm-filtered biofloc (20 %). The highest mortality was in shrimp reared in biofloc but challenged in clear water (73.3 %; $P < 0.01$). The results demonstrate that biofloc can protect whiteleg shrimp from VP_{AHPND} and that the management of biofloc in aquaculture ponds can assist in controlling bacterial infections.

Keywords: acute hepatopancreatic necrosis disease**,** bacterial community, biofloc, quorum quenching, shrimp disease, *Penaeus vannamei, Vibrio parahaemolyticus*

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Introduction

Acute hepatopancreatic necrosis disease (AHPND) of whiteleg shrimp (*Penaeus vannamei* Boone 1931) and giant tiger prawn (*P. monodon* Fabricius 1798) has had devastating impacts on shrimp production in the People's Republic of China (NACA-FAO 2011; Panakorn 2012), Malaysia (Lightner et al. 2012; NACA, 2012; Kua et al. 2016), Mexico (Nunan et al. 2014; Soto-Rodríguez et al. 2015), Thailand (Flegel 2012; Leaño and Mohan 2012; Joshi et al. 2014; Chonsin et al. 2016; Songsangjinda and Polchana 2016), Viet Nam (Lightner et al. 2012; Tran et al. 2013; Hien et al. 2016), and most recently from the Philippines (Dabu et al. 2015; dela Peña et al. 2015) and India (Ananda Raja et al. 2017), where it has been a major cause of economic loss (Shinn et al. 2018). Elsewhere, AHPND infections are reported from Costa Rica and Honduras (see Jun et al. 2015), while mortalities of *P. monodon* with manifestations of an AHPND-like condition in Cambodian ponds that were reported throughout 2011–2013 were not verified by diagnostic testing (Lang and Sothea 2016). Infections of *Vibrio parahaemolyticus* associated with the mass mortality of Chinese white shrimp, *Fenneropenaeus chinensis* (Osbeck 1765) have also been reported (Zhang et al. 2014).

Since its advent, there has been a concerted research effort to better understand the causative agent of AHPND – the bacterium *Vibrio parahaemolyticus* with a toxin gene-bearing plasmid (Han et al. 2015a; Lee et al. 2015; Hirono et al. 2016), its mode of action and host pathology (Lightner et al. 2012; Flegel 2012; Tran et al. 2013; Lai et al. 2015; Soonthornchai et al. 2016; Tinwongger et al. 2016), field surveys and conditions under which mortality has occurred (Sriurairatana et al. 2014; Soto-Rodríguez et al. 2015; Chonsin et al. 2016; Hastuti and Haryadi 2016), and investigations into practices that either mitigate against infection, curb its spread or affect its treatment (Panakorn 2012; Han et al. 2015b; Bondad-Reantaso 2016; Jun et al. 2016; Zheng et al. 2016).

With respect to the latter, biosecurity, management of the culture environment, husbandry practices and the application of appropriate probiotics have been central issues (De Schryver et al. 2014). The beneficial effects of biofloc culture (i.e. floculated aggregations of protein-rich organic material consisting of bacterial biomass, microalgae, faecal material, protozoa etc.) on the growth rate, robustness and immune parameters of shrimp are well documented (Wasielesky et al. 2006; Crab et al. 2012; Xu et al. 2013; Ekasari et al. 2014; Suita et al. 2015, 2016a, b). The use of biofloc in intensive closed culture systems is favoured, as organic material can be recycled by microorganisms into microalgae and bacterial biomass which consequentially aggregates to form flocculated material. This biofloc stimulates the growth of heterotrophic and autotrophic microorganisms to process organic residues, converting carbon and nitrogenous waste (e.g. ammonia) into new bacterial biomass (Avnimelech 1999; Luis-Villaseñor et al. 2015). The need for water exchange in the grow-out phase of commercial marine shrimp production, therefore, is reduced (Suita et al. 2016a).

A reduction in the abundance of *Vibrio* has been reported in biofloc systems (De Souza et al., 2012), while the application of selected probiotics on the nutritional profile of biofloc in the culture of a range of shrimp species, including whiteleg shrimp, has been studied (De Souza et al. 2012; Silva et al. 2012). These changes in the microbial community and in the shrimp status have led to better growth and survival rates (Crab et al. 2010; Xu et al. 2013; Aguilera-Rivera et al. 2014). The use of disinfectant without the proper preconditioning of ponds prior to stocking shrimp can lead to impoverished microbial communities, creating conditions that may facilitate the proliferation and domination of certain bacterial species like *V. parahaemolyticus* (De Schryver et al. 2014). The current study, therefore, set out to investigate the potential protective effects of biofloc and shrimp culture conditions prior to and during infection with a pathogenic strain of *V. parahaemolyticus* (VP_{AHPND}).

Materials and Methods

Culture of Experimental Animals

The effect of biofloc on the survival of whiteleg shrimp when exposed to VP_{AHPND} was determined. A single cohort of postlarvae (PL) stage 12 *P. vannamei* was obtained from a specific pathogen free (SPF) facility (details withheld for confidentiality) within Thailand and transferred to the quarantine facility within Fish Vet Group Asia Limited's (FVGAL) research aquarium in Chonburi. On receipt, the PL were disinfected in 100 ppm povidone iodine, stress tested and then held under quarantine while a subsample of 150 PL were tested for the microsporidian *Enterocytozoon hepatopenaei* (EHP), for AHPND and for five viral agents: infectious hypodermal and haematopoietic necrosis virus (IHHNV), infectious myonecrosis virus (IMNV), Taura syndrome virus (TSV), white-spot syndrome virus (WSSV) and yellow head virus (YHV) following World Organisation for Animal Health (OIE) approved methodologies (10 batches of 15 PL samples tested in 70 separate polymerase chain reaction (PCR) reactions). Once the PL had been demonstrated to be free of these diseases, they were stocked into 400 L tanks containing a 2-week-old culture of biofloc in 15 ppt pretreated seawater held at ambient conditions (28–29 °C). The biofloc was started using a regime of feeding tanks with rice bran, commercial shrimp feed and sugar that was adjusted daily over the 14 days prior to the receipt of the shrimp. A system of inverted air pipes was used within each tank to provide a moderate level of water movement such that dissolved oxygen levels did not fall below 5.5 $mg.L^{-1}$. The shrimp were initially stocked at 100 $PL.L^{-1}$ and then graded as they grew.

Trial One

Experimental groups for assessment

When the shrimp were approximately 1 ± 0.05 g, 60 shrimp were transferred to a 200 L tank containing 15 ppt pretreated, non-biofloc, seawater. The tanks contained a mature biofilter that was preconditioned on ground shrimp feed and sugar only for 10 days prior to the receipt of shrimp.

The shrimp were maintained at 5 % body weight per day on Charoen Pokphand (CP) Starbird 5093 S feed for 10 days; approximately 10 % of the water in each tank was exchanged daily. A second 200 L tank was stocked with 60 shrimp five days after the transfer of the first tank from the same cohort of shrimp; tank conditioning, maintenance and husbandry followed that applied for the first tank. A challenge with a pathogenic strain of VP_{AHPND} (isolate FVG0001) was conducted 10 days after the stocking of the first tank and five days after the second. For the challenge, a total of 180 1.5 L glass vessels were used and set up in a preconditioned temperature-controlled room within the challenge facility. The temperature was 27.48 ± 0.32 °C (average ± 1 S.D.; range = 26.68–28.36 °C) and monitored for three days prior to the start of the trial and then monitored throughout via the use of two Onset HOBO® UA-001-64 (Bourne, MA, USA) data loggers in each room which recorded the temperature every 15 min.

Bacterial challenge with Vibrio parahaemolyticus

For the bacterial challenges, four experimental groups were set up: 1) shrimp reared in biofloc and then maintained in clear water for 5 days before challenge; 2) shrimp reared in biofloc and then maintained for 10 days in clear water before challenge; 3) shrimp reared in biofloc then challenged in biofloc; and 4) shrimp reared in biofloc and then immediately transferred to clear water (< 4 h) before challenge. Each test vessel was filled with 400 mL of the relevant water, then stocked with a single shrimp; the shrimp were transferred approximately four hours prior to challenge. Fifteen shrimp were individually challenged in each group; the experiment was conducted using two doses of VP_{AHPND} (i.e. a total of 120 shrimp). The challenged groups were run against a corresponding set of controls with a total of 60 shrimp.

To prepare the bacterial inoculum, the VP_{AHPND} was cultured in tryptone soya broth (TSB) supplemented with 2 % NaCl and incubated under shaking conditions (i.e. 250 rpm) at 28 °C for 12 h. Thereafter, the bacteria were precipitated by centrifugation at 900 × g for 10 min at 10 °C and the resultant bacterial pellet subsequently resuspended in sterile 15 ppt brackish water. The bacterial cell number of the resultant VP_{AHPND} medium was estimated by measuring the optical density at 600 nm (OD_{600}), where for VP_{AHPND}, an OD value of 1.0 corresponded to approximately 1.0×10^8 cfu.mL^{-1}. The bacterial cell number was then adjusted and verified by viable plate counts following standard methods.

For the challenge, each shrimp was held in a glass jar containing 400 mL of 15 ppt (either biofloc or in pretreated clear) aerated seawater at 27.5 °C and then given either a 1.2 mL.vessel^{-1} (dose 1) or a 1.8 mL.vessel^{-1} (dose 2) of VP_{AHPND} (isolate FVG0001; OD_{600} = 1.012; initial concentration of the inoculum was 1.28×10^8 cfu.mL^{-1}). The bacterial doses used were determined from pretests conducted with three doses of VP_{AHPND} using three shrimp per dose held under the same experimental conditions as those used for the main challenge. Shrimp from the same population as those used for the main challenge were used for the pretests; the pretests were conducted 48 h before the main challenge. Approximately five minutes after the shrimp had been challenged, 1 mL water samples were taken from a minimum of three test vessels in each experimental group.

The average bacterial dose per vessel was determined by taking 10 µL of a 10-fold dilution of the water sample and dropping this onto a TCBS plate, incubating it at 28 °C overnight and then making manual colony counts. The shrimp were maintained for 24 h, after which a further 400 mL of the liquid medium appropriate to each test condition was added (i.e. clear water or biofloc). After 48 h post-challenge, 50 % of the water in each test vessel was replaced with fresh medium. Each shrimp was given a daily ration of 5 feed pellets. The shrimp in each jar were evaluated every 3 h post-challenge and any mortalities were noted. The trial was terminated 96 h post-challenge after the frequency of mortalities had stabilized.

Trial Two

To verify the results, a second trial was conducted using similar experimental conditions to those used for trial one. Five challenge conditions were used; shrimp that were reared in biofloc to an average weight of 1.762 ± 0.085 g (mean ± S.D.) were either: 1) maintained in clear water for 7 d before challenging in clear water; 2) maintained in clear water for 7 d before challenging in biofloc; 3) maintained in biofloc and challenged in biofloc; 4) maintained in biofloc and challenged in biofloc filtered through a < 2 µm felt bag used as a standard for water filtration on commercial shrimp farms; or 5) maintained in biofloc but challenged in clear water. For each experimental condition, a total of 15 individually held shrimp were used and assessed against non-VP_{AHPND} unchallenged shrimp; temperature in the challenge room was 28.25 ± 0.66 °C (average ± 1 S.D.; range = 25.90–29.25 °C). For the challenge, only a single dose was explored, i.e. 4 mL VP_{AHPND} inoculum in each vessel (isolate FVG0001; OD_{600} = 1.046; initial concentration of the inoculum was 1.28×10^8 cfu.mL^{-1}). The challenge dose was determined following a series of pretests on shrimp from the same population, 48 h prior to the main challenge. The total suspended solids in the biofloc medium was determined to be approximately 670 mg.L^{-1} by filtering 1 L of the shrimp culture water through preweighed No. 93 Whatman filter paper and then drying the residue for 24 h before weighing.

Analysis of Biofloc

Samples of biofloc used for each experimental trial were taken from the shrimp culture tanks and evaluated. For the first trial, a 1.5 L sample was taken and subsequently passed through a 0.45 µm cellulose membrane, after which 8 mm discs were cut and placed onto trypticase soy agar (TSA) plates supplemented with 2 % (w/v) NaCl and inoculated with 100 µL of the VP_{AHPND} (concentration = 1.28×10^8 cfu.mL^{-1}). Three biofloc filter discs were placed on two TSA plates which were subsequently incubated at 28 °C for 14–16 h, after which the plates were visually assessed for clearance zones around each disc.

Statistical Analysis

Pairwise Kaplan-Meier survival analyses with subsequent Mantel-Cox log-rank tests conducted in Excel 2016 were applied to the mortality data to calculate the survival probabilities and to compare the survival distributions of the shrimp in each experimental group. Statistical significance was set at $P < 0.05$.

Ethics Statement

These experimental procedures were reviewed by and conducted under the approval of Fish Vet Group's internal ethical review board. Scientists conducting the aquatic pathogen trials hold licences for the use of "Animals for Scientific Purposes" issued by the Institute for Animals for Scientific Purpose Development (IAD), National Research Council of Thailand (NRCT). Fish Vet Group's laboratories and challenge facilities are registered with the relevant Thai authorities and are inspected as required under current Thai legislation.

Results

Trial One

Following the experimental challenge with VP_{AHPND}, water samples were taken from a random selection of the test vessels and used to verify the dose of bacteria added (Table 1). The shrimp were subsequently assessed every three hours and any mortalities were noted; the cumulative mortality curves are presented in Figure 1. The trial was terminated 96 h post-infection after the pattern of mortalities had stabilized.

Two doses of VP_{AHPND} were used to challenge the shrimp (i.e. 2.11–2.63 × 10^5 cfu.mL^{-1} and 2.84–4.54 × 10^5 cfu.mL^{-1}; n = 60 shrimp per dose) against a control group (n = 60 shrimp; Table 1). The highest mortalities were seen in the shrimp groups that had been most recently transferred from biofloc, i.e. those that were immediately transferred from biofloc into clear water and challenged (Bio-CW Vp; mortalities of 80 % and 60 %) and those that had been reared in biofloc but maintained for 5 days in clear water and then challenged (5D Vp; mortalities of 60 % and 73.3 %; Fig. 1).

The rates of mortality were lower (i.e. 33.3 % and 20 %) in the shrimp that were transferred from biofloc and then held in clear water for 10 days (10D Vp) before they were challenged in clear water, but the lowest mortality of 0 % and 6.7 % were in the two groups of shrimp that were reared and challenged in biofloc (Biofloc Vp). All shrimp were handled identically, in that they were transferred from their culture environment into their relevant vessels approximately 4 hr prior to experimental challenge.

Significant differences between the culture conditions are presented in Table 2. The lower rates of mortality in the shrimp reared and challenged in biofloc were statistically lower ($P < 0.05$) than those determined in the other experimentally challenged groups.

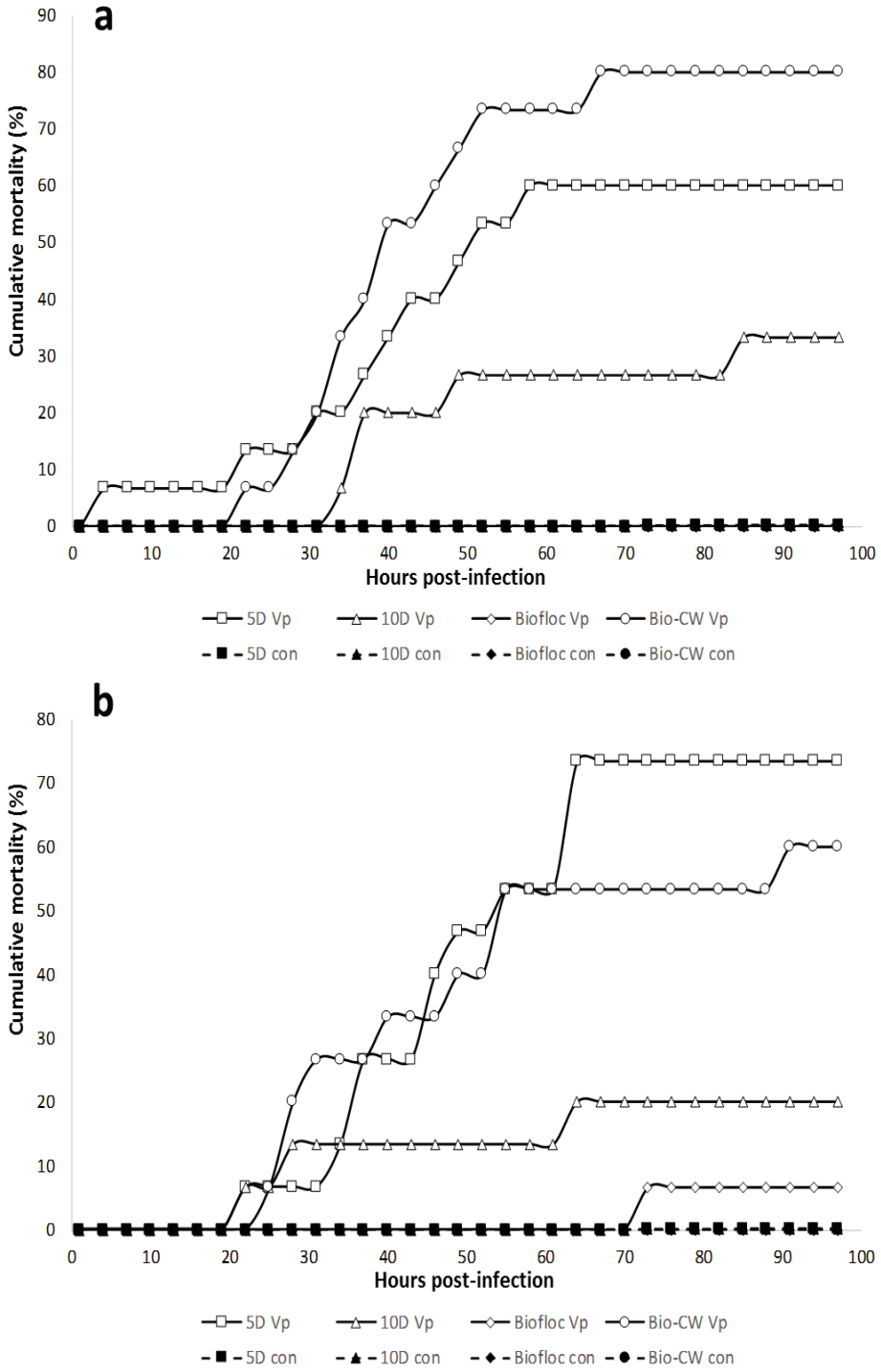

Fig. 1. Cumulative mortality curves for *Penaeus vannamei* reared in biofloc and then subjected to a water treatment before being challenged with two different doses of *Vibrio parahaemolyticus* (Vp isolate FVG0001) and compared against corresponding controls. Figure 1a = 1.2 mL VP_{AHPND} culture.vessel^{-1} (dose 1), while Figure 1b = 1.8 ml VP_{AHPND} culture.vessel^{-1} (dose 2), where the initial concentration of the inoculum was 1.28 $\times 10^8$ cfu.mL^{-1}. Shrimp were either held for five (5D Vp) or ten days (10D Vp) in non-biofloc, clear, pretreated 15 ppt seawater before challenge or were transferred immediately from biofloc to clear non-biofloc pretreated water (Bio-CW Vp). A fourth group of shrimp was reared and challenged in biofloc (Biofloc Vp). A further four groups of shrimp, 5D con, 10D con, Biofloc con and Bio-CW con are the corresponding control groups to each VP_{AHPND} -challenged group.

Table 1. The average bacterial dose (cfu) per experimental vessel and per water condition. Following the challenge of the experimental *Penaeus vannamei* with a pathogenic isolate of *Vibrio parahaemolyticus* (isolate FVG0001), the bacterial load was determined from 1 mL water samples taken from a random selection of test vessels in each water treatment (5-days = 5 days in clear water before challenge in clear water; 10-days = 10 days in clear water before challenge in clear water; Biofloc = reared in biofloc and challenged in biofloc; Bio-CW = reared in biofloc and then transferred to clear water immediately before challenge).

Condition	Water treatment	Number of bacteria (cfu.mL^{-1}) on TCBS	
		Green colonies	Yellow colonies
Vp Dose 1	5-days	2.62×10^5	
	10-days	2.63×10^5	
	Biofloc	3.90×10^5	
	Bio-CW	2.11×10^5	
Vp Dose 2	5-days	2.82×10^5	
	10-days	3.26×10^5	
	Biofloc	5.96×10^5	
	Bio-CW	4.54×10^5	
Control	5-days	1.60×10^2	4.00×10
	10-days	4.00×10	2.00×10
	Biofloc	2.60×10^3	9.20×10^3
	Bio-CW	2.00×10	2.00×10

Table 2. Pairwise Mantel-Cox log-rank tests applied to the Kaplan-Meier survival probabilities for each test group of shrimp (n = 15 replicates). Survival of *Penaeus vannamei* challenged with different doses of *Vibrio parahaemolyticus* was assessed against a control group. Shrimp reared to ca. 1 g in biofloc were either maintained in non-biofloc, pretreated 15 ppt seawater for ten days (10D), five days (5D) or were immediately transferred (approximately 4 h) before being challenged (Bio-CW). A fourth group of shrimp reared in biofloc was also challenged in biofloc (Biofloc). Figures in a bold font highlight significant differences ($P < 0.05$) in shrimp survival between the different test conditions.

		Dose 1				Dose 2			
		5D	10D	Biofloc	Bio-CW	5D	10D	Biofloc	Bio-CW
Dose 1	5D								
	10D	0.136							
	Biofloc	**< 0.001**	**0.017**						
	Bio-CW	0.272	**0.009**	**< 0.001**					
Dose 2	5D	0.852	**0.047**	**< 0.001**	0.364				
	10D	**0.030**	0.482	0.073	**0.002**	**0.009**			
	Biofloc	**0.001**	0.068	0.317	**< 0.001**	**< 0.001**	0.267		
	Bio-CW	0.897	0.147	**< 0.001**	0.217	0.634	**0.038**	**0.002**	
Control	5D	**0.001**	**0.004**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
	10D	0.068	0.175	**0.017**	**0.017**	0.267	0.562	0.073	0.073
	Biofloc	0.317	0.150	1.000	1.000	1.000	**< 0.001**	0.317	0.317
	Bio-CW	**<0.001**	**<0.001**	**< 0.001**	**< 0.001**	**0.011**	**0.023**	**0.004**	**0.004**

Trial Two

The second trial, using VP_{AHPND} doses of 1.33–1.44 × 10^6 cfu.mL^{-1} (Table 3), yielded similar results to that of the first (Figure 2). The trial, however, included filtered biofloc as a test condition. Again, the highest level of mortality (73.3 %) was seen in the shrimp transferred from biofloc to clear water prior to challenge, which differed significantly ($P < 0.001$) from those reared and challenged in biofloc which had the lowest level of mortality (13.3 %). A high level of mortality (53.3 %) was also seen in the shrimp that were reared in clear water for 7 days prior to challenge. Lower levels of mortality were seen in the other groups challenged in biofloc, i.e. 26.7 % for those reared in clear water for 7 days but then challenged in biofloc, and only 20 % mortality among the shrimp reared in biofloc but challenged in < 2µm filtered biofloc. Significant differences in the survival rates of the shrimp in the different experimental groups are given in Table 4.

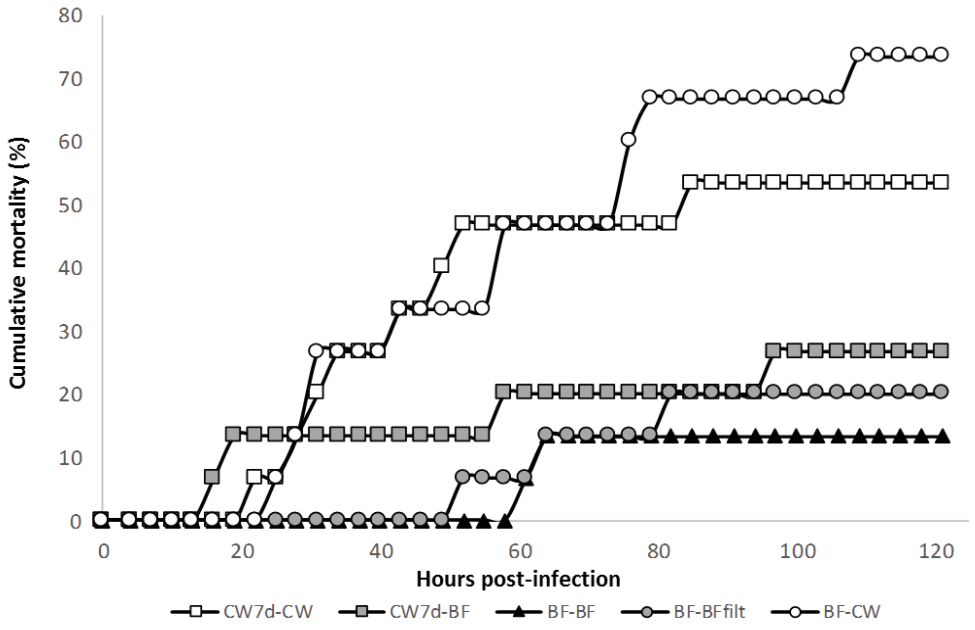

Fig. 2. Cumulative mortality curves of *Penaeus vannamei* challenged with *Vibrio parahaemolyticus* in the second biofloc-based experiment CW7d-CW = shrimp held for seven days in clear water before challenge in clear water; CW7d-BF = shrimp held for seven days in clear water and then challenged in biofloc; BF-BF = shrimp reared and challenged in biofloc; BF-BFfilt = shrimp reared in biofloc but challenged in filtered biofloc; and BF-CW = shrimp reared in biofloc but challenged in clear water. Each group consists of 15 shrimp; corresponding controls were used for each challenge condition. The control curves are not shown but there was the loss of two shrimp in the CW7d-CW control at 49 h post-inoculation (p.i.) and three shrimp in the CW7d-BF control at 49 h (1 shrimp) and 97 h p.i. (two shrimp). There was no other loss of control shrimp.

Table 3. Average bacterial dose (cfu.mL^{-1}) per experimental vessel and per water condition in the second biofloc-based *Vibrio parahaemolyticus* challenge trial. Following bacterial challenge, the bacterial load was determined in a random selection of test vessels within each water treatment (CW7d-CW = 7 days in clear water before challenge in clear water; CW7d-BF = 7 days in clear water before challenge in biofloc; BF-BF = reared in biofloc and challenged in biofloc; BF-BFfilt = reared in biofloc and then challenged in filtered biofloc water; BF-CW = reared in biofloc and then challenged in clear water).

Condition	Water treatment	Number of bacteria (cfu.mL^{-1}) on TCBS	
		Green colonies	Yellow colonies
Vp	CW7d-CW	1.33×10^6	3.33×10^4
	CW7d-BF	1.44×10^6	6.67×10^3
	BF-BF	1.92×10^6	
	BF-BFfilt	1.27×10^6	
	BF-CW	1.46×10^6	
Control	CW7d-CW	2.00×10	6.00×10
	CW7d-BF	5.00×10^2	6.20×10^2
	BF-BF	2.60×10^2	4.60×10^2
	BF-BFfilt	3.80×10^2	2.40×10^2
	BF-CW		

Table 4. Pairwise Mantel-Cox log-rank tests applied to the Kaplan-Meier survival probabilities from *Penaeus vannamei* challenged with *Vibrio parahaemolyticus* under different water conditions (CW7d-CW = 7 days in clear water before challenge in clear water; CW7d-BF = 7 days in clear water before challenge in biofloc; BF-BF = reared in biofloc and challenged in biofloc; BF-BFfilt = reared in biofloc and then challenged in filtered biofloc water; BF-CW = reared in biofloc and then challenged in clear water). Figures shown in a bold font highlight significant differences ($P < 0.05$) in the survival of shrimp between the different water conditions.

		Vp challenged				
		CW7d-CW	CW7d-BF	BF-BF	BF-BFfilt	BF-CW
Vp chal	CW7d-CW					
	CW7d-BF	0.148				
	BF-BF	**0.014**	0.340			
	BF-BFfilt	**0.038**	0.634	0.644		
	BF-CW	0.452	**0.021**	**0.001**	**0.003**	

		Control				
		CW7d-CW	CW7d-BF	BF-BF	BF-BFfilt	BF-CW
Vp chal	CW7d-CW	**0.018**	**0.034**	**0.001**	**0.001**	**0.001**
	CW7d-BF	0.365	0.603	**0.035**	**0.035**	**0.035**
	BF-BF	0.957	0.668	0.150	0.150	0.150
	BF-BFfilt	0.690	0.949	0.073	0.073	0.073
	BF-CW	**0.001**	**0.002**	**<0.001**	**<0.001**	**<0.001**

Control	CW7d-CW					
	CW7d-BF	0.679				
	BF-BF	0.157	0.076			
	BF-BFfilt	0.157	0.076	NS		
	BF-CW	0.157	0.076	NS	NS	

Analysis of Biofloc

No demonstrable antimicrobial activity (i.e. no clear zones around the discs through which the biofloc was filtered) was detected in the samples of biofloc taken from the shrimp culture tank.

Discussion

Biofloc has also been shown to have a positive impact on the survival, growth and the digestive enzyme activities of *P. vannamei* (Xu et al. 2013). The studies conducted by the latter authors found that the levels of digestive enzyme activity were significantly higher in the stomachs of biofloc-reared shrimp than in those of shrimp raised in non-biofloc water.

These findings were also in agreement with an earlier study conducted by Moss et al. (2001), who had found that the activity of digestive enzymes in the hepatopancreas was higher in *P. vannamei* reared in ponds than in those raised in well water. Suita et al. (2015) stated that biofloc positively affects intestinal peristalsis and the synthesis of digestive enzymes by the hepatopancreas, with an increased thickness to the hepatopancreatic tubules and a rise in the number of enzyme-producing B cells. Changes to the hepatopancreas may be associated with essential amino acids, vitamins and minerals present in biofloc (Decamp et al. 2002).

The current study set out to compare the survival rates of whiteleg shrimp when reared in two main water types (i.e. clear water or in biofloc) for different periods of time and then challenged with $VP_{AHPND.}$ The mortality of shrimp within each water condition can be ranked as follows:

Trial 1a: Biofloc (0 %) > 10D (33.3 %) > 5D (60 %) > Bio-CW (80 %)

Trial 1b: Biofloc (6.7 %) > 10D (20 %) > Bio-CW (60 %) > 5D (73.3 %)

Trial 2: Biofloc (13.3 %) > Filtered biofloc (20 %) > CW7d-Biofloc (26.7 %) > CW7d - CW (53.3 %) > BF-CW (73.3 %)

These findings show that the shrimp, when challenged in biofloc regardless of their prior culture conditions, have lower levels of mortality than those challenged in clear water. Within the biofloc-challenged group, those that were reared and infected in biofloc had the lowest mortality (i.e. 0 %, 6.7 % and 13.3 %). There appears to be little difference in the mortality of the shrimp that were reared in biofloc and then challenged in < 2 μm filtered biofloc (20 %) and those reared for 7 days in clear water but then transferred to biofloc ca. 4 h prior to challenge (26.7 %) – a difference in mortality of only a single shrimp. For the shrimp challenged in clear water, there is a suggested correlation in mortality and the duration spent in clear water prior to challenge, i.e. 10D (ave. 26.65 %) > 5D (ave. 66.7 %) > 4 h (ave. 71.1 %). All shrimp were initially reared in biofloc until they were transferred into clear water.

The results suggest that the movement into clear water places a stress on the shrimp that has an immediate impact on their feeding activity and intake, with potential consequential impacts on enzymatic activity within the hepatopancreas and the immune status of the shrimp. The change in culture environment will also effect a change in the intestinal bacterial community of the gut, changing the shrimp's resistance to pathogenic bacteria by, for example, affecting virulence by quorum quenching (Pande et al. 2013; Luis-Villaseñor et al. 2015; Zheng et al. 2016). The lower rates of mortality correlated to the time spent in clear water suggest that accommodation to the new environment is required in: 1) re-establishing a stable intestinal microflora; and, 2) switching from the option to feed continuously on biofloc supplemented with a commercial pelleted diet to a regime where diet is not continuously available. On termination of the trial, the shrimp maintained in biofloc had dark guts indicating that they had continued to feed throughout the challenge, whereas the shrimp maintained in clear water had very little within their guts despite being fed throughout.

From the current trial, shrimp that were reared in biofloc but then were transferred to clear water for the VP_{AHPND} challenge had the highest rates of mortality (Figs. 1 and 2). This suggests that the preculture in biofloc, and the shrimp's intestinal bacterial community, offers no protection when transferred and then challenged in clear water under the experimental conditions used here. In a marked contrast to this, the benefits of biofloc appear to be immediate. There was only a 26.7 % mortality in the shrimp that were reared in clear water for 7 days but then were immediately transferred to biofloc for the VP_{AHPND} challenge, whereas there was a 73.3 % mortality in the shrimp reared in biofloc but then transferred to clear water for the VP_{AHPND} challenge (Fig. 2). Under the conditions used here, it would appear that biofloc has an impact on the virulence of VP_{AHPND}, theoretically by disrupting quorum sensing. This appears to be unaffected by filtration. In conclusion, *P. vannamei* challenged with VP_{AHPND} in biofloc had the highest rates of survival (ave. 86.7 % survival for those challenged in biofloc versus ave. 43.4 % for shrimp challenged in clear water). The benefits appear to be immediate; however, the protection is immediately lost once shrimp are transferred into clear water and challenged. The findings suggest that careful management of the microbial community within aquaculture ponds can assist in controlling bacterial infections.

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Risk Factors Associated with Acute Hepatopancreatic Necrosis Disease (AHPND) Outbreak in the Mekong Delta, Viet Nam

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Abstract

The Vietnamese shrimp farming industry has experienced massive production losses due to Acute Hepatopancreatic Necrosis Disease (AHPND) caused by *Vibrio parahaemolyticus* (Vp_{AHPND}) since 2011. The objective of this study was to identify factors associated with AHPND occurrence on shrimp farms in the Mekong Delta in Viet Nam. A retrospective cross-sectional study was carried out on shrimp farms in four districts in the Mekong Delta area, Viet Nam from January 2012 to May 2013. Data were collected from 1920 ponds belonging to 1195 farms. Factors related to farm characteristics, farm management, pond and water preparation and management, feed management, postlarval (PL) shrimp, and stock management were evaluated. Multivariable logistic regression analysis was used to determine factors affecting the occurrence of AHPND at the pond and farm levels. The following characteristics were identified as significant farm-level risk factors: (i) having a larger culture area in terms of hectarage, (ii) using the sun-dried sediment method for cleaning pond bottom during the pond preparation process, and (iii) being sited close to other farms using the same AHPND-affected water source. Ponds with the following features were associated with increased risk of AHPND occurrence: (i) water depth of 1.2 m or less, (ii) extremely change of weather events occurring during the first 35 days of culture (DOC) or until the first signs of AHPND, and (iii) use of fertilizers and probiotics for water treatment. Moreover, ponds that were supplied with PL from some specific hatcheries were more likely to be infected with AHPND than were others. On the other hand, the risk of AHPND occurring was reduced in ponds that used minerals and algaecide for water treatment.

Keywords: *Vibrio parahaemolyticus*, acute hepatopancreatic necrosis disease (AHPND), risk factor, farm management

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Introduction

Acute Hepatopancreatic Necrosis Disease (AHPND) has been responsible for huge losses in the shrimp aquaculture industry of many countries since 2010. The disease was first reported in the People's Republic of China in 2010, followed by Viet Nam and Malaysia in early 2011 (Lightner *et al*., 2012), and then Thailand in late 2011 (Flegel 2012). AHPND has a bacterial aetiology (Tran et al. 2013). It is caused by a particular strain of *Vibrio parahaemolyticus* (Vp_{AHPND}), which contain a ~70-kbp plasmid (pVA1) with genes homologous to those coding for the *Photorhabdus* insect-related (Pir) binary toxin (PirA^{vp}/PirB^{vp}) that induces cell death (Kondo et al. 2015; Lee et al. 2015).

The disease typically affects newly stocked penaeid shrimp (i.e. 20–30 days after stocking), including *Penaeus monodon* Fabricius 1798, *P. vannamei*, and *P. chinensis* Osbeck 1765 (NACA/FAO 2011; NACA 2012a). Affected shrimp display signs of lethargy and anorexia, and a pale and atrophied hepatopancreas as a prominent gross lesion, and commonly experience secondary infections with opportunistic bacteria. Shrimp mortality gradually increases, with cumulative pond mortality regularly reaching 100% within a week (Lightner et al. 2012). A polymerase chain reaction (PCR) test for the detection of bacterial isolates that cause AHPND has been available since late 2013 (Flegel and Lo 2014) and nested PCR was reported in 2015 (Sirikharin et al. 2015). However, before the PCR method became available, the diagnostic procedure relied on clinical signs and histopathological finding matching the case definition as given by the AHPND disease card (NACA 2012b).

AHPND was firstly recognized in Viet Nam in Soc Trang Province in late 2010 and continuously occurred and spread to other provinces in the Mekong Delta such as Tien Giang, Ben Tre, Tra Vinh, Soc Trang, Bac Lieu, Kien Giang and Ca Mau provinces in 2011. It caused approximately 50 % reduction of total shrimp production in Viet Nam. The Food and Agriculture Organization of the United Nations (FAO) Project TCP/VIE/3304 was funded to assist the Government of Viet Nam, particularly the Competent Authority on aquatic animal health to achieve a better understanding of the unknown disease that was affecting cultured shrimp and causing significant losses in the Mekong Delta. The objective of this study was to measure the prevalence of AHPND in the Mekong Delta of Viet Nam during January 2012 to May 2013 and understand the pattern of its spread. Moreover, the risk factors related to various farm practices, such as pond preparation, water quality management, PL and stock management were evaluated for their association with the occurrence of AHPND at the pond and farm levels.

Material and Methods

Study area

The study areas were chosen based on information provided by field officers of the Department of Animal Health (DAH). The areas were selected based on the AHPND problem experienced (i.e. AHPND still occurred in the area) and the scale of farm operation in the area. Therefore, districts in three provinces of the Mekong Delta were chosen: Dam Doi District of Ca

Mau Province, Hoa Binh District of Bac Lieu Province, and Vinh Chu and Tran De districts of Soc Trang Province (Fig. 1).

Fig. 1. Four districts included in the study area. Note: DD = Dam Doi District of Ca Mau Province, HB = Hoa Binh District of Bac Lieu Province, VC = Vinh Chu District and TD = Tran De District, both of Soc Trang Province.

Study population and pond selection

Black tiger shrimp (*P. monodon)* and Pacific white shrimp (*P. vannamei*) were the subjects of the study. Both species are cultured in the Mekong Delta area and were experiencing problems with AHPND. A cross-sectional study design was used. The study population consisted of shrimp cultured in ponds during the period January 2012 to May 2013. The list of shrimp farms was provided by DAH officers and used for the sampling frame. To reduce the problem of forgotten or loss of data, the last crop data of ponds in farms that had several crops during the study period were included in the study.

As this study used a retrospective study design, the pond-level case definition used for further analysis was based on clinical signs of AHPND-affected shrimp (presence of a prominent, pale and atrophied hepatopancreas), affected ponds having a cumulative mortality of more than 40 % within 5–7 days after occurrence of clinical signs, and the problem having occurred before 35 DOC. The farm-level case definition was based on the AHPND history of the farm, i.e. an affected farm must have had at least one operation cycle with a pond affected by AHPND from 1 January 2012 until the date of the study.

Sampling size calculation and study type

Sampling size was calculated using the Survey Toolbox software program (Cameron 2002). The prevalence of AHPND in the study areas as reported by Corsin (2012) was used to calculate the sampling size required for each district. Two-stage sampling was used to sample farm and pond data. The 1st stage sampling was randomly selected farms using the method of probability proportional to size. The 2nd stage sampling chose ponds by the simple random sampling method. The number of farms and number of ponds per farm used in this study are indicated in Table 1.

Table 1. Number of studied farms, number of ponds per farm and expected total ponds used in this study

Province	District	Number of farms	Prevalence (%)	Average farm size (# of ponds)	# of farms	# of ponds per farm	Expected total ponds
Ca Mau	DD	2117	29.2	2.67	297	2	594
Bac Lieu	HB	2500	46.9	4.88	332	2	664
Soc Trang	VC	2041	30.4	3.04	304	2	608
Soc Trang	TD	1398	30.4	4	316	2	632

Note: DD = Dam Doi District, HB = Hoa Binh District, VC = Vinh Chu District and TD = Tran De District

Data collection

Data were collected by means of questionnaires administered through face-to-face interviews. Interviewers were trained on how to use the questionnaires prior to carrying out the data collection. The questionnaires were pre-tested to ensure understanding and practical availability of information. Data collected included the following: (i) respondent (farmer) information, (ii) general farm information, (iii) general pond information, (iv) farm characteristics, (v) shrimp species, (vi) culture system, (vii) water management: water and pond preparation, water exchange, (viii) feed, (ix) source of PL shrimp, (x) shrimp age at stocking, (xi) PL stocking density, and (xii) weather conditions during the week prior to disease occurrence (for case) or before shrimp reached 35 DOC (for control).

Statistical analysis

Data were checked for missing values, and mean calculation was carried out for each district. Dummy variables were created for summary of information contained in the record prior to the analysis. Variables were identified by farm and pond levels. Farm-level factors included the following: farm size (number of available ponds), production size (number of active ponds), reservoir area size (measured in hectares), ratio of reservoir to culture area, bottom type, source of water supply, water management system, shrimp species, pond preparation methods, chemical treatment for water preparation, and shrimp health status of nearby farms. Pond-level factors included pond size and depth, water and pond preparation, source of PL, shrimp age at stocking, PL stocking density, feed practice, water quality and exchange rate, other animals in the pond, aerator application, use of probiotics, and weather conditions.

The presence or absence of AHPND at the farm and pond levels was considered the outcome variable. The analytical processes were accomplished using the statistical software package STATA (Version 14.0, Stata Corp, College Station, TX). Multivariable logistic regression was used to evaluate factors associated with AHPND outbreak at farm and pond levels, separately. The assumptions for the logistic regression were assessed (Dohoo et al. 2009). Unconditional associations between each predictor and outcome variables were evaluated using univariable logistic regression. All factors with *P*-value of less than or equal to 0.2 were included in the multivariable analysis. Categorized variables were generated if the relationship between a continuous predictor and log-odds of the outcome was not linear.

The cut-offs were decided at the point of the most change in log-odds when the independent variables changed, and these were incorporated with biological factors prior to the multilevel logistic regression analysis**.** District was considered a potential confounding factor and remained in the model as fixed effect**.** Shrimp species was included as random effect when assessing the farm**-**level model**.** Farm was included as random effect to account for potential farm effect when assessing the pond**-**level model**.** The best-fit model was found by a manual backward selection process in which the likelihood-ratio test (LRT) was used to test the significance (P-value < 0.05) of subtracting one variable at a time from the models. Interactions effect among significant variables that notice biological synergism plausible were also tested**.**

Results

Survey data were collected from a total of 1254 farms and 2508 ponds in the four study areas (Table 2). We could not complete all of the questionnaires because some farms were no longer in operation. Moreover, some farms had only one pond, so we had only one pond record, which is less than the target number (2 ponds per farm). Therefore, the data collected for farm and pond were 95.3 % and 76.6 % of the goal, respectively.

Table 2. Number and proportion of farms and ponds used for collection of data.

Province	District	# Target farm	# Target pond	#Complete farm	#Complete pond
Soc Trang	VC	309	618	289 (93.5 %)	466 (75.4 %)
Soc Trang	TD	316	632	323 (102 %)	387 (61.2 %)
Bac Lieu	HB	332	664	305 (91.9 %)	586 (88.3 %)
Ca Mau	DD	297	594	278 (93.6 %)	481 (81 %)
Total		1254	2508	1195 (95.3 %)	1920 (76.6 %)

Note: DD = Dam Doi District, HB = Hoa Binh District, VC = Vinh Chu District and TD = Tran De District

Farm-level data analysis

Farm characteristics and farm management

Farm which cultured *P. monodon, P. vannamei* and both species were 84.8 %, 7.1 % and 8.1 %, respectively. Almost half of the farms culturing *P. monodon* (53.6 %) were semi-intensive farms, while intensive farms and improved extensive farms comprised 33.2 % and 13.2 % of the total. respectively.

Farms culturing *P. vannamei* were mainly intensive farms, (90.4 %). Farm managing with open, closed, semiclosed, recycled and mix water management systems were 12.2 %, 53.4 %, 29.5 %, 1.2 % and 3.7 %, respectively.

Descriptive statistics and unconditional associations for each factor

More than half of the interviewed farms (i.e. 78.5 %) experienced AHPND. Prevalence of AHPND in *P. monodon*, *P. vannamei* and both secies farms was 79.2 %, 65.7.0 % and 82.5 %,

respectively. Prevalence of AHPND in *P. monodon* farms was 78.5 %, 78.2 % and 86.7 % for intensive, semi-intensive and improved extensive farms, respectively. Prevalence of AHPND in intensive and semi-intensive *P. vannamei* farms was 74.2 % and 78.6 %, respectively. The results from unconditional association analysis are presented in Table 3.

Table 3. Descriptive statistics and unconditional associations for factors with the potential to affect AHPND occurrence in shrimp farms.

	Control			Case			
Variable name	**n**	**%**	**Mean (SD)**	**n**	**%**	**Mean (SD)**	***P*-value***
District							<0.001
- Dam Doi (DD)	59	27.8		132	17.0		
- Hoa Binh (HB)	97	45.8		194	25.1		
- Tran De (TD)	45	21.2		198	25.6		
- Vinh Chu (VC)	11	5.2		250	32.3		
Number of culture ponds	211		3.1 (2.9)	774		3.7 (6.0)	0.107
Total culture area (hectare)	211		1.2 (1.1)	771		1.7 (4.6)	0.004
Water reservoir							0.178
Not available	104	49.1		339	43.9		
Available	108	50.9		434	56.1		
Total reservoir area (hectare)	212		0.2 (0.6)	771		0.2 (0.4)	0.481
Ratio of reservoir to culture area	211		0.3 (0.8)	770		0.2 (0.4)	0.033
Source of water supply							0.000
Fresh water (<0 ppt)	0	0.0		3	0.4		
Brackish water (5−15 ppt)	51	24.1		321	41.5		
Sea water (>15 ppt)	142	67.0		405	52.3		
More than one source	19	8.9		45	5.8		
Water management system							0.005
Open	27	12.7		93	12.0		
Closed	85	40.1		442	57.1		
Semi-closed	87	41.1		204	26.4		
Recirculation	3	1.4		9	1.2		
Mixed system	10	4.7		26	3.3		
Water supply and drainage							0.01
Same water inlet and outlet	164	80.4		537	71.4		
Separate water inlet and outlet	40	19.6		215	28.6		
Shrimp culture species							0.735
P. monodon	174	82.1		662	85.5		
P. vannamei	24	11.3		46	6.0		
Both species	14	6.6		66	8.5		
Culture system (*P. monodon*)							0.146
Intensive (>20 PL/m^2)	65	34.8		237	32.8		
Semi-intensive (10−20 PL/m^2)	106	56.7		381	52.8		
Improved extensive (<10 PL/m^2)	16	8.5		104	14.4		
Culture system (*P. vannamei*)							0.724
Intensive (≥ 60 PL/m^2)	34	91.9		98	89.9		
Semi-intensive (< 60 PL/m^2)	3	8.1		11	10.1		
Pond bottom type							0.658
Soil	205	97.2		746	96.8		
Plastic lining (slope or all area)	6	2.8		25	3.2		

Table 3. Continued**.**

Variable name	Control			Case			*P*-value*
	n	%	Mean (SD)	n	%	Mean (SD)	
Cleaning pond with at least one method							0.195
not applied	2	1.0		2	0.3		
applied	210	99.0		771	99.7		
Cleaning pond bottom by flushing							0.32
not applied	100	47.2		335	43.3		
applied	112	52.8		438	56.7		
Cleaning pond bottom by soil removal							0.006
not applied	105	49.5		302	39.1		
applied	107	50.5		471	60.9		
Cleaning pond bottom by sun drying							0.002
not applied	24	11.3		40	5.2		
applied	188	88.7		733	94.8		
Duration of sun drying (days)	152		20.5 (16.8)	459		29.0 (19.8)	0.000
Cleaning pond bottom by ploughing							0.725
not applied	198	93.4		727	94.0		
applied	14	6.6		46	6.0		
Liming pond							0.966
not applied	19	9.0		70	9.1		
applied	193	91.0		703	90.9		
Carricide control program during pond preparation							
not applied	3	1.4		10	1.3		0.894
applied	209	98.6		761	98.7		
- Filtration inlet water (not used)	15	7.1		54	7.0		0.982
Filtration inlet water (used)	197	92.9		714	93.0		
- Chlorine (not used)	104	49.1		428	56.6		0.051
Chlorine (used)	108	50.9		328	45.4		
- Insecticide (not used)	195	92.0		699	92.5		0.817
Insecticide (used)	17	8.0		57	7.5		
- Saponin (not used)	106	50.0		389	51.5		0.708
Saponin (used)	106	50.0		367	48.5		
- Other carricide agent (not used)	171	80.1		612	81.4		0.812
Other carricide agent (used)	41	19.9		140	18.6		
Application of probiotic bacteria during pond preparation							
not applied	157	74.1		562	74.6		0.865
applied	55	25.9		191	25.4		
Water treatment before using							
not applied	18	8.9		117	15.9		0.013
applied	184	91.1		617	84.1		
- Holding water (not used)	28	13.9		171	23.4		0.004
Holding water (used)	174	86.1		561	76.6		
- Chlorine (not used)	139	68.8		501	68.6		0.961
Chlorine (used)	63	31.2		229	31.4		
- Other disinfectant (not used)	170	84.2		637	87.5		0.216
Other disinfectant (used)	32	15.8		91	12.5		

Table 3. Continued**.**

Variable name	Control n	Control %	Control Mean (SD)	Case n	Case %	Case Mean (SD)	*P*-value*
Duration water withheld in reservoir (days)	201		11.9 (10.6)	722		10.2 (9.8)	0.026
Feed brand	70 different brands were used, with none showing a significant relation to AHPND outbreak						0.434
Feed broadcast manner							
Hand without feeding tray	61	28.9		343	44.9		0.000
Hand with feeding tray	150	71.1		417	55.1		
Mechanical by automatic feeder	0	0.0		4	0.0		
Feed storage condition							
Cool shaded storage	9	4.4		20	2.7		0.741
On-shelf storage	2	1.0		25	3.4		
Both methods	195	94.6		700	93.9		
Duration feeds stored in farm until using (days)	200		7.5 (3.6)	690		7.1 (3.2)	0.104
Nearby farms using same water source that is affected by or not affected by AHPND							
Not affected	95	49.7		130	17.9		0.000
Affected	96	50.3		596	82.1		

Note: *factors with *P*-value of less than or equal to 0**.**2 were included in the multivariable analysis**.**

Table 4. Final multiple logistic regression model for factors associated with AHPND occurrence in shrimp farms.

	OR	SE	Z	*P*-value	95 % CI
- Total culture area (hectare)	1.3	0.10	3.56	0.000	(1.1, 1.5)
- Cleaning pond bottom by sun-dry method	2.0	0.64	2.11	0.035	(1.1, 3.7)
- Nearby farms using same water source that is affected by EMS/AHPND	5.1	0.97	8.46	0.000	(3.5, 7.4)
District					
Dam Doi (DD)			Reference		
Hoa Binh (HB)	0.6	0.13	-2.48	0.013	(0.3, 0.9)
Tran De (TD)	1.5	0.38	1.53	0.126	(0.9, 2.5)
Vinh Chu (VC)	8.3	3.03	5.75	0.000	(4.0, 17.0)
Constant	0.4	0.14	-2.61	0.009	(0.2, 0.8)

Note: OR = odds ratio, SE = standard error, Z = standard normal deviate, 95 % CI = 95 % confidence interval

Multivariable logistic regression model

The final multivariable logistic regression model of factors associated with AHPND cases in shrimp farms in in the Mekong Delta area after controlling for the confounding effect of district is presented in Table 4. Factors with odds ratio (OR) greater than one are interpreted as increasing the risk of having AHPND in shrimp farms. Those factors with OR less than one are considered having protective effect. The prevalence of AHPND varied among districts, the highest prevalence being recorded in Vinh Chu District.

Risk factors that were related to AHPND occurrence include: (i) farm with larger culture area in terms of hectare, (ii) farm using sun-dry sediment method for cleaning pond bottom during pond preparation process, and (iii) farm site nearby other farms and using the same water source that is affected by AHPND. No significant interaction among significant variables was detected.

Table 5. Descriptive statistics and unconditional associations for factors with the potential to affect AHPND occurrence in shrimp ponds.

	Control			Case			
Variable name	**n**	**%**	**Mean (SD)**	**n**	**%**	**Mean (SD)**	***P*-value***
District							
- Dam Doi (DD)	246	36.6		201	16.7		reference
- Hoa Binh (HB)	322	47.9		260	21.6		0.925
- Tran De (TD)	79	11.8		306	25.4		0.000
- Vinh Chu (VC)	25	3.7		437	36.3		0.000
Pond characteristic							
- Pond size (hectare)	670		0.4 (0.3)	1,197		0.5 (0.4)	0.000
- Pond depth (meter)	664		1.4 (0.2)	1,170		1.3 (0.2)	0.000
Water treatment							
- Fertilizer use (not used)	437	65.8		780	66.2		0.881
Fertilizer use (used)	227	34.2		399	33.8		
- Fertilizer type							
- not used	437	66.4		780	67.1		reference
- inorganic	200	30.4		353	30.4		0.916
- organic	12	1.8		25	2.21		0.664
- both	9	1.4		5	0.4		0.037
- Application of any chemical for water quality management							0.045
- not used	98	14.8		136	11.6		
- used	563	85.2		1,040	88.4		
- Mineral application (not used)	52	8.8		66	6.2		0.048
Mineral application (used)	536	91.2		996	93.8		
- Disinfectant application (not used)	244	41.9		392	38.2		0.143
Disinfectant application (used)	338	58.1		634	61.8		
- Algaecide application (not used)	456	78.5		720	73.9		0.040
Algaecide application (used)	125	21.5		255	26.1		
- Pesticide application (not used)	565	97.3		935	97.7		0.578
Pesticide application (used)	16	2.7		22	2.3		
- Probiotic application (not used)	116	20.0		332	33.0		0.000
Probiotic application (used)	465	80.0		675	67.0		
Antimicrobial application							
- not used	406	69.9		716	73.6		0.115
- used	175	30.1		257	24.4		
Postlarval (PL) management							
- Shrimp species							0.000
- *P. vannamei*	121	18.3		111	9.4		
- *P. monodon*	541	81.7		1074	90.6		
- Stage of PL at stocking date	652		12.9 (1.9)	1123		12.9 (2.0)	0.975
- Stocking density (PL/m^2)	663		32.3(29.4)	1184		25.4(21.8)	0.000

Table 5. Continued.

Variable name	Control n	Control %	Control Mean (SD)	Case n	Case %	Case Mean (SD)	*P*-value*
Supply PL source (hatchery)							
- Small-sized hatchery	301	59.0		413	49.5		reference
- Medium-sized hatchery	24	4.71		98	11.8		0.000
- Hatchery A	22	4.3		49	5.9		0.070
- Hatchery B	40	7.8		131	15.7		0.000
- Hatchery C	52	10.2		88	10.6		0.271
- Hatchery D	71	13.9		55	6.6		0.000
Potential diseases carrier							
- Presence of wild animals in the pond							0.393
- absent	476	71.9		879	73.7		
- present	186	28.1		313	26.3		
- Crabs (absent)	545	82.7		1024	86.1		0.049
Crabs (present)	114	17.3		165	13.9		
- Finfish (absent)	621	94.5		1082	91.1		0.008
Finfish (present)	36	5.5		106	8.9		
- Wild shrimp (absent)	617	93.8		1090	91.8		0.117
Wild shrimp (present)	41	6.2		98	8.2		
Water quality							
- Morning pH	269		7.8 (0.4)	521		7.6 (0.3)	0.000
- Afternoon pH	232		8.1 (0.4)	465		8.1 (0.4)	0.022
- Salinity	189		16.8 (7.6)	252		14.0 (7.0)	0.000
Water management							
- Exchange or topping up of water during the first 35 DOC (or until the first signs of AHPND)							
- NO	629	93.6		1157	96.1		0.016
- YES	43	6.4		47	3.9		
- Aerator application (not used)	38	5.8		147	13.0		0.000
Aerator application (used)	619	94.2		984	87.0		
Environmental							
Any unusual climatic events during the first 35 DOC (or until the first signs of AHPND)							
- NO	293	59.1		423	40.8		0.000
- YES	203	20.9		615	59.2		

Note: *factors with *P*-value of less than or equal to 0.2 were included in the multivariable analysis.

Pond-level data analysis

Descriptive statistics and unconditional associations for each factor

Sixty four percent of the study ponds had experienced an AHPND outbreak. The prevalence of AHPND in ponds in Dam Doi (Ca Mau), Hoa Binh (Bac Lieu), Tran De (Soc Trang) and Vinh Chau (Soc Trinang) was 45.0, 44.6, 79.5 and 94.6 %, respectively. Pond which culture *P. monodon* and *P. vannamei* were 87.4 and 12.6 %, respectively. PL was supplied from 222 hatcheries with the frequency of supply ranging from 1–173 ponds. Due to the many categories, it was not possible to analyzed this factor.

We overcame this problem by grouping this factor by frequency of supply, so hatcheries that supplied PL to less than 20 ponds were grouped as small-sized hatcheries and used as the reference group for logistic regression analysis, those hatcheries that supplied PL for 20–30 ponds were classified as medium-sized hatcheries, while those hatcheries that supplied PL to more than 30 ponds were not grouped. The results from the unconditional association analysis are presented in Table 5.

Table 6. Final multivariable logistic regression model for factors associated with AHPND occurrence in shrimp ponds.

	OR	SE	Z	*P*-value	95 % CI
Pond characteristic					
- Pond depth (0 if >= 120 cm; 1 if < 120 cm)	1.6	0.33	2.48	0.013	(1.1, 2.4)
Water treatment					
- Fertilizers	1.4	0.24	2.20	0.028	(1.0, 2.0)
- Mineral application	0.5	0.16	-2.18	0.029	(0.3, 0.9)
- Algaecide application	0.6	0.12	-2.68	0.007	(0.4, 0.9)
- Probiotic application	1.5	0.33	2.01	0.044	(1.0, 2.3)
Environmental					
- Any unusual climatic events during the first 35 DOC (or until the first signs of AHPND)	1.9	0.31	3.84	0.000	(1.4, 2.6)
Postlarval (PL) management					
- Source of PL (hatchery)					
- Small-sized hatchery			Reference		
- Medium-sized hatchery	1.6	0.59	1.20	0.230	(0.8, 3.3)
- Hatchery A	1.9	0.65	1.86	0.063	(1.0, 3.7)
- Hatchery B	1.0	0.30	0.03	0.979	(0.6, 1.8)
- Hatchery C	0.9	0.25	- 0.34	0.733	(0.5, 1.6)
- Hatchery D	1.8	0.50	2.02	0.044	(1.0, 3.1)
District					
DD			Reference		
HB	0.5	0.11	-3.14	0.002	(0.3, 0.8)
TD	5.3	2.20	4.05	0.000	(2.8, 12.0)
VC	14.4	5.74	6.71	0.000	(6.6, 31.5)
Constant	1.0	0.33	0.02	0.982	(0.5, 1.9)

Note: OR = odds ratio, SE = standard error, Z = standard normal deviate, 95 % CI = 95 % confidence interval

Multivariable logistic regression model

Water quality data could not be included in the multivariable logistic regression analysis due to a lot of missing data, ranging from 58.0–99.9 % of records. The pond depth factor was transformed into categorized variables with cut-off at ≤120 cm. and 120 cm. prior to being included in the regression model. The final multivariable logistic regression model of factors associated with AHPND cases in shrimp ponds in the Mekong Delta area of Viet Nam after controlling for the confounding effect of districts and farms is presented in Table 6. The prevalence of AHPND varied among the districts, with the highest prevalence in Vinh Chu District. The factors related to increased risk of AHPND occurring were the following: ponds with water depth equal to 1.2 m or below, ponds which experienced abnormal weather events

during the first 35 DOC or until the first signs of AHPND, and ponds that used fertilizers and probiotics for water treatment.

Moreover, ponds that were supplied with PL from a specific hatchery (i.e. hatchery D) were more likely to have outbreaks of AHPND than ponds supplied from the other hatcheries. On the other hand, ponds that were supplied with minerals and algaecides for water treatment were shown have a reduced risk of AHPND occurring. No significant, biologically plausible interactions were detected.

Discussion

Farm characteristics and farm management

Every hectare increase in culture area of the farm increased the risk of AHPND occurrence by 1.32 times. The larger culture area might relate to more difficulty in pond management and reduced cost-effectiveness of using disinfectants to prevent the entry of the pathogen into the farm. This finding was similar to that of Corsin (2012) during an investigation of an unknown disease outbreak (now known later as AHPND) in Viet Nam in 2012. Farms being located close to other farms using the same AHPND-infected water source increased the risk by 5.07 times, and this was the most important farm-level risk factor. The same finding was reported in a similar study investigating an outbreak of AHPND in eastern Thailand (Kasornchandra et al. 2014; Boonyawiwat et al. 2017).

These findings indicate that the transmission of AHPND occurs via water. Pond preparation through sediment drying was associated with an increased risk of AHPND when compared to other pond preparation methods (such as flushing, ploughing or soil removal). This finding differed from the results of the study by Corsin (2012), also in Viet Nam, which the first time of his study indicated that washing the pond during pond preparation increased the risk of AHPND occurring. During the AHPND outbreak, shrimp farmers reduced their economic losses by delaying their crop operation. Leaving the pond empty and disinfecting the pond bottom by exposure to sunlight are some of the most cost-effective and convenient measures in this situation. About 92.7 % of study farms used these methods for cleaning their ponds. Such methods might be good for degradation of remaining organic matter in pond soil, but the depth of the degradation process is limited by the level of oxygen in the soil.

Usually, a high degree of organic matter degradation occurs only to a depth of 10–15 cm below the bottom surface, depending on type of soil. The organic matter under that level may remain and be a problem after adding water or during the crop operation. Sun-drying of the pond bottom may have eliminated all the microbes (Austin and Austin 1999) but this method created unequilibium of microbial community and a consequent lack of competition. After filling pond with water the fast-growing bacteria (such as many pathogenic *Vibrio* spp.) will have a good opportunity to have access to the rich nutrients and growth without competing with other microbes (Lavilla-Pitogo et al. 1998; Schryver et al. 2014). Therefore, the *Vibrio* spp. will dominate and recolonizing the environment. (Attramadal et al. 2015).

Pond management

Ponds with a water depth of 1.2 m. or below have a higher risk of AHPND due to the rapid change of water quality (e.g. water temperature, salinity, alkalinity, etc.), especially during periods of abnormal weather events. However, their interaction effect was not found to be significant, although a higher proportion of AHPND cases during unusual weather occurred in ponds with low water level (Fig. 2).

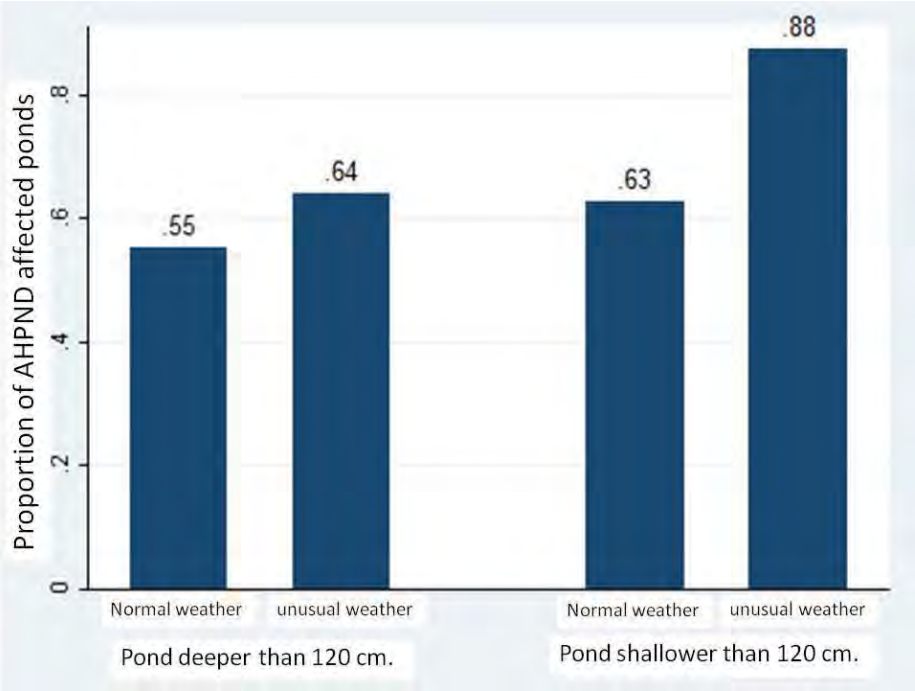

Fig. 2. Proportions of AHPND cases to total assessed ponds for shrimp ponds when unusual weather occurred with different pond water level.

Postlarval (PL) shrimp

Previous risk factors studies conducted in Viet Nam (Corsin 2012) and Thailand (Boonyawiwat et al. 2017) have found that source of PL is associated with an increased risk of AHPND outbreak at the pond level. Such findings suggest that the occurrence of AHPND in grow-out ponds may be related to stocking of infected PL (particularly PL infected with *Vibrio parahaemolyticus)* from hatchery and nursery facilities. Bacteria could be introduced to the facilities via various pathways, for example, through infected nauplius, contaminated water or feed, or water pipelines contaminated with bacteria. Moreover, bacteria could rapidly multiply when inappropriate management conditions occur in hatchery operations (i.e. high PL density, high organic load and high temperature). Disinfection water prior to use is a common practice in this stage of shrimp culture, but it lead to increase nutrient availability, reduced microbial number, lack of competition and predation induced the recolonization by heterotrophic bacteria (*r*-strategists) (MacArthur and Wilson 1967; Hess-Erga et al. 2010). Therefore, the numbers of bacteria in the water have been noted to increase after a few days post-treatment. Such bacterial loads may result from the growth of bacteria present in the water pipelines.

As a survival mechanism, bacteria belonging to the family Vibrionaciae can form biofilms when in a harmful environment, such as when in contact with a disinfectant (Gode-Potratz and McCarter 2011). The increase in the number of bacteria in hatchery and nursery facilities makes it possible for PL to become infected through these contaminated sources.

Water management

Water management through fertilization to promote phytoplankton growth is a common process in grow-out farms. However, the use of organic fertilizers (e.g. chicken manure) may promote the growth of harmful bacteria in the shrimp pond. In addition, applying a large amount of fertilizer to the pond may promote an increase in phytoplankton, increased water pH, a wide range of daily pH fluctuation, and high nitrogen content. *Vibrio parahaemolyticus* is an enteric bacterium that prefers high pH and high levels of nitrogen. Adding an algaecide may reduce phytoplankton numbers, changing the water to a suboptimal condition for *Vibrio* spp. Shrimp need to absorb minerals (i.e. calcium, magnesium and potassium) from water to meet their requirements for growth. Thus adding minerals to the pond is another way to support their health status and promote normal growth performance.

This study also found that the use of probiotics is another risk factor for AHPND outbreak. This is in agreement with the study done by Corsin (2012), but did not support suggestions that why the application of probiotics has a positive impact on the survival of shrimp grown in AHPND-affected areas (Panakorn 2012; Schryver et al. 2014). Probiotic technology can change microbial species composition in shrimp ponds by adding selected bacterial species that displace deleterious normal bacteria. While, probiotic bacteria may do well in terms of competition with ubiquitous or opportunistic bacteria. But the application of recent commercial strain of probiotics gave uncertain results with regard to Vp_{AHPND} infection. Therefore, the studies to find a specific bacterial strain that can effectively control Vp_{AHPND} bacteria were need, including the dose and frequency of application to prove its efficacy.

Measures to control disease spread have been widely implemented on shrimp farms in Thailand, including the improvement of on-farm and shrimp health management practices. However, the effectiveness of applying probiotics, traditional herbal medicines and molasses has not been evaluated (FAO 2013b). Simultaneously, a number of research collaborations have initiated to control the occurrence of AHPND on shrimp farms and to identify risk factors associated with AHPND cases (FAO 2013).

A preliminary study of AHPND conducted in eastern Thailand (Kasornchandra 2014) has suggested that the source of PL shrimp, the total amount of feed used before the disease event, and water disinfection with chlorine are associated with AHPND occurrence on shrimp farms. While the findings have initiated discussion around farm management practices, there has yet to be a comprehensive study to identify management practices that may affect the risk of AHPND on shrimp farms. A better understanding of farm management practices related to disease would inform AHPND policies on shrimp farms, control strategies, and risk management plans.

Conflict of interest

There were no known conflicts of interest**.**

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Mortality Outbreaks in Whiteleg Shrimp (*Penaeus vannamei* Boone 1931) Cultured in Peninsular Malaysia

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Abstract

The whiteleg shrimp (*Penaeus vannamei* Boone 1931) was introduced for farming in Malaysia in early 2002. In 2009, reports of early mortality syndrome (EMS) were noted in the People's Republic of China and Viet Nam. One form of EMS, acute hepatopancreatic necrosis disease (AHPND), has now spread to several shrimp-growing countries in Asia. In 2011, Malaysia recorded a mortality outbreak that prompted an investigation of 20 farms where 204 moribund shrimp samples were analyzed. On average, 64 % of the affected shrimp showed haemolymph clotting time longer than 1.5 min, and 80 % had pale hepatopancreas, soft body and empty gut. Multiple bacterial infections, particularly *Vibrio* spp. and *Photobacterium damsela,* were isolated from the haemolymph and hepatopancreas of affected shrimp. *Vibrio parahaemolyticus* was detected positive for the *tox*R gene.

Histopathology showed massive sloughing of the epithelial cells of the hepatopancreatic tubules and multifocal septic and melanized hepatopancreatic tubules that were encapsulated by haemocytes. Tests by polymerase chain reaction (PCR) were negative for infectious myonecrosis virus (IMNV) (0/110) and only a low prevalence (7/196) of infectious hypodermal and haematopoietic necrosis virus (IHHNV) was recorded. Infected shrimp also tested positive for paralytic shellfish poison (PSP) (24/24), and rearing water samples showed ammonium, nitrate, sulfide and iron levels above the optimal range for culture purposes.

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In 2012, samples were detected positive for EMS/AHPND using the IQ2000 Ems2 detection kit. The findings from this investigation showed that shrimp had multiple bacterial infections and pathological changes consistent with AHPND; some affected shrimp were positive for IHHNV and PSP toxin. These findings support the conclusion that mortalities were due to EMS/AHPND.

Keywords: acute hepatopancreatic necrosis disease, bacteria, early mortality syndrome, Malaysia, *Penaeus vannamei,* shrimp

Introduction

The shrimp culture industry in Malaysia has inevitably suffered major epizootics due to viral infections. In 1996, white-spot syndrome virus (WSSV) affected many farms culturing giant tiger prawn (*Penaeus monodon* Fabricius 1798) in northern Peninsular Malaysia, crippling the industry. The operators were forced to shift to fish culture or, in some cases, cease operation. In 1999, specific pathogen free (SPF) whiteleg shrimp (*P. vannamei* Boone 1931) were introduced, and this encouraged most farmers in Southeast Asia to abandon giant tiger prawn culture in favour of whiteleg shrimp. Malaysia was no different, and whiteleg shrimp was introduced in early 2002 (Briggs et al. 2004). After a few cycles of cultivating whiteleg shrimp, production was reported to have increased significantly; the production of whiteleg shrimp in 2005 was 11 497 tonnes, which increased to 18 601 tonnes in 2006, exceeding that of giant tiger prawn (DOF 2005, 2006). In 2010, total production of whiteleg shrimp reached 69 084 tonnes, 50-fold more than giant tiger prawn, indicating that whiteleg shrimp is a better species to culture (DOF 2010). *Penaeus vannamei* was seen to have faster growth rate, was perceived to have better tolerance to ammonia and nitrite toxicity, and showed higher survival. Generally, three to four crops a year could be produced, as each crop requires only 80–90 days.

Penaeus vannamei is non-indigenous to Asia, and concern about negative impacts such as the introduction of Taura syndrome virus (TSV), infectious myonecrosis virus (IMNV) and infectious hypodermal and haematopoietic necrosis virus (IHHNV) accompanied its introduction. TSV, IMNV and IHHNV are diseases listed by the World Organisation for Animal Health (OIE). TSV and IMNV have caused high mortalities compared to IHHNV (Lightner 1996) and may pose a risk to culture sites and the local shrimp industry. Following the availability of SPF *P. vannamei* postlarvae (PL) and the ability to adapt to a wide range of salinity (0.5 to 28 ppt), culture of whiteleg shrimp spread rapidly (Pan et al. 2007). Unfortunately, both IMNV and TSV have been detected and have caused mass mortality in cultivated *P. vannamei* in Indonesia (Taukhid and Nur'aini 2009). In 2006, Indonesia was the first country in the Asia-Pacific to report mass mortality of *P. vannamei* because of IMNV, the gross signs of which include white necrotic areas or reddening in the muscle of the distal abdominal segments and the tail fan.

After viral disease outbreaks, farms in Asia observed rapid mortalities of *P. vannamei* in the first 30 days of culture. Initially called early mortality syndrome (EMS), affected shrimp were lethargic, anorexic and showed severe damage in the hepatopancreas (Lightner et al. 2012). EMS was first detected in cultivated shrimp in the southern part of the People`s Republic of China in 2009. Slow mortality occurred during the early days of culturing (20–30 days after stocking), and mortality could reach 100 %. In April 2011, farms in Viet Nam experienced 65 to 90 % mortality of *P. vannamei* during the first 45 to 50 days following stocking. Similar scenarios were seen in Thailand in 2012, but mortalities there started at 15 days post-stocking and lasted until 40 days of culture. Sirikharin et al. (2015) reported that a unique strain of the bacterium *Vibrio parahaemolyticus* capable of producing soluble toxins has been identified as the causative agent of acute hepatopancreatic necrosis disease (AHPND). Histopathological observations revealed massive sloughing of the epithelial cells of the hepatopancreatic tupules as a result of the toxins released by this unique bacterial strain.

In 2011, farms culturing *P. vannamei* in the Malaysian states of Perak, Pahang and Penang reported problems similar to AHPND. During investigation, it was found that there were multiple bacterial infections which included *V. parahaemolyticus*. In addition, massive sloughing of the epithelial cells of the hepatopancreatic tubules was also seen in Perak, where 60 % mortality was reported in *P. vannamei* at 20 days of culture (DOC). The remaining stock survived for 50 days, but mortality had reached 90 % by then. In Pahang and Penang, slow mortality was observed at 30–60 DOC. The number of samples from shrimp disease outbreaks submitted to the National Fish Health Research Division (NaFisH) in Penang has been increasing since 2011. Hence, investigations were carried in the states of Perak, Pahang and Penang, northern Malaysia, to confirm the cause of mortalities and determine the factors associated with these outbreaks.[1]

Materials and Methods

Sources of Penaeus vannamei *and Gross Observations*

Two investigations (Phases I and II), with 2–3 months duration each were carried out at different periods in late 2011 and early 2012 (Table 1).

[1] During the period covered by this investigation (2011–2013), we confirmed disease outbreak due to AHPND based on our histological findings (massive sloughing of the hepatopancreatic tubule epithelial cells) and the presence of *V. parahaemolyticus*. Subsequently, in 2014, the samples were confirmed positive using the IQ 2000 ems 2 kit.

Table 1. Investigations carried out during the study period.

Phase	Period	Type of investigation
1	Nov – Dec 2011	Case history
		Gross observation
		Haemolymph clotting time
		Bacteriology
		Histopathology
2	Jan – Mar 2012	Virology (infectious myonecrosis virus (IMNV), Penaeus vannamei nodavirus (PvNv) & infectious hypodermal and haematopoietic necrosis virus (IHHNV)
		Cross-sectional study on the chemical parameters of water quality with special reference to day of culture (DOC) and un-ionized ammonia (NH_3)
		Detection of paralytic shellfish poison (PSP) by enzyme-linked immunosorbent assay (ELISA)

Table 2. Sample information collected from Perak, Pahang and Penang.

Location	DOC upon sampling[1]	Mortality period (DOC)	Source of PL/health status	Survival rate (%)	Total production (tonnes)
Perak					
Farm 1	40	27 & 40	Hatchery 1/unknown	7.0	1.7
	43	30	Hatchery 2/unknown	37.0	4.4
Farm 2	47	20 & 47	Hatchery 3/SPF	10.0	0.4
	48	20	Hatchery 3/SPF	49.0	3.9
Farm 3	40	unknown	Hatchery 4/SPF	36.0	6.6
Farm 4	33	30	Hatchery 5/unknown	29.0	4.4
Pahang					
Farm 1	52	unknown	Hatchery 4/SPF	44.0	3.0
	56	unknown	Hatchery 4/SPF	44.0	3.0
	56	unknown	Hatchery 4/SPF	27.0	3.0
Farm 2	65	45	Hatchery 3/SPF	83.0	8.0
	48	unknown	Hatchery 3/SPF	25.0	2.0
	54	36	Hatchery 4/SPF	14.0	0.5
	52	31	Hatchery 4/SPF	34.0	2.0
Farm 3	84	40	Hatchery 4/SPF	94.0	6.0
	85	40	Hatchery 4/SPF	33.0	2.0
	89	40	Hatchery 4/SPF	24.0	2.0
	89	40	Hatchery 4/SPF	27.0	3.0
Penang					
Hatchery 1	unknown	unknown	Penang	unknown	unknown
Farm 1	50	48	Hatchery 1	unknown	unknown
	50	48	Hatchery 1	unknown	unknown

[1]Abbreviations: DOC = day of culture, PL = postlarvae, SPF = specific pathogen free.

A total of 204 moribund *P. vannamei* samples from 20 farms were collected from the three states in northern Malaysia and processed for haemolymph clotting time, bacteriology, virology and histopathology after gross observations were recorded (Table 2; Fig. 1).

Fig. 1. Map of Peninsular Malaysia showing the three states (*) from which samples were collected during the current study.

Water Quality

The physical parameters of the pond water (i.e. temperature, pH, salinity and dissolved oxygen) were taken *in situ* using a YSI portable meter, while the chemical parameters (i.e. ammonium, nitrate, sulfide and iron content) were analyzed in the laboratory by transporting the samples in a cool box with ice. Ammonium and nitrite were measured using Nessler and diazotization methods, respectively. Other chemical parameters were determined by using reagent kits and read by the Hach spectrophotometer 8038.

Haemolymph Clotting Time

Approximately 0.1–0.2 mL of haemolymph from each shrimp was withdrawn using a 1 mL sterile syringe. Samples were immediately dispensed in drops on a clean slide for observation of haemolymph clotting time. A clotting time of between 1 and 1.5 min was considered normal, while a clotting time higher than 1.5 min was considered as abnormal. Five to ten shrimp from each pond were tested.

Bacteriology

A drop of shrimp haemolymph was inoculated on trypticase soy agar (TSA) plates (Oxoid Ltd., England) and dominant bacterial colonies were subcultured on TSA to obtain pure bacterial isolates. Gram-staining of purified isolates was done and Gram-negative bacteria were subjected to presumptive classification test using *Vibrio* selective medium (TCBS, Merck, Germany), oxidation-fermentation test (OF), vibriostat 0/129 reaction (Oxoid Ltd., England), oxidase reaction using detection paper (Premier Diagnostics Ltd., Malaysia) and motility. Identification of isolates was done using the API 20E and 20NE (BioMerieux, France) identification strips, and bacterial profiles were determined using APIWEB software (BioMerieux, France) and the methods described by Holt et al. (1993). For identification of Gram-positive bacteria, pure cultures of were subjected to a catalase enzyme test using H_2O_2 as a substrate to differentiate between the *Staphylococcus* and the *Streptococcus* group. API 20 STAPH and API 20 STREP identification strips (BioMerieux, France) were inoculated, and bacterial profile was similarly determined by APIWEB software and the methods described by Holt et al. (1993).

Histopathology

Histology was done following Bell and Lightner (1988). A total of 85 live juvenile and adult *P. vannamei* were injected with Davidson's fixative and processed by an automatic tissue processor (Leica ASP 300) following standard procedures. Paraffin sections were affixed on slides and stained with haematoxylin and eosin (H&E).

Molecular Biology

The samples were tested for possible association with viruses known to infect *P. vannamei* in farms. Detection of IHHNV and EMS/AHPND infections was conducted by polymerase chain reaction (PCR) using the IQ2000 kit protocol, while testing for the presence of IMNV and PvVN was performed using the IQ Plus and IQ Real quantitative system distributed by Farming IntelliGene Tech. Corp., respectively. The samples were also tested for *toxR* gene according to Kim et al. (1999).

Cross-Sectional Study on the Chemical Parameters of Water Quality with Special Reference to Culture Period and Un-ionized Ammonia (NH_3)

Five farms in Perak State with different culture periods were selected for a cross-sectional study. Water samples were taken from the ponds and transported inside a cooler box with ice to the laboratory. The parameters that were investigated during the study were ammonium, nitrite, nitrate, sulfide and iron.

Statistical Analysis

The data for physical and chemical parameters were analyzed using One-way ANOVA (SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.). When significant differences were found, the Tukey method for multiple comparisons of means was applied to identify the differences between parameters ($P < 0.05$).

Results

Mortality was observed in all farms in the states of Perak, Pahang and Penang that were visited and where shrimp samples were taken. Gross signs observed in surviving shrimp during sampling included black gill, white faeces, black spot/patches on the exoskeleton, white muscle, white tail/body, reddish body, soft body, yellow discolouration in the head, enlarged hepatopancreas and swimming at pond edges, as well as slow growth.

Shrimp Samples from Perak State

Gross observation of affected shrimp showed signs of poor feeding, swimming at pond edges, white faeces, black spot or patches on the exoskeleton, yellowish head, slow growth, white body/tail and reddish body. However, no black gills were observed among the affected shrimp from four of the farms. Approximately 90–100 % of the examined shrimp showed whitish patches in the abdominal segments, and 80 % had pale hepatopancreas, soft body and empty gut. All ponds had dead shrimp at 20 and 40–50 DOC except for Farm 3 from Sg. Limau, Perak. Seventeen of 20 tested shrimp showed haemolymph clotting time longer than 1.5 min, indicating that 85 % of the tested shrimp were under stress (Table 3).

Table 3. Percentage of haemolymph clotting time tested in affected *Penaeus vannamei* obtained from Perak, Pahang and Penang.

Location	Number of shrimp tested	No. shrimp with haemolymph clotting time exceeding 1.5 min	Percentage of stressed shrimp
Perak	20	17	85
Pahang	30	15	50
Penang	22	14	63

Vibrio spp. and *Photobacterium damsela* were isolated from the haemolymph of the affected shrimp samples from three of the four sites in Perak. Early and terminal phases of EMS pathology were observed in the hepatopancreas and muscle of shrimp from all three sites (Table 4).

The proximal part of the hepatopancreas lacked B, F and R cells, and showed sloughing and necrosis of hepatopancreatic cells (Fig. 2). Multifocal septic and melanized hepatopancreatic tubules with haemocyte encapsulation were also seen in some of the specimens (Fig. 3A). Focal acute necrosis with no obvious agent associated with the lesions was seen in the muscle (Fig. 3B). Water quality parameters such as temperature, pH, salinity and dissolved oxygen were within acceptable ranges for marine shrimp culture (Table 5). However, ammonium, nitrate, sulfide and iron exceeded the normal range recommended for shrimp culture. A cross-sectional study on different days of culture from one farm and five farms showed high ammonium levels on a different DOC (Fig. 4; Table 6). A similar scenario of high levels of ammonium and nitrite also occurred at less than 25 DOC (Fig. 5).

Table 4. Diagnostic results in affected *Penaeus vannamei* obtained from Perak, Pahang and Penang.

Location	Bacteriology	Molecular biology (PCR)[1]	Pathology (%)
Perak	*Vibrio* spp.		Early & terminal stage of EMS (100)
	Vibrio parahaemolyticus	7/64 +ve IHHNV	
		3/3 +ve *tox*R	
	Photobacterium damselae	3/3 +ve EMS/AHPND	
Pahang	*Vibrio* spp.	0/110 +ve IHHNV, PvNv & IMNV	Early & terminal stage of EMS (100)
Penang	Nil	0/22 +ve IHHNV	Early & terminal stage of EMS (100)

[1]Abbreviations: AHPND = acute hepatopancreatic necrosis disease, EMS = early mortality syndrome, IHHNV = infectious hypodermal and haematopoietic necrosis virus, IMNV = infectious myonecrosis virus, PCR = polymerase chain reaction, *tox*R = toxin operon gene.

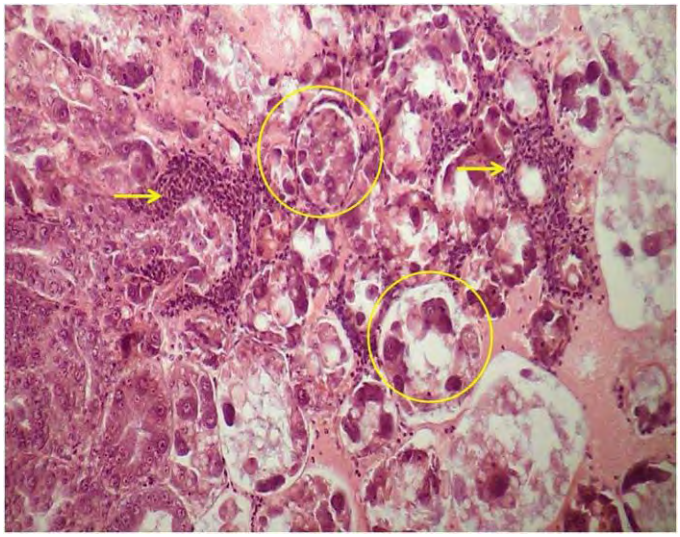


Fig. 2. Proximal hepatopancreas with no B, F or R cells, sloughing (in circle) and necrosis of hepatopancreatic cells with some haemocyte-encapsulated necrotic tubules (arrows).

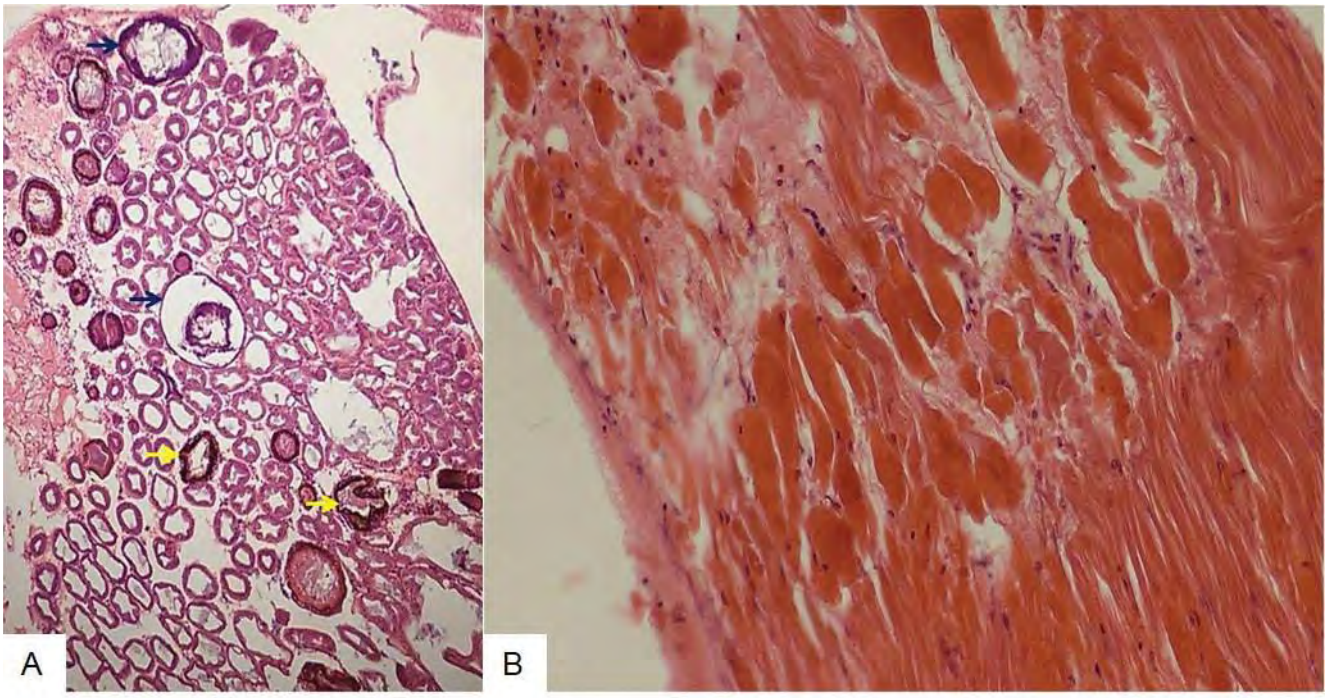
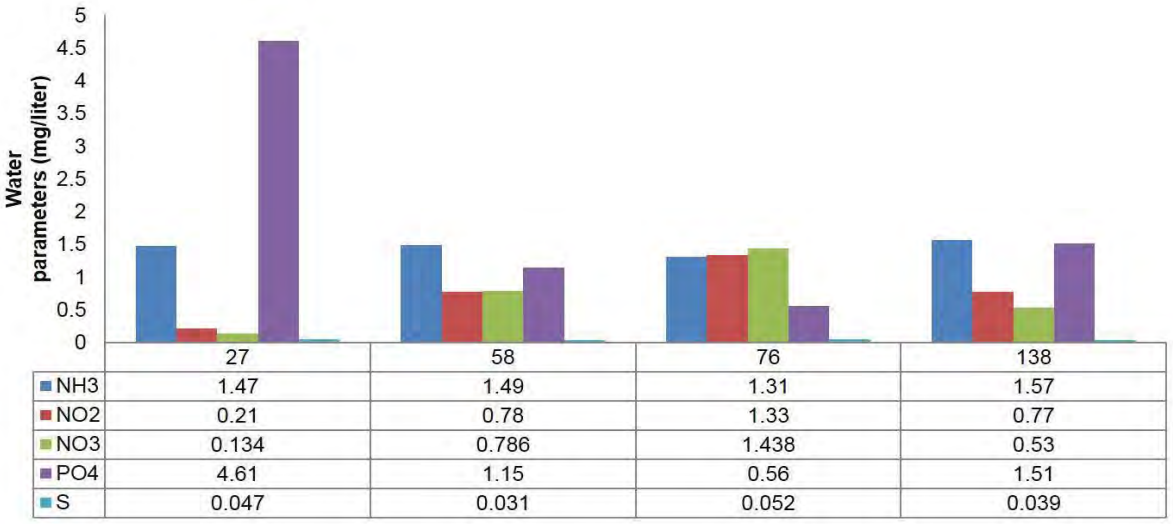


Fig. 3. (A). Hepatopancreatic cells with multifocal septic (black arrow) and melanized hepatopancreatic tubules with haemocyte encapsulation (yellow arrows) and (B). A focal acute necrosis with no obvious agent associated with the lesions in muscle.

Table 5. Water quality parameters in three farms in Perak compared with optimal water quality for shrimp culture.

Water Quality	Perak			Optimal water quality for shrimp culture
	Farm 1	Farm 2	Farm 3	
Temperature (°C)	31.0 – 31.1	31.7	–	25 – 30
Dissolved oxygen (mg.L^{-1})	8.7 – 9.6	8.5	3.5 – 5.5	> 4
pH	7.9 – 8.5	7.7	7.7 – 7.9	7.5 – 8.5
Salinity (ppt)	18.0 – 19.0	21.0	20.0 – 30.0	10.0 – 25.0
Nitrite (mg.L^{-1})	0.02 – 0.05	0.0	0.11	< 1.0
Un-ionized ammonia (mg.L^{-1})	3.9 – 5.0	4.8	3.65	< 0.1
Iron (mg.L^{-1})	0.3 – 1.0	0.7	0.5	–
Un-ionized hydrogen sulfide (mg.L^{-1})	29.0 – 52.0	65.0	33	< 0.005

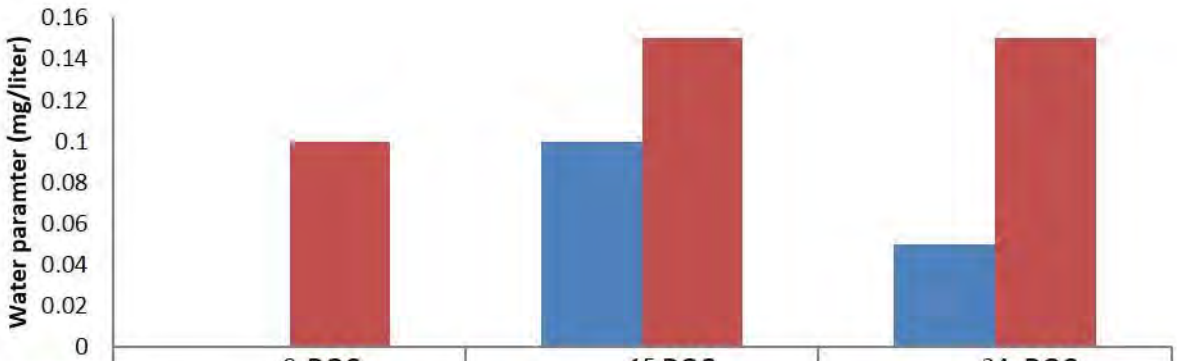


	27	58	76	138
NH3	1.47	1.49	1.31	1.57
NO2	0.21	0.78	1.33	0.77
NO3	0.134	0.786	1.438	0.53
PO4	4.61	1.15	0.56	1.51
S	0.047	0.031	0.052	0.039

Fig. 4. Cross-sectional study on chemical parameters of water quality in different day of culture (DOC) in one farm in Perak.

Table 6. Cross-sectional study on chemical parameters of water quality in different days of culture (DOC) in five farms in Perak.

Day of culture (DOC)	Farm	Pond	Water quality parameters (ppm)				
			Ammonia	Nitrate	Nitrite	Phosphate	Sulfide
32	1	B4	1.27	0.9	0.58	1.83	37
33	2	AA	2.06	0.41	2.96	1.88	40
42	3	2	1.14	0.27	0.19	1.46	39
58	4	A3	1.2	1.58	1.46	0.16	53
68	5	B3	1.31	0.08	0.04	1.32	70



	8 DOC	15 DOC	24 DOC
NH3	0	0.1	0.05
NO2	0.1	0.15	0.15

Fig. 5. Chemical parameters of water quality in a pond cultivating *Penaeus vannamei* in Perak.

Shrimp Sampling in Pahang and Penang States

Samples from Pahang and Penang were processed similar to samples from Perak. All shrimp samples (100 %) showed whitish patches in the abdominal segments, and 80 % had pale hepatopancreas, soft body and empty gut. All samples came from ponds with a history of mortality at 50 DOC except those from the hatchery at Balik Pulau, Penang. Haemolymph clotting time in 50 and 60 % of the shrimp from Pahang and Penang, respectively, exceeded 1.5 min, showing that they were under stress (Table 3). No farm tested positive for IHHNV infection. Pahang samples showed bacterial infection (*Vibrio* spp. and *Photobacterium damsela*). Samples from Pahang and Penang showed similar pathology in the hepatopancreas typical of developing EMS. However, in samples from Pahang, haemocytic infiltration and necrosis in the abdominal muscles were observed. This pathology is similar to lesions observed in IMNV or *Penaeus vannamei* nodavirus (PvNv). However, tests by IQ Plus for IMNV and PvNvin tissues obtained from live samples were negative (Table 4).

Discussion

The haemolymph clotting time test indicated 85, 50 and 64 % of the sampled shrimp were under stress in Perak, Pahang and Penang, respectively. Such stress could be triggered by disease occurrence, drastic changes in water quality parameters, poor diet or improper management. All of these factors, especially poor water quality, were present in all the investigated ponds. Stress due to consistent exposure to a high ammonium level during culture could be a contributing factor. Salinity, pH and dissolved oxygen (DO) levels were within acceptable ranges for shrimp culture, except for ammonium, nitrite and phosphate (Cheng et al. 2003). Throughout the cross-sectional study, the mean of ammonium concentrations ranged from 1.2–2.0 ppm, levels that were higher than the optimal range for cultured shrimp. Chien (1992) reported that ammonia is toxic to shrimp at high concentration, and Kasnir et al. (2014) highlighted that most shrimp can tolerate ammonia at a concentration of <0.1 $mg.L^{-1}$ NH_3–N. Data from one farm showed that at 27 DOC, rearing water for smaller shrimp has lower ammonium levels compared to that for larger shrimp at 58, 76 or 138 DOC, respectively. In another study, data from five farms registered higher ammonia levels ranging from 1.2–2.0 ppm, irrespective of the DOC at 32, 33, 42, 58 and 68. The increase in NH_4^+-N concentrations at certain times over the cultivation period could be due to the increased size of shrimp and the feeding rates (Guerrero-Galván et al. 1999).

At shorter DOC or smaller size, shrimp exposed to higher ammonium levels may not be able to tolerate such stress, which could reduce their immunity to infection. We believe that larger shrimp or those cultured for a longer period (i.e. > 40 DOC) are able to cope well compared with those at a shorter DOC (i.e. < 40). Prolonged exposure to stress due to a high concentration of ammonium could inhibit shrimp growth, as it could cause deterioration of the hepatopancreas and subsequently lead to increased susceptibility to EMS or AHPND.

Besides being associated with prolonged stress due to high concentrations of ammonium, EMS also could have another toxic etiology. Lightner et al. (2012) highlighted that degenerative pathology of the hepatopancreas is frequently a result of toxin. However, laboratory experiments conducted by Lightner et al. (2012) on crustacide and commercial feeds did not produce a consistent result similar to EMS pathology. The present study showed the presence of PSP toxin in infected shrimp; however, the concentration of toxin was lower than the human lethal dose of 2 mg (Hwang et al. 1992). The presence of PSP toxin in organs indicates that the affected shrimp ingested the toxin through food web transfer. The possibility of the toxin being present in commercial feeds, in common bacteria in the environment or in plankton could thus not be ruled out. In another study, Furio et al. (2012) showed that among several PSP-causative species of *Alexandrium*, *A. minutum* was found in low-salinity brackish environments in Viet Nam, Thailand, the Philippines and Malaysia. We believe that the presence of PSP toxin in EMS-affected shrimp could come the ingestion of diatoms, dinoflagellates or other micro-organisms in the water.

As the affected shrimp were under stress, we believe that they were more susceptible to all kinds of common pathogens, and particularly susceptible to multiple bacterial infections by members of the *Vibrio* group. Under these conditions, the shrimp were also exposed to other living organisms, including the dinoflagellate, which showed some PSP toxin. There is also the possibility that the toxin seen in affected shrimp showing acute pathology of the hepatopancreas could be protein based, as that toxin can be detected by the ELISA method used in the present study.

During the investigations conducted during Phases I and II (see Table 1), we observed that all samples tested for IMNV were negative, indicating that IMNV was not the cause of mortality despite the appearance of whitish abdominal muscles. According to the farm operators, most of the PL used originated from SPF broodstocks, and the affected shrimp tested negative for IMNV and TSV before being stocked into the ponds. Most of the samples also tested negative for IHHNV, with only seven of 64 samples from Perak being positive. IHHNV infection is known to cause "runt deformity syndrome", irregular and reduced growth, and cuticular deformities in *P. vannamei* (Kalagayan et al. 1991; Brock and Main 1994; Lightner 1996). During the investigation period, a single positive case of IHHNV in the affected shrimp would not have caused high mortality in cultivated *P. vannamei*. Vibriosis was recorded in samples from Perak and Pahang, but not in Penang. *Vibrio* species are part of the natural microflora of wild and cultured shrimp (Sinderman 1990) and become opportunistic pathogens when the natural defense mechanisms of shrimp are suppressed (Brock and Lightner 1990).

Mortalities due to vibriosis also occur when shrimp are stressed by factors such as poor water quality, crowding, high water temperature, low DO and low water exchange (Lewis 1973; Lightner and Lewis 1975; Brock and Lightner 1990). Vibrios are among the most important bacterial pathogens found in cultured shrimp, and they are responsible for a number of diseases

where mortalities may be up to 100 % (Jayasinghe et al. 2008). Shrimp-pathogenic vibrios are mainly *V. harveyi*, *V. fluvialis, V. parahaemolyticus*, *V. damsela* and *V. vulnificus* (Chythanya and Karunasagar 2002). The present study revealed that multiple bacterial infection consistently showed the presence the of *V. parahaemolyticus* in affected the shrimp.

Histopathological analysis showed a typical histopathology of AHPND in the hepatopancreatic tubules. Karyomegaly, sloughing of epithelial cells from hepatopancreatic tubules, multifocal septic tubules and melanized hepatopancreatic tubules with haemocyte encapsulation were seen in some specimens, suggesting the acute and terminal stages of AHPND. This provides evidence that the disease outbreaks in shrimp ponds in Perak, Pahang and Penang were due to AHPND.

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Recent Research on Acute Hepatopancreatic Necrosis Disease (AHPND) and *Enterocytozoon hepatopenaei* in Thailand

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Abstract

This review (up to 20 October 2016) covers two newly emerging, serious diseases in Asian shrimp aquaculture since 2009. The first (recognized in 2013) is acute hepatopancreatic necrosis disease (AHPND) caused by isolates of *Vibrio parahaemolyticus* (VP_{AHPND}) that carry a pVA plasmid containing genes for $PirA^{vp}$ and $PirB^{vp}$ toxins. The second is hepatopancreatic microsporidiosis (HPM) caused by *Enterocytozoon hepatopenaei* (EHP). AHPND causes high, early mortality, but prevalence may be overestimated if mistakenly equated with early mortality syndrome (EMS), a practice that is not problematic for farmers or the popular press, but is unacceptable for science. Progress on AHPND research in Thailand has focused on characterization of VP_{AHPND} isolates and development of molecular detection methods based their toxin proteins and respective genes. Additional work on AHPND outbreak ponds has revealed bacterial partners that have a potent synergistic effect on VP_{AHPND} virulence. Unlike AHPND, losses from HPM result from growth inhibition rather than mortality. However, this was not immediately recognized because growth inhibition from HPM is not easily detectable until the second or third month of cultivation, after the period of highest risk for AHPND. Work in Thailand has focused on the characterization and detection of EHP in broodstock and postlarvae (PL), and on development of laboratory infection models.

Keywords: acute hepatopancreatic necrosis, AHPND, EHP, EMS, *Enterocytozoon hepatopenaei,* HPM, Pir^{vp} toxins, shrimp disease, Thailand, *Vibrio parahaemolyticus*

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Introduction

Acute hepatopancreatic necrosis disease (AHPND) of shrimp is caused by unique isolates of *Vibrio parahaemolyticus* (VP_{AHPND}) (Tran et al. 2013) and is one of several diseases that can cause early mortality in shrimp rearing ponds. Thus, it is included under the heading "early mortality syndrome" (EMS) by shrimp farmers. This farmer practice can lead to confusion, if the term EMS is taken as equivalent to AHPND. For example, it can result in overestimation of the prevalence of AHPND outbreaks and underestimation or ignorance regarding early mortality caused by other agents or pathogens.

At least in scientific forums we must be aware of the distinction. The critical feature in diagnosing AHPND by histological analysis using the currently accepted case definition is the occurrence of massive sloughing of hepatopancreatic (HP) tubule epithelial cells in the absence of bacterial cells or other possible pathogens (Fig. 1). If this feature is not observed for at least one individual shrimp in a sample set, then the sampled population cannot be confirmed histologically as a case of AHPND.

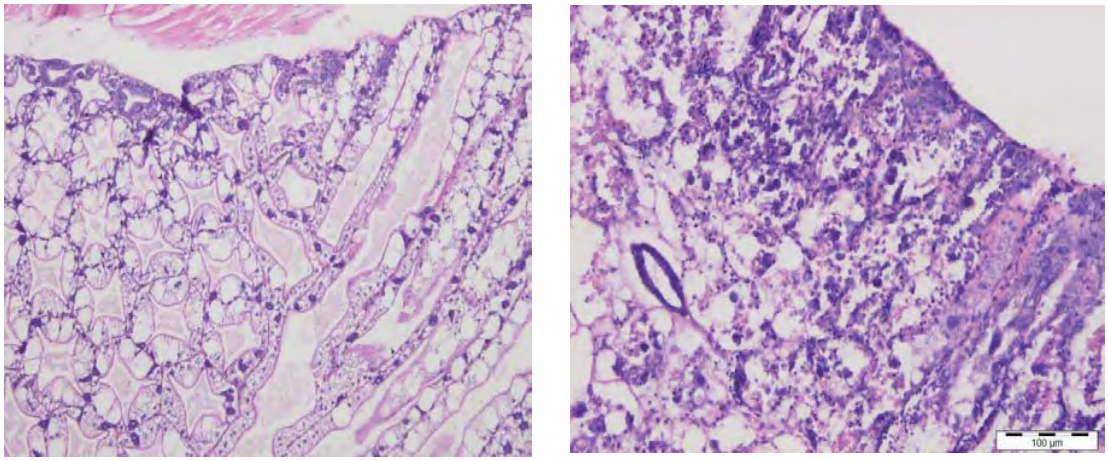

Fig. 1. Photomicrographs of normal histology of the shrimp hepatopancreas (left) compared to the pathognomonic lesion of AHPND (right) characterized by massive sloughing of tubule epithelial cells in the absence of bacteria or other pathogens.

Since the Food and Agriculture of the United Nations (FAO) meeting on EMS/AHPND in Panama in June 2015, the countries reporting the occurrence of AHPND outbreaks (Thitamadee et al. 2016) have changed only by the addition of Australia, where two AHPND outbreaks were reported from giant tiger prawn (*Penaeus monodon* Fabricius 1798) cultivated in Queensland. In the Australian report to the World Organisation for Animal Health (OIE) in early 2016, the bacterium isolated was identified as *V. harveyi* carrying the Pir^{vp} toxin genes, but possibly on the chromosome rather than in a pVA plasmid as reported for VP_{AHPND} isolates (Lee et al. 2015). However, there are rumours that outbreaks have also occurred but not been reported from India and from countries in Central America in addition to Mexico (Enríquez-Espinoza et al. 2016; Nunan et al. 2014; Soto-Rodriguez et al. 2015).

There is also one published report of a *Vibrio harveyi* (VH) isolate that causes AHPND and carries a pVA plasmid containing the $PirA^{vp}$ and $PirB^{vp}$ binary toxin genes (Kondo et al. 2015), possibly as a result of horizontal gene transfer from a VP_{AHPND} isolate. This suggests that additional isolates of VH and perhaps isolates of other *Vibrio* species or even species of other bacterial genera may eventually be found to carry this plasmid and cause AHPND.

In addition, there are two reports from Thailand that describe presence of the pVA plasmid in many different serotypes of VP from environmental samples (Chonsin et al. 2015; Kongrueng et al. 2015). These results suggest that pVA transfer among bacteria is relatively frequent. The review of work on hepatopancreatic microsporidiosis (HPM) caused by *Enterocytozoon hepatopenaei* (EHP) presented at the FAO meeting on EMS/AHPND in Panama in June 2015 cited reports of its occurrence in Thailand (Chayaburakul et al. 2004; Tangprasittipap et al. 2013; Thitamadee et al. 2016; Tourtip et al. 2009), the People's Republic of China *(*Liu et al. 2016) and Viet Nam (Ha et al. 2010), but it has now been reported also from India (Biju et al. 2016; Santhoshkumar et al. 2016; Rajendran et al. 2016) and Indonesia (Tang et al. 2016).

These and earlier reports of unidentified but morphologically similar hepatopancreatic microsporidians in *P. monodon* from Malaysia (Anderson et al. 1989; Baticados and Enriques 1989) and in *Penaeus japonicus* Spence Bate 1888 from Australia (Hudson et al. 2001) suggest that this pathogen is enzootic in the region and has been transmitted to imported, exotic stocks of *Penaeus vannamei* Boone 1931 that were derived from specific pathogen free (SPF) stocks known to be free of EHP.

Acute Hepatopancreatic Necrosis Disease (AHPND)

Variation in VP_{AHPND} Virulence

Recent work has shown variation in the virulence of VP_{AHPND} isolates for reasons as yet unknown (Joshi et al. 2014; Lai et al. 2015). It has been suggested that such variation may be related to differences in pVA copy number (e.g. 7 to 121 copies per cell) (Han et al. 2015) or to other VP_{AHPND} virulence factors that may or may not be carried by the pVA plasmid (Sirikharin et al. 2015). However, Dr Bruno Gomez-Gil (personal communication, 2016), whose group has sequenced full genomes of many VP_{AHPND} isolates has stated that the read frequencies in their next generation sequencing data for all of the isolates indicated only one pVA plasmid copy in each isolate.

There is evidence that there are other factors (e.g. toxins) that may act to potentiate the Pir^{vp} binary toxins or may kill shrimp directly without causing the pathognomonic histopathology characteristic of AHPND (see Fig. 1). Support for this proposal can be found in a newly characterized VP isolate from Viet Nam (unpublished). It contains a mutated $Pir^{vp}A/Pir^{vp}B$ gene region on its pVA plasmid and does not produce either of the Pir^{vp} toxins, but still causes 50 % shrimp mortality without characteristic AHPND histopathology. Instead, the moribund shrimp show collapsed HP tubule epithelia (like those in Fig. 2).

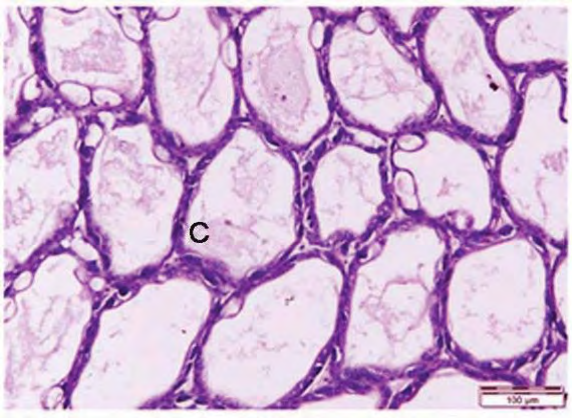

Fig. 2. Photomicrograph of a lesion resulting from shrimp challenged with a virulent VP_{AHPND} mutant that produces no Pir^{vp} toxins but still causes 50 % shrimp mortality. The lesions are characterized by collapsed HP tubule epithelia rather than the pathognomonic AHPND lesions shown in Fig. 1.

Similarly, VP isolate (2HP) obtained from an AHPND outbreak pond in Thailand also causes only 50 % shrimp mortality accompanied by lesions characterized by collapsed HP tubule epithelia (Joshi et al. 2014) similar to those seen in Fig. 2 for the Vietnamese VP_{AHPND} mutant. Thus, it is being further characterized to determine whether it is also a VP_{AHPND} mutant. At the same time, it is known that dilution of VP_{AHPND} isolate 5HP results in reduced shrimp mortality with the moribund shrimp also showing collapsed HP tubule epithelia (like those in Fig. 2) instead of pathognomonic AHPND lesions (Joshi et al. 2014).

Similar results are obtained when mixed, heterologously expressed $Pir^{vp}A$ and $Pir^{vp}B$ toxins are diluted in shrimp challenge tests (Sirikharin et al. 2015). More important, the concentration of the combined, heterologously expressed $Pir^{vp}A$ and $Pir^{vp}B$ toxins needed to cause AHPND (20 $\mu g.g^{-1}$ shrimp) was 20 times higher than the 1 $\mu g.g^{-1}$ of crude ammonium sulfate protein precipitate fraction obtained from the culture broth of the VP_{AHPND} isolate (5HP) used as the positive control (Sirikharin et al. 2015). Since the crude precipitate fraction contained many proteins, the proportion of the total 1 $\mu g.g^{-1}$ attritutable to $Pir^{vp}A$ and $Pir^{vp}B$ toxins would necessarily be much less than 1 $\mu g.g^{-1}$ each. All these examples support the proposal that other proteins produced by VP_{AHPND} potentiate the virulence of its $Pir^{vp}A/B$ toxins.

Obviously, it will be important to identify and characterize the other virulence factors that may act synergistically with $Pir^{vp}A$ and $Pir^{vp}B$ in isolates that cause AHPND. A number of putative toxin genes have been identified in the pVA plasmid (Han et al. 2015), and these would be prime candidates to study for possible synergistic activity when combined with the Pir^{vp} toxins. In addition, the description of pathology from AHPND bacteria should be expanded to include information indicating that low bacterial or Pir^{vp} toxin concentrations may lead to reduced mortality accompanied by collapsed HP tubule epithelia rather than massive sloughing of HP tubule epithelial cells seen with acute AHPND. In addition, we will need to decide whether or not VP isolates that carry mutant pVA and do not produce Pir^{vp} toxins but still kill shrimp should be included under the heading AHPND.

These examples add to the data reinforcing the need to stop using the term "early mortality syndrome" (EMS) interchangeably with AHPND. We already know that some proportion of early mortality in shrimp ponds is not the result of AHPND. For example, it may be caused by viruses such as white-spot syndrome virus (WSSV) and yellow head virus (YHV), by environmental factors (e.g. low dissolved oxygen, pesticides) or by bacterial pathogens other than those carrying an intact pVA plasmid. Thus, farmer reports of EMS cannot be equated with AHPND, and reports of AHPND must be considered suspect if they are not accompanied by confirmatory laboratory tests.

Bacterial Partners that Potentiate VP_{AHPND} Virulence

In addition to variation in virulence of AHPND bacteria themselves, it has been reported that some bacterial genera not previously associated with shrimp occur in a higher proportion in EMS ponds than in normal ponds (FAO 2013). Using this information as a starting point, a new species of bacterium from the Order Berkholderiales has been isolated from AHPND ponds in Thailand (unpublished). This bacterium (tentatively called *Delftia*-like) is lethal to shrimp with virulence equal to that of VP_{AHPND} isolate 5HP (i.e. 100 % mortality in three days at a concentration of 10^5 cfu.mL^{-1} in bath culture). However, the *Delftia*-like isolate does not cause AHPND histopathology but instead a unique histopathology of its own. This consists of vacuolated cells in the epithelium of the anterior midgut caecum (AMC**) (**Fig. 3) and sometimes also in other tissues. However, when the two isolates were mixed together in a bath challenge at 10^3 cfu each (i.e. 2 000 cfu total), 100 % mortality also occurred within three days. The 50 times lower cfu required to obtain 100 % mortality revealed a synergistic effect on virulence by mixed bath challenge. Furthermore, this mortality was not accompanied by pathognomonic AHPND histopathology and might not be diagnosed as a case of AHPND based on histological analysis of moribund shrimp in an outbreak of early mortality. It is also possible that the level of VP_{AHPND} in such a sample might be too low for detection by one-step polymerase chain reaction (PCR) analysis.

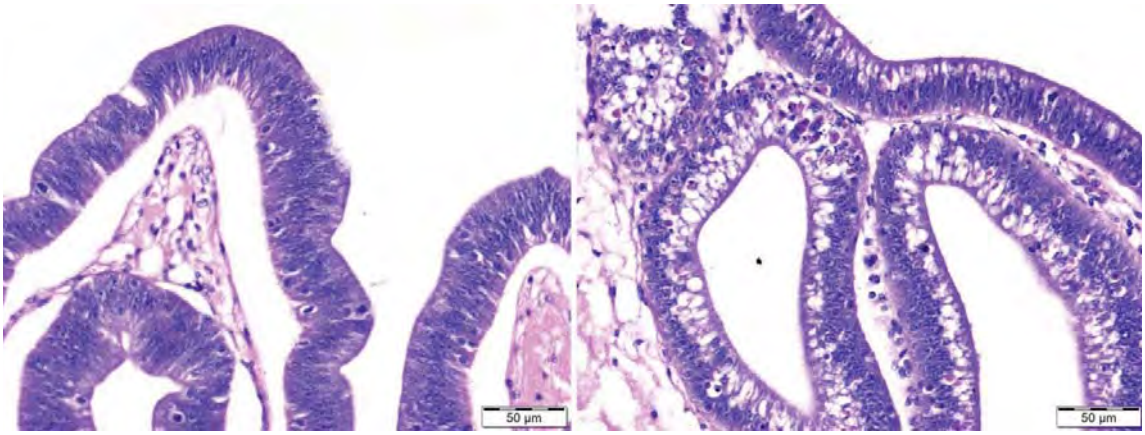

Fig. 3. Distinctive lesions found in moribund shrimp challenged by bath exposure to the *Delftia*-like bacterium. (A) Normal non-vacuolated epithelial cells of the anterior midgut caecum from negative control shrimp. (B) Abnormal vacuolated epithelial cells from a moribund shrimp specimen challenged with the *Delftia*-like bacterium.

Similar to the *Delftia*-like isolate, an isolate of *Shewanella* obtained from an AHPND pond in Thailand (unpublished) acts in a synergistic manner to increase the virulence of 5HP even though it is not itself virulent for shrimp. The result of the mixed challenge also differs in that it does cause pathognomonic AHPND histopathology, but together with a histological feature consisting of vacuolated E-cells in the HP tubule epithelium, similar to the vacuolated cells seen in the AMC with the *Delftia*-like isolate above (see Fig. 3). Thus, unlike the case of the *Delftia*-like/5HP mixture, histological analysis of moribund specimens infected with a *Shewanella*/VP_{AHPND} would be diagnosed correctly as a case of AHPND in any EMS outbreak. However, if an accompanying PCR test gave a negative result for VP_{AHPND} because of its low-level concentration, it might seem to contradict the histological diagnosis. Both the *Shewanella* and *Delftia*-like isolates lacked pVA and gave negative PCR test results for the $PirA^{vp}$ and $PirB^{vp}$ toxin genes. Thus, we suspect that they have their own virulence factors or virulence potentiaters that increase the virulence of AHPND bacteria. These factors also need to be identified. Whole genome information of the *Shewanella* isolate has recently been obtained and should assist in unravelling this phenomenon.

In summary, the pathology of AHPND and virulence of VP_{AHPND} isolates appear to be somewhat complex and many questions remain to be answered. The situation may be further complicated by the possibility of pVA plasmid transfer to other *Vibrio* species or even other genera of bacteria.

Hepatopancreatic Microsporidiosis (HPM)

Hepatopancreatic microsporidiosis (HPM) caused by *Enterocytozoon hepatopenaei* (EHP) is the other disease of major concern in Asia at this time. EHP differs from the only other microsporidian (*Agmasoma penaei*) (Flegel et al. 1992; Pasharawipas et al. 1994) earlier reported to infect *P. monodon, P. merquiensis* and *P. vannamei* (Laisutisan et al. 2009) in the region. However, unlike EHP, *A. penaei* does not infect shrimp tissues of endodermal origin such as cells of the HP tubule epithelium and cells of the midgut epithelium that are targeted by EHP. On rare occasions, severe infections of *A. penaei* may include expansion into connective tissue between the tubules of the hepatopancreas, but the tubule epithelial cells (endodermal origin) never become infected. In addition, *A. penaei* is not transmitted horizontally among shrimp. Instead, they are proposed, based on PCR testing, to be infected by spores originating from an alternative fish host (Pasharawipas and Flegel 1994).

The proposal is supported by successful control of the pathogen by elimination of suspected fish species from the shrimp culture system. HPM from Thailand was first reported in 2004 as an unidentified hepatopancreatic microsporidian in *P. monodon* that morphologically resembled microsporidians reported from *P. monodon* in Malaysia (Anderson et al. 1989; Baticados and Enriques 1989) and *P. japonicus* from Australia (Hudson et al. 2001). In Thailand, no correlation was found between this infection and shrimp growth at that time. It was later characterized and named as a new microsporidian species (*Enterocytozoon hepatopenaei*) (Tourtip et al. 2009).

Although it was subsequently found in shrimp exhibiting white faeces syndrome (WFS) in Thailand, it did not appear to be the direct cause of WFS (Tangprasittipap et al. 2013). However, there is evidence that HPM is associated with retarded shrimp growth that may not become clearly visible until the second or third month of cultivation. It has also been found that there is no apparent impact on growth if the copy number by quantitative PCR (qPCR) is not above 10^3 per ng DNA, after which the degree of retardation is directly proportional to the increase in copy number (Liu et al. 2016) and may result in progressive, slow mortality at numbers above 10^8 per ng DNA (Robins McIntosh, personal communication, 2016). The best way to determine EHP severity of infection is to determine its copy number using quantitative PCR (Liu et al. 2016). Estimation by counting infected HP cells containing EHP spores by normal light microscopy is not recommended for two reasons. First, the spores are very small and it is not easy to detect and confirm their presence by normal light microscopy because an oil emersion lens must be used (Fig. 4A-C). Second and more important is the fact that estimation from counting infected HP cells containing EHP spores is unreliable in determining the severity of infection because heavily infected specimens sometimes show few or no cells containing spores.

This has been revealed by staining tissue sections with haematoxylin & eosin (H&E) or chromotrope stain and comparing the results to adjacent sections assayed by *in situ* hybridization (Fig. 5). Thus, counting infected cells by presence of spores alone can lead to a great underestimation of the severity of infections. An additional revelation from *in situ* hybridization was that the distribution of EHP-infected cells in the HP can be very uneven, such that a small HP tissue sample taken from different portions of the same hepatopancreas may give opposite results (i.e. positive and negative). With qPCR, this problem must be avoided by homogenization of the whole HP followed by removal of a measured proportional sample, so that a qPCR infection index can be calculated for the whole HP of each sample. This would be particularly important for studies on the effect of infection level on shrimp growth and for comparison of infections between PL and juveniles or between small and large juveniles.

The high prevalence of EHP was not realized until an epidemiological study of EMS outbreaks was carried out in Thailand covering the period 19/08/2013 to 23/04/2014 (Boonyawiwat et al. 2016). The study included analysis of ten shrimp samples (sufficient to detect pond prevalence of EHP at 26 % or more from each of 196 shrimp cultivation ponds randomly selected prior to shrimp stocking. The results revealed an unexpected overall pond prevalence of 119/196 (61 %) that prompted an immediate warning (Sritunyalucksana et al. 2015) and follow-up studies to determine the source of infections and their impact on shrimp cultivation. More information is available in a recent review (Thitamadee et al. 2016).

There is a danger that living shrimp stocks exported from Asia or translocated within Asia for aquaculture may carry EHP, so it should be added to the list of pathogens to be monitored by quarantine authorities. PCR detection methods based on the ssu rRNA gene of EHP are available (Tourtip et al. 2009; Tangprasittipap et al. 2013; Tang et al. 2015; Itsathitphaisarn et al. 2016; Liu et al. 2016) and are suitable for testing PL and hepatopancreatic tissue of cultivated shrimp specimens.

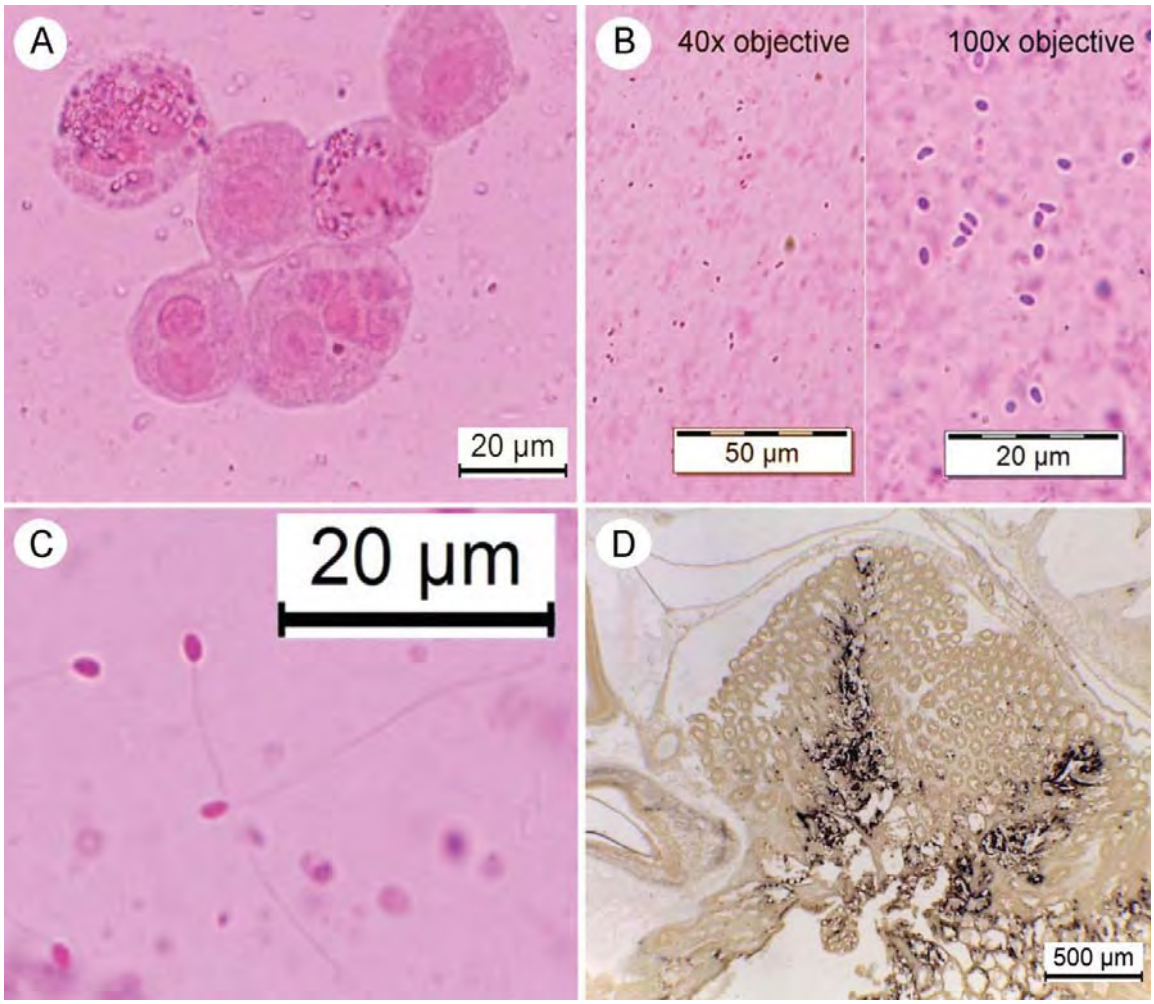

Fig. 4. Photomicrographs of EHP spores in HP squash mounts from infected PL and of *in situ* hybridization detection in HP tissue sections from an infected juvenile shrimp specimen. (A) Squash mount of HP tissue stained with 2 % phloxine B in distilled water and showing EHP spores in tubule epithelial cells. (B) Squash mount stained with phloxine B showing released spores using the 40x and 100x objectives. (C) Squash mount showing EHP spores with extruded polar tubes (also called polar filaments). (D) Positive *in situ* hybridization reactions (dark staining) with a probe targeting the EHP SSU rRNA gene and showing that the distribution of infected cells in the HP is not uniform such that the whole HP should be homogenized before subsampling to do qPCR.

However, we now know that closely related microsporidians give cross reactions with these detection methods and that they are therefore not suitable for use in testing shrimp feeds or feed ingredients when doing surveys for potential carrier species or for other environmental samples, since they may give false positive results. Instead, a recently developed nested-PCR method based on the spore wall protein gene of EHP is recommended. The method is described in detail at the website of the Network of Aquaculture Centres in Asia-Pacific, Bangkok, Thailand (Itsathitphaisarn et al. 2016) and a manuscript describing the development has been published (Jaroenlak et al. 2016).

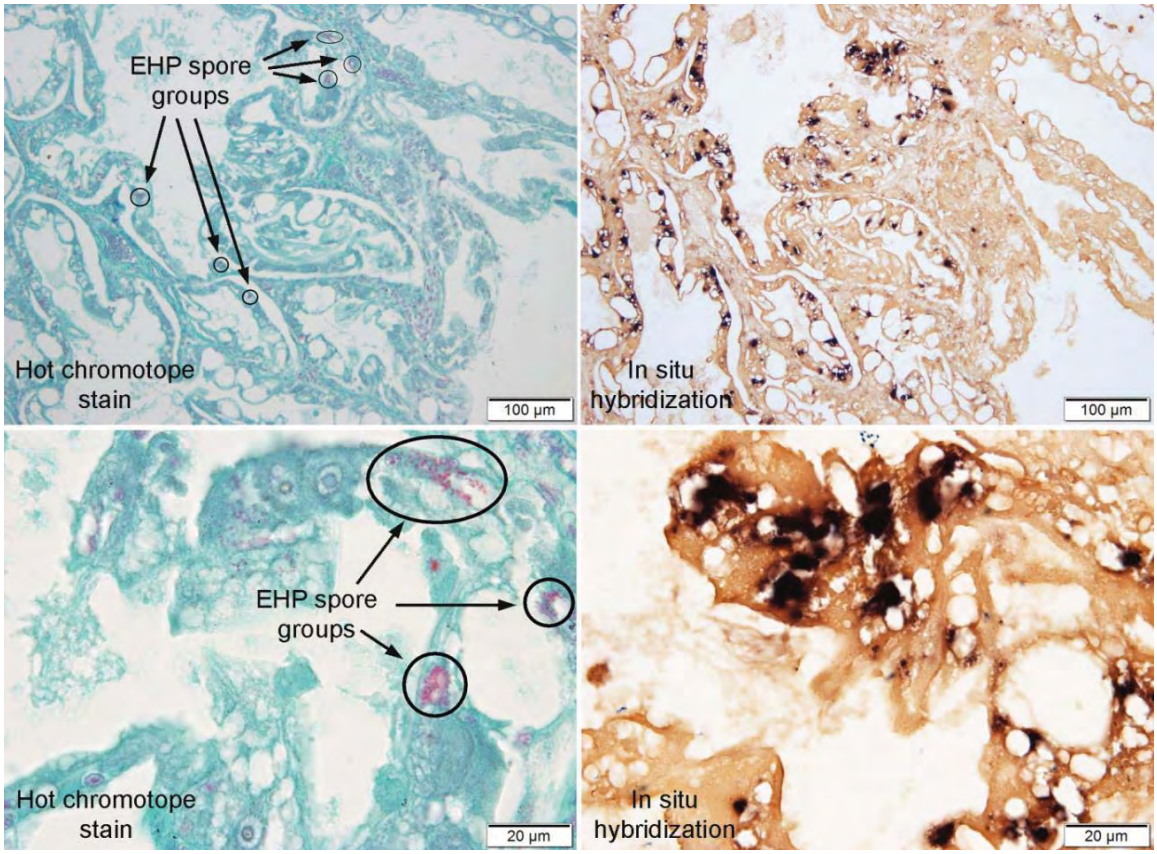

Fig. 5. Photomicrographs HP tissue of a juvenile shrimp specimen stained to reveal EHP-infected cells by using the chromotrope method (Weber et al. 1992; Moura et al. 1997) (green counterstain) for detecting the presence of spores (red staining) and contrasted with an adjacent HP tissue section stained to reveal EHP-infected cells by positive (dark staining) *in situ* hybridization reactions (Bismark brown counterstain). It is clear that using spore detection alone would give a gross underestimation of the number of infected cells in this specimen. The lower pair of photomicrographs are simply enlargements of matching portions of the upper pair.

It has been tested with DNA from microsporidian species closely related to EHP and does not cross-react with them as the ssu rRNA method does. The spore-wall protein gene sequence was obtained from DNA extracted from purified EHP spores obtained from infected shrimp by density gradient separation. The purified DNA was used to prepare a draft genome that has been submitted for publication (Boakye et al. submitted). It is hoped that sequence information will provide insights suitable for use in developing potential targets for HPM prevention and therapy.

The availability of the new spore wall protein PCR (SWP-PCR) method makes it possible to screen not only cultured shrimp but also suspected carriers and environmental samples for EHP with good assurance that false positive results will not be obtained from closely related microsporidians. At the same time, any PCR-positive, putative carriers should be tested by *in situ* hybridization to determine whether they are infected or mechanical carriers. This has not yet been done with the currently suspected carriers such as polychaetes but should now be possible using PCR-positive polychaetes from natural sources or using the co-habitation method of infected shrimp separated from uninfected polychaetes.

So far, there is no recommended or approved chemotherapy for HPM, and the main focus for control is on supply of PCR-negative PL cultivated in ponds where appropriate biosecurity measures have been taken to exclude EHP. However, EHP is horizontally transmissible by feeding of infected HP tissue or by cohabitation of infected shrimp separated by a mesh cage from naïve shrimp in the laboratory. The latter method is particularly suitable for testing of proposed chemicals and reagents designed to prevent transmission. Other methods for control of EHP in shrimp hatcheries and on farms have been summarized elsewhere (Sritunyalucksana et al. 2015; Thitamadee et al. 2016).

In summary, a highly specific and reliable nested PCR detection method is now available for use in screening broodstock, PL and juvenile shrimp for EHP infections and for testing environmental samples for EHP reservoirs. Thus, it should be possible to produce EHP-free PL to supply shrimp farmers by the proper use of SPF shrimp stocks with a history for freedom from EHP. SPF stocks of *P. vannamei* or *P. monodon* imported into Asian countries for production of PL in local hatcheries are at great risk of contamination by EHP unless held under strict hatchery quarantine. Work is urgently needed to find potential reservoirs for EHP and to test methods of prevention or therapy using the cohabitation model.

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Research progress on Acute Hepatopancreatic Necrosis Disease (AHPND) in Viet Nam

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Abstract

The work by conducted in Vietnam the ShrimpVet Laboratory on early mortality syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND) of penaeid shrimp is summarized, and includes evaluation of diagnostic methods (histology, bacteriology, polymerase chain reaction), challenge studies to evaluate the efficacy of various products in controlling AHPND in shrimp (e.g. probiotics, acidifiers, immunostimulants, bacteriophages, quorum quenching, feed additives, toxin absorbents, essential oils, herbal extracts), and approaches to field practices (e.g. better selection of PL and PCR tests for PL, nursery phase, polyculture, using "mature" water from fish ponds for stocking and water exchange, sludge removal, water discharge with central drainage, using probiotics to remove excessive organic matter, more water exchange and more reservoir area, avoiding eutrophication and excessive algal bloom, better natural food bloom before stocking, using gut probiotics, including organic acids in feed, and using herbs such as garlic and turmeric). To reduce the risk of AHPND in shrimp farming, a very holistic approach is needed that includes: biosecurity, PL quality, good farming practices, a more diversified microflora in both the shrimp gut and shrimp pond, sustainable farming practices and better environmental management. In short, shrimp farming should be considered as a value chain in which every part of the chain is equally important.

Keywords: AHPND, challenge studies, disease diagnosis, EMS, field practices, Viet Nam

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Introduction

Since the emergence of early mortality syndrome (EMS) in Viet Nam in 2010, these disease outbreaks were further identified as due to a toxin-mediated disease produced by strains of *Vibrio parahaemolyticus* (Tran et al. 2013a, b). The disease was later named acute hepatopancreatic necrosis disease (AHPND), and research and measures for control became more focused on dealing with the bacterial etiology.

Laboratory Research

The ShrimpVet Laboratory, founded in 2014 by Loc Tran and his team in Vietnam, has been involved in research on shrimp diseases, with a strong focus on EMS/AHPND, both in terms of academic studies and field application.

Diagnostics

So far at the ShrimpVet Laboratory, three main methods of disease diagnosis have been applied to screen for and confirm the presence of EMS/AHPND in affected shrimp or suspected materials.

- *Histology:* Based on the cased definitions proposed by Lightner et al. (2012), histology is a principle method used to confirm the presence of pathology in field specimens, as well as infections in infected shrimp in challenge studies.
- *Bacteriology:* So far, the ShrimpVet Laboratory has a collected over 40 EMS/ AHPND-causing bacterial strains (confirmed by both polymerase chain reaction (PCR) and bioassay (Koch's postulate)). Using the API 20 NE strips bio-chemical test, 36 of the 40 strains were identified as *V. parahaemolyticus*, while 4 were not *V. parahaemolyticus* . Among the EMS/AHPND-causing *V. parahaemolyticus*, there was no special biochemical characterization confirmed. Therefore, bacteriology alone is not recommended for confirmation of AHPND.
- *Polymerase Chain Reaction (PCR):* the ShrimpVet Laboratory has applied both AP3 primers released by Dr Tim Flegel (1-step PCR) in detecting the toxin gene of AHPND since 2014. The results so far have shown that 1-step PCR alone is not sensitive enough to detect AHPND, either in fixed samples of postlarvae (PL) or in pond water. The ShrimpVet Laboratory has conducted a series of challenge studies showing that, shrimp start to die when the bacterial load (AHPND *V. parahaemolyticus*) exceeds 10^4 CFU.g^{-1} shrimp. This also means that if the shrimp sample is extracted for DNA (only 2 mg of sample to be extracted), and it is presumed that the sample has 10^4 CFU.g^{-1}, then only be10^2 bacterial DNA genomes will be extracted. That means that this number of copies extracted might not be enough to be detected. Even with samples of moribund shrimp from challenge studies with AHPND, the PCR analysis often gave negative results.

In our clients' PCR samples of alcohol-fixed shrimp that are submitted to the laboratory, we seldom see PCR-positive results, even with parallel samples confirmed with AHPND using histology. However, if the fresh samples are used to culture bacteria in broth media for 4 hr, the PCR results for AHPND can be positive.

With parallel samples tested with AP4 (nested PCR) or real-time PCR, more positive results were obtained. This indicates that with fresh shrimp samples, it is best to have a 4-hr bacterial culture before running the PCR. With fixed samples of shrimp, only nested PCR or real-time PCR is sensitive enough to detect AHPND.

Challenge Studies

The ShrimpVet Laboratory has done several AHPND challenge studies to evaluate the efficacy a variety of different products in controlling AHPND in shrimp. Probiotics, acidifiers, immunostimulants, bacteriophages, quorum quenching, feed additives, toxin absorbents, essential oils, herbal extracts etc. have been tested.

Our studies indicate that products that have a direct effect on the bacterial population in the shrimp gut seem to work best. Several "designed" probiotics that have been selected from strains that can inhibit the growth of vibrios have been checked for their effect against AHPND. Many challenge studies have shown that several gut probiotics can improve the survival rate above that of the positive control (i.e. shrimp challenged with AHPND-causing *Vibrio parahaemolyticus*), survival rates being increased from 20 to 30 % to 60 to 70 %. Some acidifiers mixed with proper dosages in feed also conferred quite good tolerance to AHPND in the challenge studies. Many other challenge studies with other substances also provided some promising results.

Field Practice

Since the disease has been identified as directly related to the pathogenic bacterium, farmers are paying much more attention to keeping bacteria under control. Several approaches have been applied in the field including: better selection of PL and PCR tests for PL, nursery phase, polyculture, using "mature" water from fish ponds for stocking and water exchange, sludge removal, water discharge with central drainage, using probiotics to remove excessive organic matter, more water exchange and more reservoir area, avoiding eutrophication and excessive algal bloom, better natural food bloom before stocking, using gut probiotics, including organic acids in feed, and using herbs such as garlic and turmeric. Checking for AHPND quality using PCR has become more common in both hatchery and farm practices. Samples analyzed from hatcheries include broodstock faecal matter; live feeds such as bloodworm, squid and oyster; PL and nauplii. So far, we have had a significant number of samples of bloodworm and broodstock faeces testing positive. This indicates that bloodworm can be an important source of AHPND contamination and that finding a replacement for this particular live feed that is caught locally near the faming areas is an urgent need.

Applying a nursery phase in order to improve the health of juvenile shrimp, increase their size before stocking, and shorten the grow-out phase has become a common practice. The nursery phase usually happens in a small, controlled environment such as concrete tanks, fiberglass tanks or plastic-lined ponds. The culture period for this phase usually varies from two to four weeks. Then the shrimp will be transferred to the grow-out ponds. The clear-water system (with a lot of water exchange), recirculation system, or biofloc system is then applied to control the water quality during culture.

Polyculture is also a common practice that has been proven to be quite effective in reducing AHPND (Tran et al. 2014). In Viet Nam, polyculture with tilapia is very common, and includes the mixing of tilapia with shrimp, separating fish in hapas, using water from and exchanging water with tilapia ponds, and tilapia-shrimp crop rotations. The effects of tilapia can include: cleaning up of the pond bottom; consumption of dead shrimp, stopping disease transmission via cannibalism; encouraging beneficial bacteria and algae in the pond; and treating the waste released. By having a more diverse natural microflora in the shrimp pond, the harmful pathogenic bacteria seem to have less likelihood to bloom and cause shrimp mortalities. Measures to control conditions that favour fast-proliferating bacteria like *Vibrio* spp. are the main approach. Removing the pond sediments and sludge and having a more precise feeding programme are also important parts of controlling the excessive nutrients that potentially favour vibrios.

In general, in order to reduce the risk of AHPND in shrimp farming, we must have a very holistic approach that including: biosecurity, PL quality, good farming practices, a more diversified microflora in both the shrimp gut and shrimp pond, sustainable farming practices and better environmental management. In short, shrimp farming should be considered as a value chain in which every part of the chain is equally important.

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Acute Hepatopancreatic Necrosis Disease (AHPND) in Vietnam

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Abstract

Acute hepatopancreatic necrosis disease (AHPND) is the most severe disease currently affecting brackish-water shrimp aquaculture in Viet Nam. The disease causes huge losses to shrimp farmers in the Mekong Delta, where more than 70 percent of Vietnamese shrimp production originates. Losses due to AHPND were recorded in about 59 000 ha of farms in the Mekong Delta when the disease first appeared in 2011, and at present, the disease has spread to 294 communes belonging to 86 districts in 25 provinces throughout the country. However, the shrimp industry is now recovering from the disease and is continuing to play an important role in the country's aquaculture sector. This paper reviews the significant work related to AHPND that has been done by Vietnamese scientists under programmes designed by the National Task Force for Shrimp Disease. It includes a history of the disease in Viet Nam, reviews the various research projects that have been conducted and the management actions that have been implemented by governmental authorities, as well as the activities taken by the farmers to recover from the disease.

Keywords: AHPND, disease management and prevention, shrimp disease, Viet Nam

Introduction

A serious shrimp disease outbreak causing high mortalities in giant tiger prawn (*Penaeus monodon*) and whiteleg shrimp (*P. vannamei*) that was later called acute hepatopancreatic necrosis disease (AHPND) was officially documented in Soc Trang province in southern Viet Nam in April 2011. However, it was believed that the disease had been occurring in some provinces in the Mekong Delta area from the end of 2010. The pattern of spread and the clinical signs of disease were not similar to those associated with any previous shrimp disease outbreak in Viet Nam.

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Affected shrimp consistently showed abnormal hepatopancreas that appeared either shrunken or swollen, and discoloured. Records at the Department of Animal Health (DAH) of Viet Nam show that the total affected area of shrimp farms in the Mekong Delta was 58 812 ha in 2011, including the provinces of Tra Vinh (6 200 ha), Soc Trang (20 648 ha), Ca Mau (15 000 ha) and Bac Lieu (16 964 ha). In Soc Trang province, about 68.5 percent of the shrimp-producing area was affected and farmers were suffering from production losses. In Bac Lieu and Ca Mau provinces, an estimated 30 000 farming households were affected.

In 2012, the disease spread to 19 provinces throughout the country. The total AHPND-infected area, based on the clinical signs shown by shrimp samples, was 46 093 ha (or 45.7 percent) of the total area under culture. The most-affected province was Soc Trang with 23 371 ha (56.6 percent of the total culture area), followed by Bac Lieu with 16 919 ha (41.9 percent of the total culture area). The affected area in Tra Vinh was 12 224 ha (49.5 percent of the total culture area), while Ben Tre province had 2 237 ha affected (29 percent of the total culture area) (D-Fish 2012). In the year 2017, AHPND has spread to 294 communes belonging to 86 districts in 25 provinces throughout the country (DAH 2017).

Because of the significant impacts of the disease, the Government of Viet Nam took the following actions to respond to this disaster:

- established the National Task Force responsible for prevention and control of shrimp diseases;
- performed an intensive epidemiological survey;
- carried out screening for shrimp pathogens to narrow down the suspected causative agents of AHPND;
- carried out diagnostic investigations done by national and regional laboratories;
- distributed water treatment materials/disinfectants to provinces affected by AHPND outbreaks;
- granted funds for emergency research to be done by national institutions and universities; and
- solicited technical assistance from regional and international organizations.

The DAH, on behalf of the Government of Viet Nam, sent a request for technical assistance to the Food and Agriculture Organization of the United Nations (FAO), and subsequently, a Rapid Deployment Team (RDP) fielded by FAO visited the Mekong Delta provinces in July 2011 to conduct a quick assessment of the then unknown disease. Viet Nam then received support from FAO through project TCP/VIE/3304(E) “Emergency assistance to control the spread of an unknown disease affecting shrimps” (see FAO 2013).

Research Activities

Recognizing the severity of the disease, the Ministry of Agriculture and Rural Development (MARD) gave full support to the study of the causative agent and the development of prevention strategies to mitigate the disease. A National Task Force for Shrimp Disease was established by Decision No. 1254/QD-BNN-TCTS dated May 28, 2012 with the mandate to identify the causative agent and minimize the problem caused by AHPND. The approach applied in this massive study included screening of all possible causes of the mortality, as well as taking a closer look at any farm not affected by the disease. Figure 1 shows how the study on AHPND was approached.

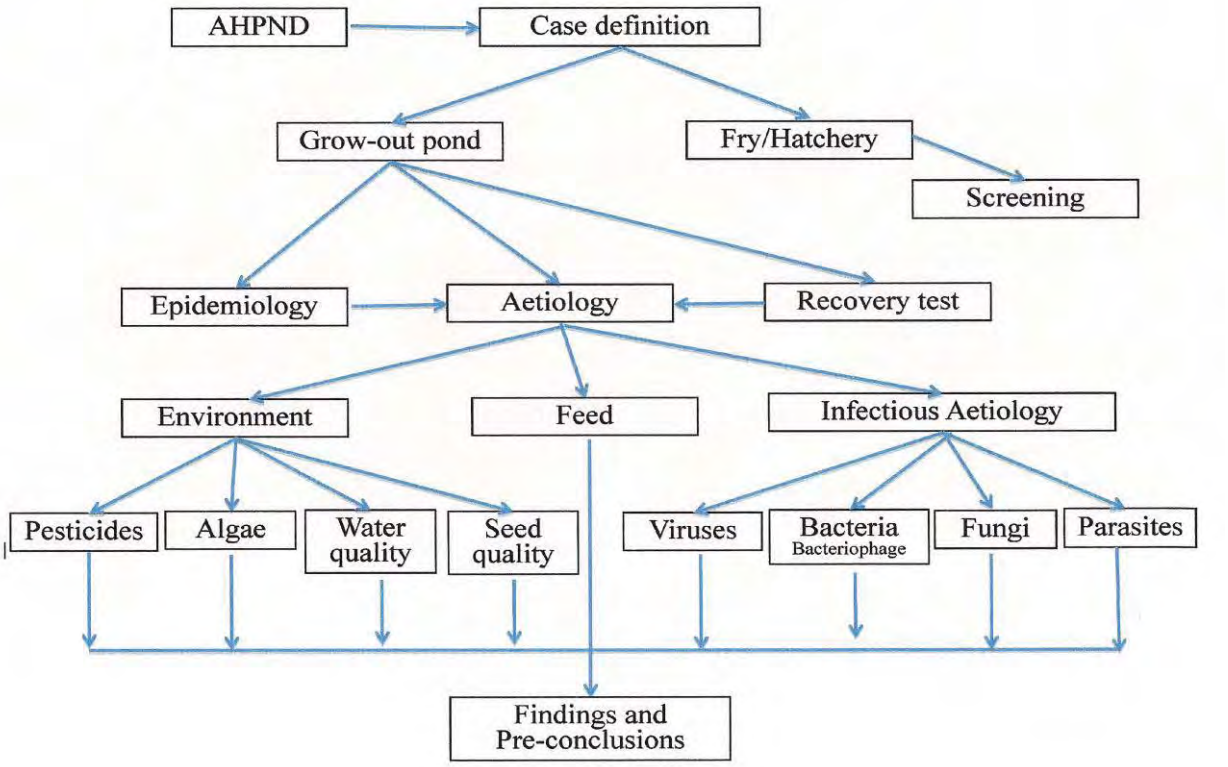

Fig. 1. Approach to the study on acute hepatopancreatic necrosis disease (AHPND)

Case Definition of AHPND

For the purpose of monitoring and early detection of the disease, as well as to assist in accurate reporting, a case definition of AHPND was developed at both the animal and pond levels. This was officially documented on June 21, 2012 (Document No. 970/TY-TS). The case definition is as follows:

- Susceptible host species: *P. monodon* and *P. vannamei.*
- Susceptible host stages: All grow-out stages are potentially susceptible, but mortality mainly occurs during 10–45 days poststocking.

- Clinical signs at the pond level:

    - At the early stage of the disease, the clinical signs are unclear. Shrimp exhibit slow growth, stop eating, show corkscrew swimming behaviour, and moribund shrimp sink to die at the bottom of the pond.
    - During a disease outbreak, diseased shrimp often have soft shells and abnormal hepatopancreas (HP) which becomes shrunken or swollen and has pale to white discolouration.

- Clinical signs at the animal level (based on Lightner et al. 2012):

    - Affected shrimp show acute progressive degeneration of the HP accompanied initially by decrease of R (Restzellen), B (Basenzellen), F (Fibrillenzellen) and E (Embyonalzellen) cells;
    - Lack of mitotic activity in E cells;
    - Proximal-to-distal dysfunction of R, B, F cells;
    - Prominent karyomegaly (enlarged nuclei), with rounding and sloughing of cells into the HP tubule lumens; and
    - Bacterial infection during advanced/terminal stages of the disease.

Table 1. Detection of acute hepatopancreatic necrosis disease (AHPND) in shrimp farms in northern Viet Nam in 2012.

Location of shrimp farm	No. samples	No. shrimp ponds	Histo-pathology findings	Pathogen isolation[1]			
				Bacteria	Bacterio-phage	Viruses	Parasites
Nghe An	321	25	AHPND	*Vibrio parahaemolyticus* *V. vulnificus* *V. cholerae* *V. harveyi* *V. ordalii* *V. mimicus*	–	IHHNV (1/25 ponds)	–
Ha Tinh	28	2	AHPND	*V. parahaemolyticus* *Photobacterium damselae*	–	–	–
Quang Ninh	20	2	AHPND	*V. alginolyticus* *V. vulnificus* *V. harveyi* *V. ordalii* *V. rotiferianus* *V. mytilis* *V. fischeri*	–	–	–
Hai Phong	146	7	AHPND	*V. parahaemolyticus* *V. vulnificus* *V. harveyi* *V. mimicus* *V. cholerae*	–	HPV, WSSV (2/7 ponds)	–

[1] – = not detected, HPV = hepatopancreatic parvovirus, IHHNV = infectious hypodermal and haematopoietic necrosis virus, WSSV = white-spot syndrome virus.

Epidemiology of AHPND

To determine if AHPND was already present in northern Viet Nam, diseased shrimp showing clinical signs of AHPND were collected from Nghe An, Ha Tinh, Quang Ninh and Hai Phong provinces, the main shrimp culture areas in the north of the country. Samples were processed for histopathological analysis and for pathogen isolation (Phan et al. 2012). Histopathological analysis confirmed that AHPND has spread to the northern part of Viet Nam (Table 1), indicating a nation-wide occurrence.

Monitoring of shrimp postlarvae (PL) for AHPND was also done by collecting more than 300 PL samples (about 50 PL/sample) from northern, central and southern Viet Nam for analysis based on the case definition. Results showed that AHPND was present in PL in Viet Nam (Table 2).

Table 2. Detection of acute hepatopancreatic necrosis disease (AHPND) in postlarvae obtained from various hatcheries in Viet Nam in 2012.

Location of hatchery	Number of PL samples	AHPND detection[1]	Bacterial isolation	Reference
Northern	6	–	*Vibrio* spp.	Phan et al. 2012
Central	> 200	–	*Vibrio* spp.	Vo 2012
Southern	113	3	*Vibrio* spp.	Dang 2012

[1] – = not detected.

Studies on the Causative Agent of AHPND

To determine the causative agent of AHPND, studies were conducted to analyze shrimp pond water (temperature, pH, DO, salinity, NH_4-N, NH_3, NO_2-N, NO_3-N, PO_4-P, chemical oxygen demand (COD), H_2S and Fe) and sediment quality (pH, percentages of total N, P and C). Also analyzed were toxic algae in pond water, pesticides used in shrimp ponds and feed quality, as well infectious organisms associated with AHPND-affected shrimp. Analysis of water and sediment quality of ponds with AHPND-infected shrimp showed that parameters were within normal levels except for high concentrations of NH_4-N (2.5 $mg.L^{-1}$) and PO_4-P (0.4 $mg.L^{-1}$) in pond water and high levels of total N (0.96 percent) and P (476.50 $mg.kg^{-1}$) in pond sediments, indicating high organic loads in some of the AHPND-infected ponds (Phan et al. 2012).

A total of 44 water samples were collected from AHPND-infected shrimp ponds and AHPND-uninfected ponds located in Nghe An, Ha Tinh, Quang Ninh and Hai Phong provinces and analyzed for toxic algae. Results showed that toxic algae belonging to the Dinophyta and Cyanobacteria were present in water sampled from both AHPND-infected and uninfected shrimp ponds, but in very low prevalences and densities (Table 3). These indicate that toxic algae were not the cause of AHPND.

Table 3. Toxic algae in AHPND-infected and uninfected shrimp ponds.

Toxic algae	Prevalence	Density (cells.L^{-1})
Dinophyta		
Dinophysis caudata	1/44	8
Prorocentrum minimum	3/44	10 – 62
Ceratium fucar	5/44	3 – 25
Cyanobacteria		
Oscillatoria limosa	17/44	2 – 7 292
Oscillatoria sp.	6/44	3 – 60
Annabaena cf. *spiroides*	1/44	57 500
Microcystis flos-aquae	1/44	1 300
Microcystis sp.	7/44	2 – 2 250
M. aeruginosa	6/44	6 – 65 625

For pesticide analysis, a total of 42 water samples and 23 sediment samples were collected from both AHPND-infected and uninfected shrimp ponds and analyzed by spectral scanning method using liquid chromatography mass spectrometry. The data (Table 4) showed that pesticides were present in both infected and uninfected ponds, indicating that pesticides were not the cause of AHPND.

Table 4. Analysis of pesticides in water and sediments in shrimp ponds.

Pesticide	Water (n = 42)		Sediment (n = 23)	
	Concentration (mg.L^{-1})	Prevalence	Concentration (mg.L^{-1})	Prevalence
Deltamethrin	0.001 – 0.070	23/42	0.035 – 0.097	11/23
Permethrin	0.001	1/42	0.010 – 0.063	2/23
Cypermethrin	0.001	1/42	–	0
Fenitrothion	0.001 – 0.012	14/42	0.028 – 2 316	14/23
Chlopyrifos	0.0002	1/42	–	0
Hexaconazole	0.001 – 0.074	13/42	0.020 – 0.134	09/23
Fipronil	0.001 – 0.065	16/42	0.024 – 2 597	6/23
Abamectin	0.003	1/42	0.197 – 0.839	3/23
Carbaryl	–[1]	0	0.030	1/23

[1] – = not detected.

For feed analysis, two commercial shrimp feeds used in AHPND-infected shrimp ponds were collected from Nghe An Province for pesticide residue analysis by spectral scanning method using liquid chromatography mass spectrometry.

The data showed that pesticide residues are not found in feeds, but preservatives such as ethoxyquin and butylated hydroxyl toluene (BHT) are present within allowable limits (Table 5). These results indicate that contamination of feeds with pesticides may not have a role in the occurrence of AHPND.

Table 5. Analysis of feed used for shrimp infected with acute hepatopancreatic necrosis disease (AHPND).

Feed Brand	Hexaconazole	Abamectin	Emamectinbenzoate	Ethoxyquin	Butylated Hydroxyl Toluene
			(mg.kg^{-1})		
NA - HH	–[1]	–	–	4 690	2 868
NA - QL	–	–	–	4 045	1 752

[1] – = not detected.

In terms of infectious aetiology, Table 1 shows that no bacteriophages or parasites were isolated from AHPND-diseased shrimp, but diseased shrimp were highly infected by *Vibrio* spp., mainly *V. parahaemolyticus*, *V. harveyi*, *V. vulnificus* and *V. alginolyticus*. Some shrimp were co-infected with viruses such as hepatopancreatic parvovirus (HPV), white-spot syndrome virus (WSSV) and infectious hypodermal and haematopoietic necrosis virus (IHHNV). At present, there are at least two *Vibrio* species confirmed by bioassays and molecular biology to cause AHPND in shrimp in Viet Nam: *V. parahaemolyticus* (Tran et al. 2013) and *V. harveyi* (Kondo et al. 2015; Dang et al. 2016).

Recovery from AHPND

According to the report prepared by the Department of Animal Health (DAH 2015), although AHPND had spread to 19 provinces in 2012 and to up to 22 provinces in 2015 and 25 provinces in 2017, the area affected has significantly decreased from 28 005 ha in 2012 to 9 284 ha in 2015 and 6 793 ha in 2017. The data, which were calculated based on histopathological and molecular biological analysis, indicate that AHPND still occurs in Viet Nam, but also that it seems to be managed, as reflected in the reduction in the affected area. So what has been done in Viet Nam in order to recover from the "AHPND disaster"? Based on current knowledge of AHPND and the outcomes of the research projects, several actions have been taken by the Government of Viet Nam and relevant stakeholders, including the shrimp farmers to reduce or control AHPND outbreaks.

Firstly, at the provincial and national levels, some technological programmes have been applied to shrimp culture, such as VietGAP (Vietnamese Good Aquaculture Practices) and biofloc technology (BFT). The VietGAP standard is a normative good aquaculture practice that is based on four basic criteria in order to ensure safety from epizootics, environmental safety, social security and product origin. Applying VietGAP, 100 percent of shrimp seed was tested and guaranteed for quality by authorities. Farmers worked in groups, complied with a cooperative production calendar and implemented shrimp farming based on environmental protection.

They did not discharge effluents and sludge directly to the enviroment, and applied biosecure shrimp farming by using probiotics to improve the culture environment. Thus, VietGAP standards helped in controlling diseases and made shrimp farming more sustainable. BFT is considered as an ecosystem management technology in which water exchange is limited and organic subtrates are allowed to accumulate. The microorganisms (biofloc) in the BFT system are developed by balancing carbon and nitrogen in the water, resulting in maintenance of water quality and control of bacterial infections in the ponds. Secondly, monitoring and surveillance programmes for shrimp farms have also been put in place. According to DAH information, 45 of 63 provinces have implemented programmes for the prevention and control of aquatic animal diseases, including shrimp disease. Surveillance results are reported monthly to DAH, and the directors present the results to the Minister of MARD during monthly meetings. Thirdly, the government has also funded research projects on aquatic animal diseases and assisted in the establishment of diagnostic laboratories at the provincial and district levels.

At the farm level, farmers have applied practical solutions to avoid disease, such as:

- screening PL for disease before stocking to ensure high quality;
- applying suitable stocking density;
- daily monitoring of environmental parameters (e.g. temperature, pH, salinity);
- monitoring and control of total *Vibrio* by applying probiotics;
- control of feeding, especially during the first month of stocking, by feeding shrimp several times per day in small rations; and
- applying polyculture system and/or rotational farming with shrimp-tilapia or shrimp-marine fish.

Some farmers also believe that application of a nursery system to grow larger PL before stocking into ponds can help to control AHPND outbreaks.

Future Perspectives

The AHPND crisis has been a serious challenge for the shrimp farming industry, not only in Viet Nam, but also worldwide, because the causative agents (*Vibrio parahaemolyticus* and *V. harveyi*) are common inhabitants of coastal and estuarine environments all over the world and are often found naturally associated with shrimp aquaculture systems. The transfer of plasmids carrying toxin genes between bacteria is facilitated by the aquatic environment. Therefore, innovative farm management and appropriate biosecurity are necessary to alleviate the AHPND crisis to ensure sustainable shrimp production. Applying disinfectant during pond preparation will reduce the risk of horizontal transfer. Management of sludge on pond bottoms is another important strategy, since organic matter that accumulates on pond bottoms can also serve as a substrate for *Vibrio* spp., including *V. parahaemolyticus*.

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Identification and Characterization of *Vibrio* Bacteria Isolated from Shrimp Infected with Early Mortality Syndrome/acute Hepatopancreatic Necrosis Syndrome (EMS/AHPNS) in Viet Nam

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Abstract

The outcomes of the work under FAO project TCP/VIE/3304 on the diagnosis of bacteria isolated from shrimp affected by early mortality syndrome/acute hepatopancreatic necrosis syndrome (EMS/AHPNS) in Viet Nam are presented. Field sampling was conducted in Soc Trang, Bac Lieu and Ca Mau provinces. At each sampling location, the targeted samples included three infected and three non-infected ponds. Disease signs at the pond level included pale hepatopancreas (HP), significant atrophy of the HP, guts with discontinuous contents or no content; onset of clinical signs and mortality starting as early as 10 days post stocking. At the end of the sampling campaign, shrimp from a total of 36 infected and 24 non-infected ponds had been collected. A total of 175 *Vibrio* isolates were isolated from the HP of shrimp specimens. A majority of isolates were identified as *Vibrio parahaemolyticus* by using API 20E kit and 16S rRNA sequencing. Thirty isolates of *V. parahaemolyticus* from each province were subjected to rep PCR analysis and detection of *Tlh*, *Tdh* and *Trh* genes. Rep PCR resulted in at least four different DNA profiles for the tested isolates. The *Tlh* gene was detected from all tested isolates, but neither the *Tdh* nor the *Trh* gene.

Keywords: acute hepatopancreatic necrosis syndrome, early mortality syndrome, *Vibrio parahaemolyticus*

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Introduction

Early mortality syndrome (EMS)/acute hepatopancreatic necrosis syndrome (AHPNS) first appeared in farmed penaeid shrimp in coastal provinces of the Mekong Delta of Viet Nam in 2010. In 2011 and 2012, EMS/AHPNS continued to occur and caused serious mortality in farmed shrimp in the Mekong Delta, and also appeared on shrimp farms in some northern coastal provinces. The disease occurred all year round, with greatest severity from April to July. It affected farms culturing giant tiger prawns (*Penaeus monodon* Fabricius 1798) and whiteleg shrimp (*P. vannamei* Boone 1931), mainly in areas of intensive and semi-intensive shrimp farming. The incidence of AHPNS seemed to be higher in farms with high salinity and during the dry season when high temperatures occurred.

At the time, the Food and Agriculture Organization of the United Nations (FAO) project TCP/VIE/3304 (E): Emergency Assistance to Control the Spread of an Unknown Disease Affecting Shrimps was being implemented in Viet Nam by the Ministry of Agriculture and Rural Development (MARD). This disease was considered idiopathic, and it was not known whether the cause was an infectious agent or a toxin. Earlier hypotheses suggested a range of possible causes, such as cypermethrin (an insecticide), other pesticides, pollution, contaminated feed, parasites, harmful algae, probiotics and inbreeding.

Field sampling for bacterial isolation from EMS/AHPNS-infected ponds was carried out during the FAO project. The objective of this study was to identify and characterize bacterial strains isolated from EMS/AHPNS-infected shrimp from outbreak and non-outbreak farms in the Mekong Delta to obtain information on the isolated *Vibrio* spp. with regard to their biochemical and physiological characteristics and the presence of selected specific genes.

Materials and Methods

Sampling locations and sampling times

Sampling was conducted in three provinces of the Mekong Delta of Viet Nam, i.e. Soc Trang, Bac Lieu and Ca Mau provinces. At each sampling location, the targeted samples included three infected and three non-infected ponds. Field sampling activities were conducted in three rounds starting on 11 September and finishing on 9 October, 2012. At the end of the campaign, samples had been collected from a total of 36 infected and 24 non-infected ponds. Sampling locations and collection dates are presented in Figure 1 and Table 1.

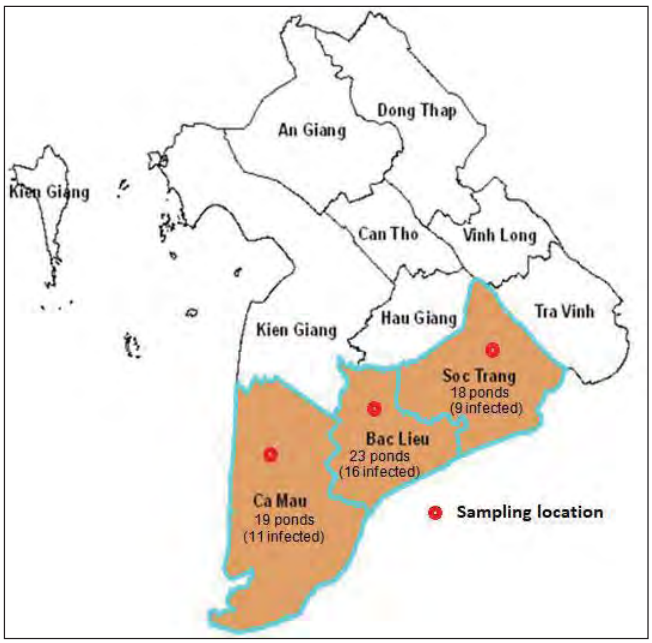


Fig. 1. Sampling locations for samples from existing outbreak and non-outbreak ponds.

Table 1. Sampling dates and locations for collection of samples from existing outbreak and non-outbreak ponds.

Sampling No.	Sampling Date	Location
1	11-12/09/2012	Soc Trang; round 1
2	14-15/09/2012	Bac Lieu; round 1
3	17-18/09/2012	Ca Mau; round 1
4	20-21/09/2012	Bac Lieu; round 2
5	23-24/09/2012	Ca Mau; round 2
6	26-27/09/2012	Soc Trang; round 2
7	29-30/09/2012	Bac Lieu; round 3
8	2-3/10/2012	Ca Mau; round 3
9	8-9/10/2012	Soc Trang; round 3

Sample collection and bacterial isolation from samples from existing outbreak and non-outbreak areas

Live shrimp were collected from each culture pond using a cast-net. Ten individuals from each pond were subjected to fresh smear examination. The external surface of the shrimp was rinsed in 70 % ethanol and an incision made over the head. The hepatopancreas (HP) was then removed, washed briefly with 70 % ethanol, and a small piece taken to make a smear on a glass slide containing a drop of Davidson's fixative (without glacial acetic acid). The preparation was then dried at room temperature, fixed in 1 % acetic acid solution, stained with Gram stain, and observed using a light microscope.

In addition, strains of *Vibrio* spp. were isolated from the HP of five individuals and incubated for 24 h at 28 °C on thiosulfate citrate bile salts sucrose (TCBS, Ovoid) agar and krypton soy agar (TSA, Ovoid) (supplemented with 1.5 % (w/v) sodium chloride) plates. Strains were stored at - 80 °C in trypton soy broth (TSB, Oxoid) containing 25 % glycerol and supplemented with 1.5 % (w/v) sodium chloride.

Table 2. Phenotypic characters of bacterial isolates from EMS/AHPNS-infected shrimp.

Character	Isolates of *Vibrio parahaemolyticus* obtained from EMS/AHPNS-infected shrimp (n = 90)[1]	ATCC 17802 (Buller 2004)
Gram stain	Negative	-
Shape	Short-rod	Short-rod
Colonies on TCBS agar	Green	Green
0/129 150 µg	+	ND
Haemolysis	+	ND
Swarming	+	ND
Motility	+	+
Catalase	+	+
Oxidase	+	+
O test	+	+
F test	+	+
Nitrate reduction	+	+
Beta-galactosidase production	-	-
Agrinine	-	-
Lysine	+	+
Ornithin	-	+
Citrate utilization	+	+
H_2S production	-	-
Urease	-	+
Tryptophane production	-	-
Indole production	+	+
Voges – Proskauer reaction	+	+
Gelatinase	+	+
Utilization of Glucose	+	+
Manitol	+	+
Inositol	-	-
Sorbitol	-	-
Rhamnose	-	-
Sucrose	-	-
Melibiose	-	-
Amygdalin	+	+
Arabinose	-	-

[1]'+' = positive strain; '-' = negative strain; ND: no data

Morphological and phenotypic characterization

Colony morphology and haemolysis were recorded after incubation for 2 days at 28 °C on blood agar plates (blood agar base (Merck) agar supplemented with 1.5 % (w/v) NaCl and 5% calf blood). Cell morphology was studied in Gram-stained preparations from the same blood agar plates according to Hucker's modification method (Barrow and Feltham 1993). Motility in broth (TSB supplemented with 1.5 % (w/v) NaCl) was studied using a drop of overnight culture on a slide and observed using a light microscope. Selected physiological and biochemical characteristics of isolates are presented in Table 2. Examination of characters was performed according to the principles of the Cowan and Steel's Manual (Barrow and Feltham 1993) using the API 20E system (BioMerieux, France) and 16S rRNA gene sequencing.

PCR analysis

An element palindromic PCR (rep-PCR) analysis, directed by the repetitive primer (GTG)5 (Bartie et al. 2006), was applied to compare isolates collected from EMS/AHPNS-infected shrimp. Detection of *Tlh, Tdh* and *Trh* genes was carried out following the protocol described by Nishibuchi et al. (1986) and Bej et al. (1999).

Results

Gross signs of EMS/AHPNS-infected shrimp

Outbreak ponds were selected as showing disease signs at the pond level, including pale to white hepatopancreas (HP); significant atrophy of HP; soft shells and guts with discontinuous contents or no content (Figs. 2B and 2b); onset of clinical signs and mortality starting as early as 10 days post stocking, and moribund shrimp coming to the pond sides or sinking to the bottom. In contrast, healthy shrimp appeared healthy with good HP and full gut (Figs. 2A and 2a). Gram-staining of fresh smears of HP from affected shrimp clearly showed the presence of Gram-negative, rod-shaped bacteria (Fig. 3B), whereas, fresh smears of HP from healthy shrimp had no bacteria (Fig. 3A).

Phenotypic characteristics of bacterial isolates

A total of 175 isolates of *Vibrio* spp. were collected from the HP of shrimp specimens. They formed large-sized (2–3 mm in diameter) green colonies after two days incubation at 28 °C on TCBS agar plates. The colonies were circular, entire and low convex, and their surface was smooth and shiny (Fig. 4A). In addition, these isolates developed swarming growth on TSA agar plates (Fig. 4B) and revealed haemolysis (beta form) on blood agar plates after 1 day of incubation at 28 °C (Fig. 4C).

The isolated bacteria were Gram-negative, short, rod-shaped, motile, positive for oxidase and catalase, fermented glucose in both aerobic and anaerobic conditions, sensitive to the vibriostatic agent O/129 using 150 μg discs, and reduced nitrate to nitrite.

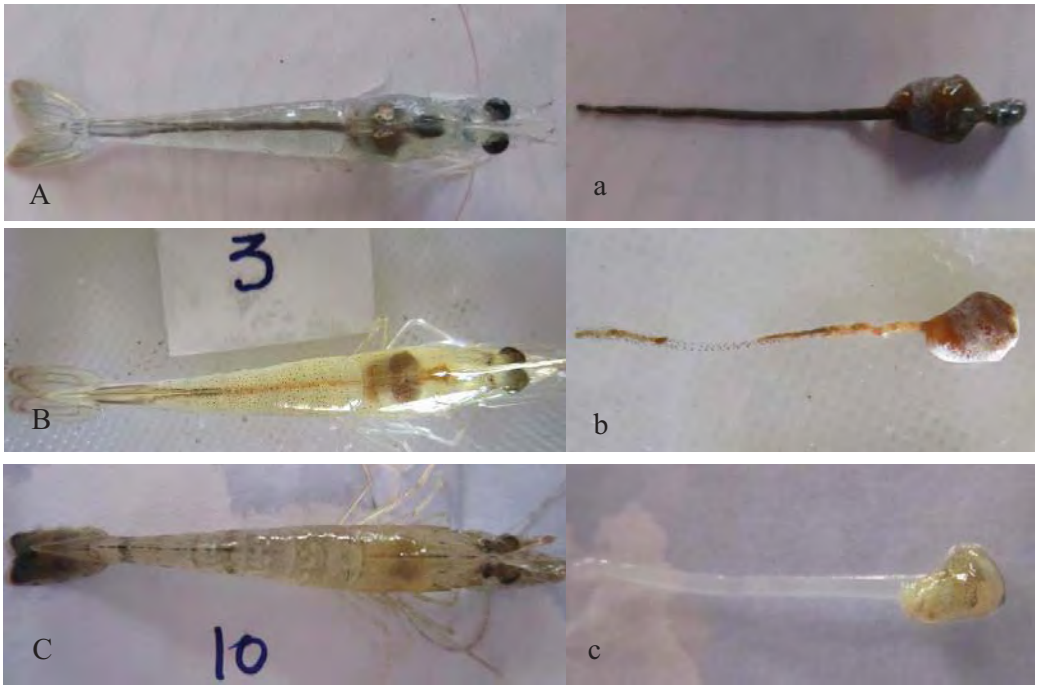


Fig. 2. Gross sign of EMS/AHPNS-infected shrimp. A. Control shrimp appear healthy; a. full gut. B and C: Infected shrimp with pale body colour; b. pale hepatopancreas (HP) and gut with discontinuous contents; c: pale HP and empty gut.

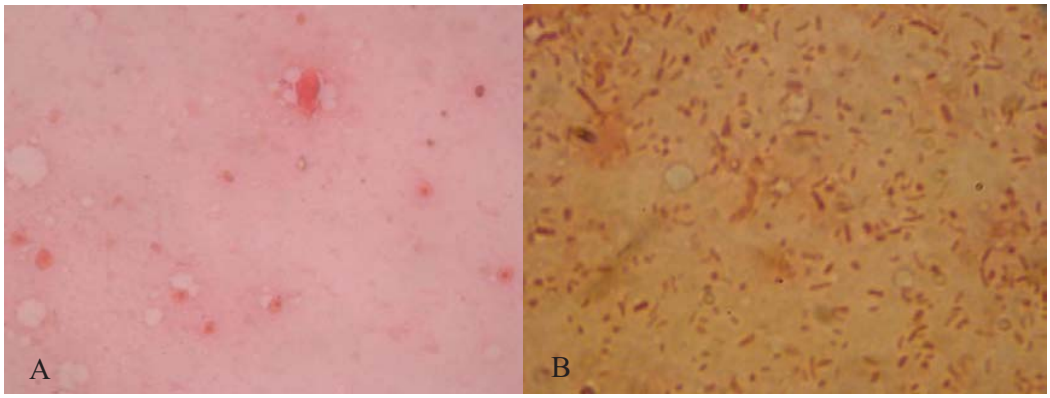
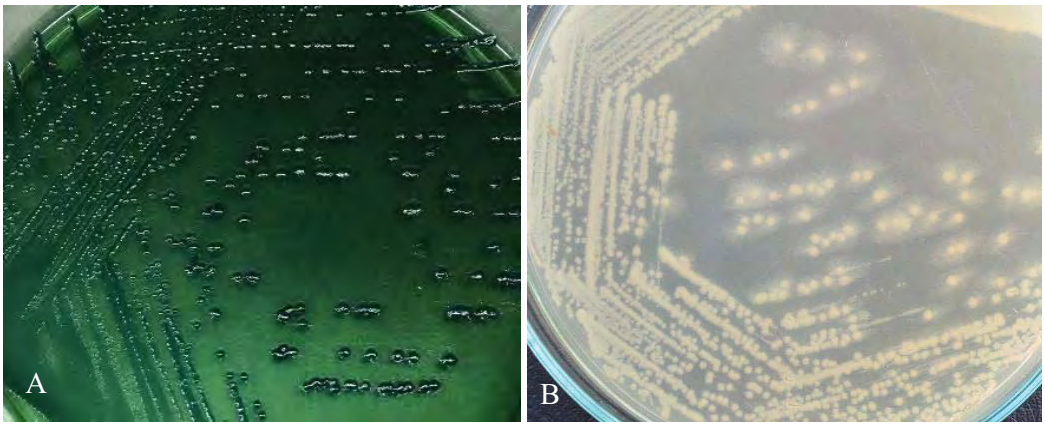


Fig. 3. Fresh smear and Gram staining of hepatopancreas from EMS/AHPNS-infected shrimp. (A) Healthy shrimp and (B) EMS/AHPNS-infected shrimp showing the presence of Gram-negative, rod-shaped bacteria (100X magnification).



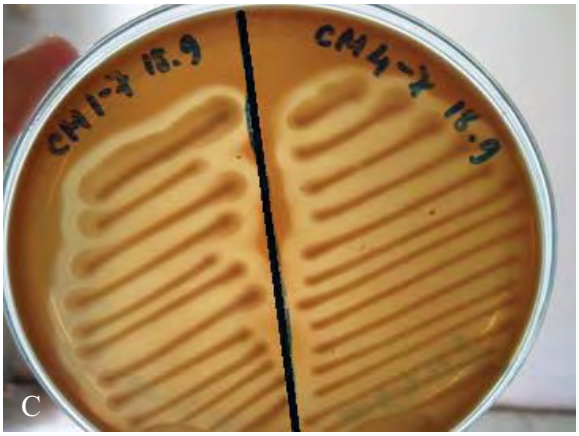


Fig. 4. Phenotypic characters of bacterial isolates from EMS/AHPNS-infected shrimp on solid agar plates. (A) green colonies on TCBS agar. (B) swarming growth on TSA agar. (C) haemolysis (beta form) on blood agar.

Isolates were identified to species level using a combination of conventional biochemical tests and API 20E kit. A majority of them were identified as *V. parahaemolyticus*; their phenotypic characteristics are presented in Table 2. Identification of isolates as *V. parahaemolyticus* was confirmed by 16S rRNA sequencing (99 % identities with GenBank *V. parahaemolyticus* AB680329 strain).

Rep-PCR analysis and detection of Tlh, Tdh and Trh genes

Ninety isolates of *V. parahaemolyticus* were selected from the three sampling locations (30 isolates each from Soc Trang, Bac Lieu and Ca Mau provinces) and subjected to rep-PCR analysis. Rep-PCR analysis resulted in at least four different DNA profiles for the tested isolates, as shown in Figures 5, 6, 7 and 8. Isolates which were isolated from EMS/AHPNS-infected shrimp from Bac Lieu Province displayed two DNA profiles of 11 and 12 bands (Figs. 5 and 6), whereas isolates which were obtained from EMS/AHPNS-infected shrimp from Soc Trang and Ca Mau provinces showed six DNA profiles with 9, 10, 11 and 12 bands (Fig. 7). Thermolabile haemolysin (*Tlh*) encoded by *Tlh* gene was detected from all tested isolates (Fig. 8), but neither the *Tdh* nor the *Trh* gene was detected.

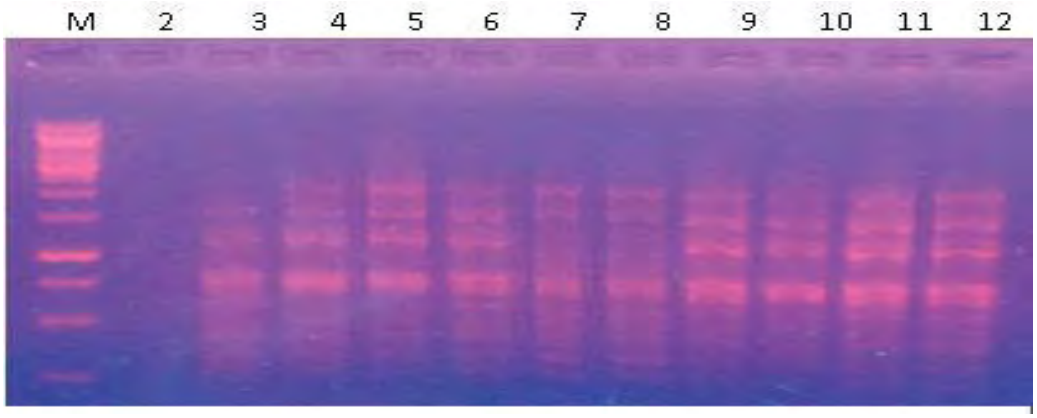


Fig. 5. Rep-PCR profiles (11 bands) of isolates of *Vibrio parahaemolyticus* obtained from EMS/AHPNS-infected shrimp from Bac Lieu Province. (M) DNA ladder 1 kb plus (Invitrogen), (2) water, (3–12) *V. parahaemolyticus* isolates.

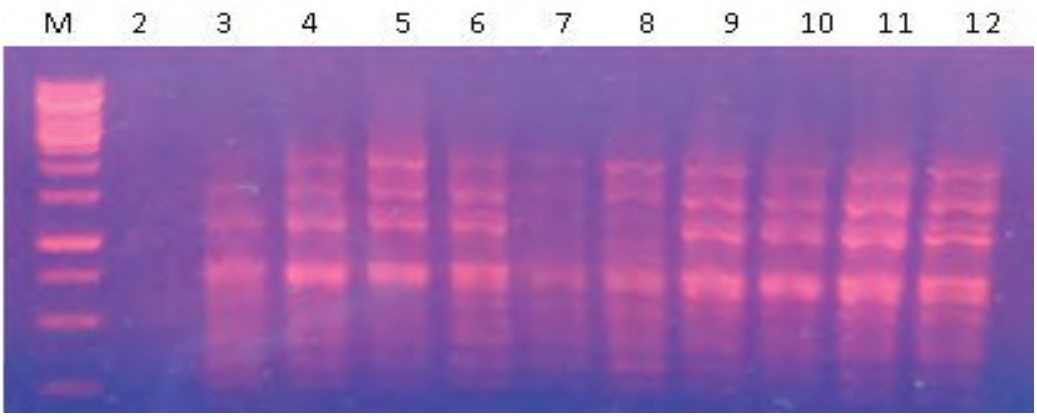


Fig. 6. Rep-PCR profiles (12 bands) of isolates of *Vibrio parahaemolyticus* obtained from EMS/AHPNS-infected shrimp from Bac Lieu Province. (M) DNA ladder 1 kb plus (Invitrogen), (2) water, (3–12) *V. parahaemolyticus* isolates.

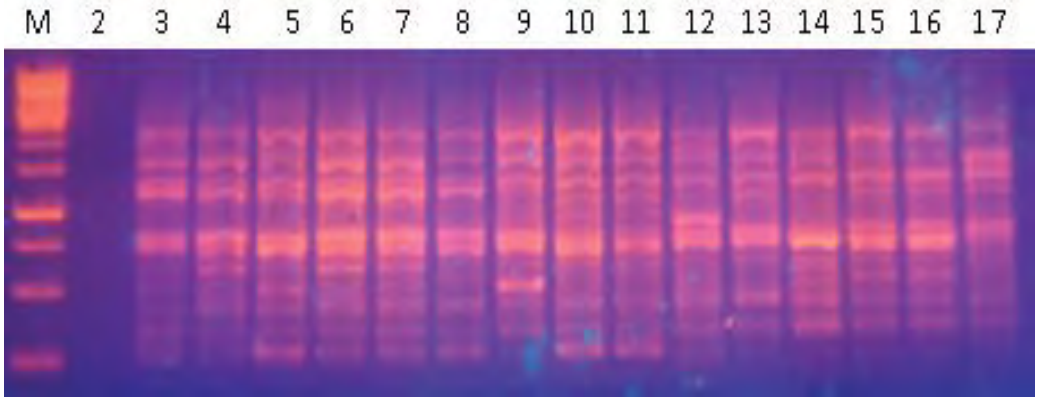


Fig. 7. Rep-PCR profiles of isolates of *Vibrio parahaemolyticus* obtained from EMS/AHPNS-infected shrimp from Soc Trang and Ca Mau provinces. (M) DNA ladder 1 kb plus (Invitrogen), (2) water, (3–17) *V. parahaemolyticus* isolates.

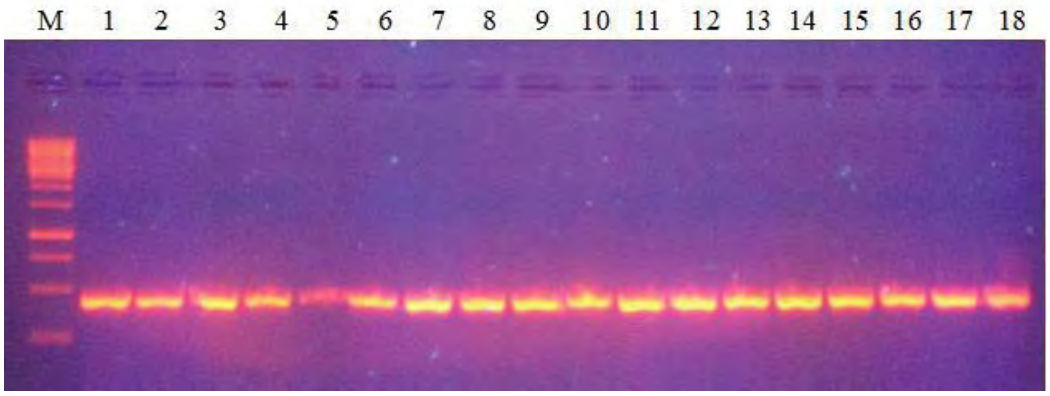


Fig. 8. Detection of *tlh* gene from isolates of *Vibrio parahaemolyticus* obtained from EMS/APHNS-infected shrimp collected during September to October 2012 field sampling activities. (M) DNA ladder 1 kb plus (Invitrogen). (1–18) representative isolates from EMS/AHPNS-infected shrimp.

Discussion

Vibrio species occur widely in aquatic environments as part of the normal flora of coastal seawater. They also exist as normal flora in fish and shellfish but have also been recognized as opportunistic pathogens in many marine animals (Austin and Austin, 1993). All bacterial isolates obtained from EMS/AHPNS-infected shrimp displayed the key phenotypical features of bacteria belonging to the genus *Vibrio*. Thus, all were motile, oxidase and catalase positive, Gram-negative rods which degraded D-glucose fermentatively, reduced nitrate to nitrite, grew on a *Vibrio* selective medium (TCBS), were sensitive to the vibriostatic agent O/129 using 150 μg discs (West et al. 1986) and gave positive results for indole production.

A majority of *Vibrio* isolates collected from EMS/AHPNS-infected shrimp were identified *V. parahaemolyticus*. The biochemical and physiological data were supported by 16S rRNA gene sequencing, while BLASTn analysis of 16S rRNA sequences from these isolates gave 99 percent identity to *V. parahaemolyticus*. *Vibrio parahaemolyticus* is a common inhabitant of coastal and estuarine environments all over the world. Therefore they are often found naturally associated with shrimp aquaculture systems. Certain environmental conditions may be more favourable for the establishment, survival and growth of these organisms, such as high pH, high temperature and high salinity, as well as tidal flushing. *Vibrio parahaemolyticus* is closely related to shrimp-pathogenic luminous bacteria such as *V. harveyi*, *V. campbelli* and *V. owensii.* These, along with other closely related *Vibrio* spp. form a "*V. harveyi* clade" (Cano-Gomez et al. 2009). Bacteria within this clade have a very high degree of similarity at both the phenotypic and genotypic levels.

The role of bacteria in EMS/AHPNS was suggested to be secondary infection, as bacterial colonization was prominent at the latter stage of the disease (Lightner et al. 2012). However, *V. parahaemolyticus* was consistently isolated from EMS/AHPNS-infected shrimp during the present study. Moreover, based on work done in Guangxi Province, P.R. China in 2010, Zhang et al. (2012) reported on a virulent strain of *V. parahaemolyticus* (strain 20100612001) which they isolated from *P. vannamei* suffering from EMS/AHPNS. The strain produced green colonies on TCBS agar, did not utilize sucrose and showed high antibiotic resistance. The results of this study suggested that further research should be focused on *V. parahaemolyticus*. Certain strains of *V. parahaemolyticus* can cause gastroenteritis in humans, and such clinical strains are characterized by the ability to produce a thermostable direct haemolysin (*Tdh*) or a *Tdh*-related haemolysin (*Trh*). The genes encoding these haemolysins (*tdh* and *trh* genes) are generally used as markers for human pathogenic strains of *V. parahaemolyticus* (FAO/WHO 2011). Human pathogenic strains possessing these markers account for 1–2 percent of environmental strains of *V. parahaemolyticus*. All strains (both clinical and environmental) produce a thermolabile haemolysin (*Tlh*) encoded by the *Tlh* gene, and this is generally used as a marker for *V. parahaemolyticus* in diagnostic tests (Keysner and Depaola 2004). Preliminary investigations of isolates of *V. parahaemolyticus* from EMS/AHPNS-infected shrimp detected the *Tlh* gene, but not the *Tdh* nor the *Trh* gene. Since the emergence of EMS/AHPNS, there has been no report of human-related disease (e.g. gastroenteritis) linked to the consumption of affected shrimp from any of the affected countries.

Acknowledgements

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Field Study on Transmission of Acute Hepatopancreatic Necrosis Syndrome (AHPNS) in Infected Ponds

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Abstract

A field transmission experiment for acute hepatopancreatic necrosis syndrome (AHPNS) in whiteleg shrimp (*Penaeus vannamei*) was set up in two AHPNS-infected ponds. Six hapas were placed in each pond; of these, three were stocked with healthy shrimp (to investigate transmission via water) and the other three were stocked with both healthy and AHPNS-infected shrimp (to investigate transmission via water and cohabitation). At 10 days post stocking, healthy shrimp in both treatments showed typical signs of AHPNS pathology as seen in naturally infected shrimp in ponds. Histopathological analysis revealed rounding and sloughing of hepatopancreatic (HP) epithelial cells, reduction in epithelium height, loss of certain cell types (B-, F- and R-cells), and severe haemocytic infiltration around HP tubules. Mortalities were noted in all experimental hapas; however, mortality rates in hapas stocked with both healthy and AHPNS-infected shrimp were higher than in hapas stocked with healthy shrimp only. Isolates of *Vibrio* bacteria obtained from infected shrimp were identified as *V. parahaemolyticus*. Polymerase chain reaction (PCR) analysis detected the *thl* gene from isolates of *V. parahaemolyticus* but not the *thd* or *trh* genes.

Keywords: AHPNS, *Penaeus vannamei*, histopathology, transmission, mortalities

Introduction

Early mortality syndrome (EMS)/acute hepatopancreatic necrosis syndrome (AHPNS) is an emerging threat in the Asian shrimp industry, causing high mortalities in shrimp aquaculture and economic losses to both small farmers and commercial producers (Eduardo and Mohan 2012). AHPNS first appeared in farmed penaeid shrimp in the coastal provinces of the Mekong Delta of Viet Nam in 2010. In 2011 and 2012, it continued to occur, causing high mortality in farmed shrimp in the Mekong Delta and also affecting shrimp farms in some northern coastal

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provinces. The disease occurred all year round, with greatest severity from April to July.

EMS/AHPNS was considered as an unknown disease before the breakthrough finding of Prof. Donald Lightner's team pointing to a strain of *V. parahaemolyticus* as its causative agent (Tran et al. 2013). It affected farms culturing both giant tiger prawn (*Penaeus monodon*) and whiteleg shrimp (*P. vannamei*), mainly in areas of intensive and semi-intensive shrimp farming. The incidence of AHPNS seemed to be higher in farms with high salinity and during the dry season with associated high temperature. The disease was considered idiopathic, and it was not known whether the cause was infectious or toxic. Earlier hypotheses suggested a range of possible causes, including cypermethrin (an insecticide), other pesticides, pollution, contaminated feed, parasites, harmful algae, probiotics and inbreeding. This field study on disease transmission in AHPNS-affected ponds was carried out during the FAO TCP/VIE/3304 (E) Emergency Assistance to Control the Spread of an Unknown Disease Affecting Shrimps, which was being implemented by Viet Nam's Ministry of Agriculture and Rural Development (MARD). The objective of the study was to determine if AHPNS is transmissible by water and by co-habitation of infected and healthy shrimp in AHPNS-affected ponds.

Materials and Methods

Experimental set up

The study was carried out from April to May, 2013, and the experiment was set up in two AHPNS-infected ponds at Cong ap 10, Tan Duyet Commune, Dam Doi District, Ca Mau Province (Fig. 1a). AHPNS-infected ponds were determined by histopathological examination of infected shrimp showing typical AHPNS pathology (Figs. 1b and 3b). Healthy broodstock of *P. vannamei* held at the College of Aquaculture and Fisheries, Can Tho University were induced to spawn, and the resulting larvae were reared to a size of ~ 1–1.5 gram. Ten individuals with apparently healthy hepatopancreas (HP) and full gut content (Fig. 2) were checked by histology to make sure that they showed no AHPNS pathology (Fig. 3a) prior to being transported to the experimental site.

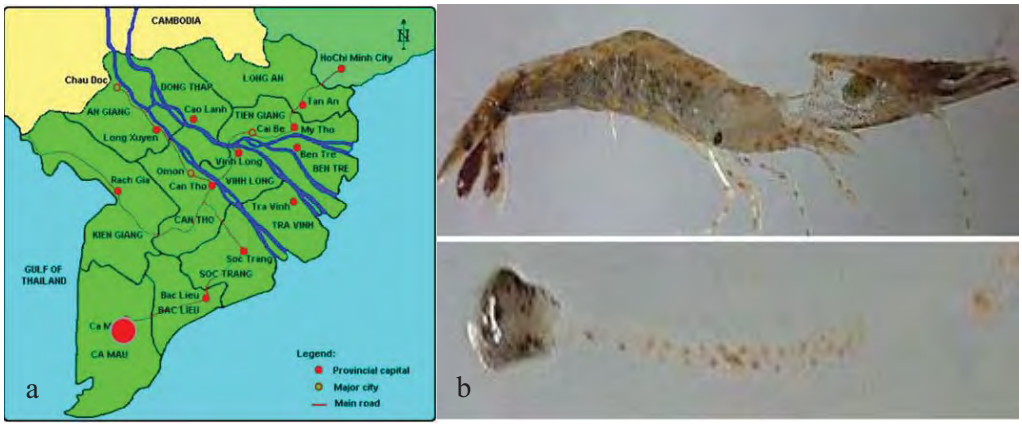

Fig. 1. (a) Location of the experimental study on AHPNS transmission, and (b) Gross signs of AHPNS in shrimp from experimental ponds.

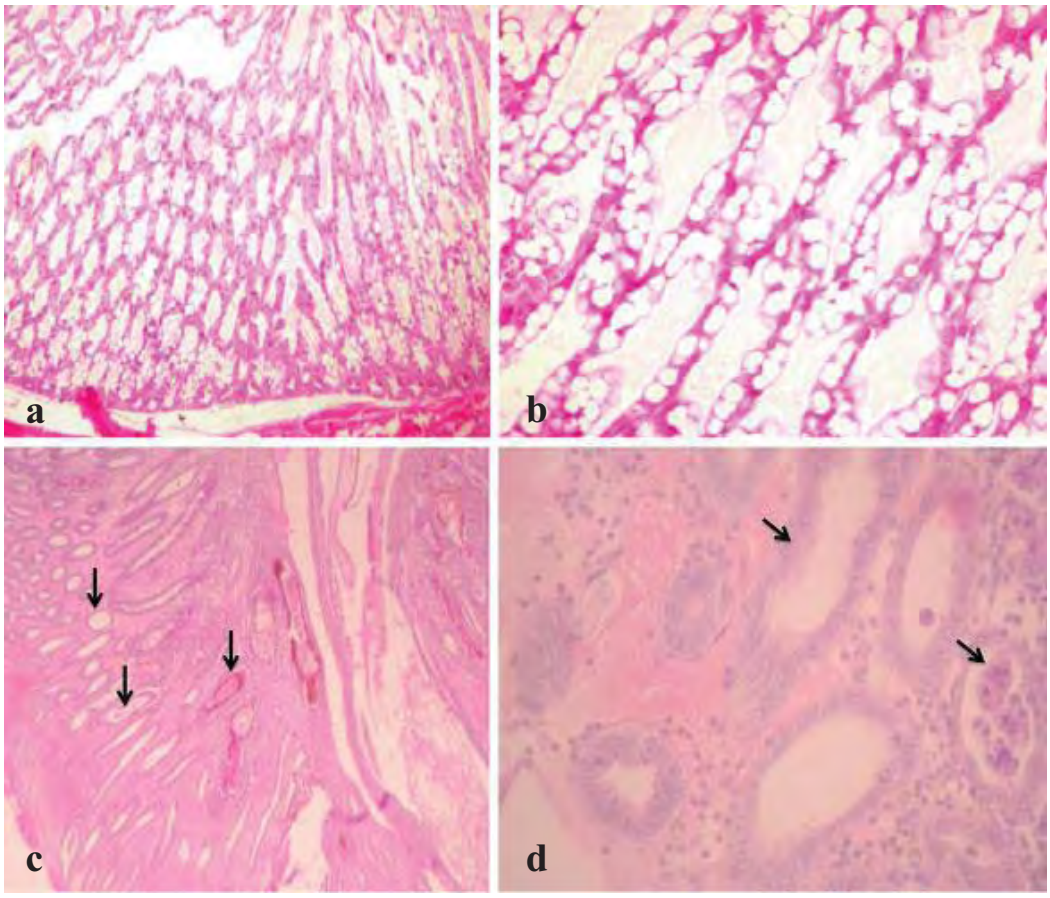

Fig. 2. Histological sections showing the hepatopancreas (HP) of healthy experimental shrimp (a: 10X; b: 40X magnification) and infected shrimp; (c) arrows (L to R): distruction of HP tubules, melanization (10X magnification) and (d) arrows (L to R): distruction of HP tubule, sloughing cells (40X magnification). H&E stain.

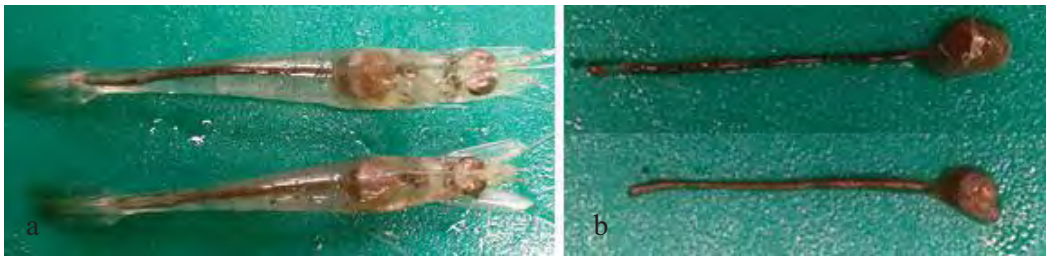

Fig. 3. (a) Healthy shrimp with full gut content used in the study and (b) hepatopancreas and gut from healthy shrimp.

Both the water transmission and the cohabitation experiments were conducted in a series of 6 hapas (1m x 1m x 1m) aligned as shown in Figure 4 (Pond 1), with a second, duplicate pond (Pond 2) also with a similar 6 hapas. Each of the ponds contained 2 experimental groups as follows:

- Group 1 (hapas 1, 2 and 3): stocked only with healthy shrimp (100 shrimp per hapa) that had been reared under laboratory conditions at Can Tho University to study of the transmission of AHPNS via water.
- Group 2 (hapas 4, 5 and 6: stocked with 100 healthy shrimp per hapa and 15 AHPNS-infected shrimp collected from an AHPNS-infected pond to study transmission of AHPNS by cohabitation.

The experiment was followed for up to 10 days post-stocking of healthy shrimp in the experimental hapas.

Fig. 4. Experimental hapas were set up from 1–6. Hapas 1, 2 and 3 contained healthy shrimp only, while hapas 4, 5 and 6 were stocked with both healthy and AHPNS-affected shrimp.

Histopathology

During the experiment, moribund shrimp (shrimp that are dying, but are still alive) were collected from experimental hapas and injected with Davidson's AFA (alcohol-formalin-acetic acid) fixative and processed and stained with hematoxylin and eosin (H&E) using the routine histological methods described by Lightner (1996). The histological sections were examined by light microscopy for AHPNS lesions in the hepatopancreas (HP). At the end of the experiment, 10 shrimp from each hapa were randomly collected and examined by histology for pathology due to AHPNS.

Bacterial isolation and characterization

Bacterial isolates (primarily *Vibrio* spp.) were obtained from the HP of shrimp specimens. Strains were stored at -70 °C in tryptic soy broth (Merck) containing 25 % glycerol supplemented with 1.5 % (w/v) sodium chloride. The strains were identified using API 20E (BioMerieux, France) and detection of the *tlh*, *tdh* and *trh* genes was carried out by PCR (Nishibuchi et al. 1986; Bej et al. 1999).

Data analysis

Differences in final mortality between experimental groups were analysed using Student's t-test ($P < 0.05$).

Results

Gross clinical signs in experimental shrimp

Experimental shrimp had changes in the HP colour from the first day after being released into the experimental hapas. From the second day, shrimp displayed the first signs of AHPNS as described by Lightner et al. (2012), including anorexia, lethargy and pale colouration of the body and HP (Fig. 5). Almost from day 4 onwards, the HP of experimental shrimp displayed gross signs similar to those seen in diseased shrimp collected from the same ponds (Fig. 6). AHPNS pathology was more evident in samples which were collected at end of the experiment (10 days after the shrimp were released into experimental hapas).

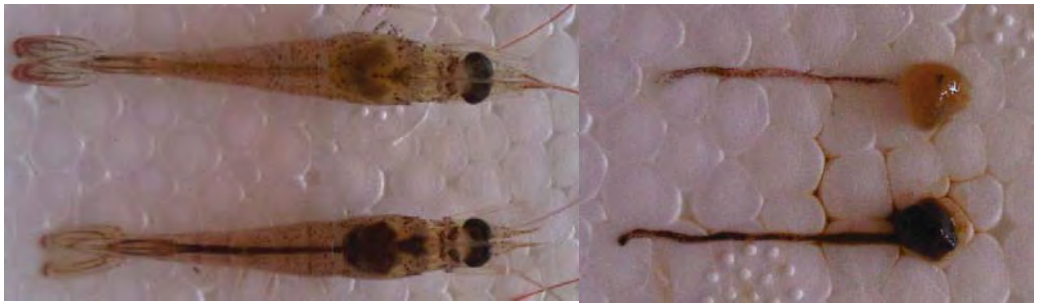

Fig. 5. Experimental shrimp collected on day 2 after release into hapas.

Fig. 6. Experimental shrimp showing change of colour of the hepatopancreas and empty gut four days after release into hapas.

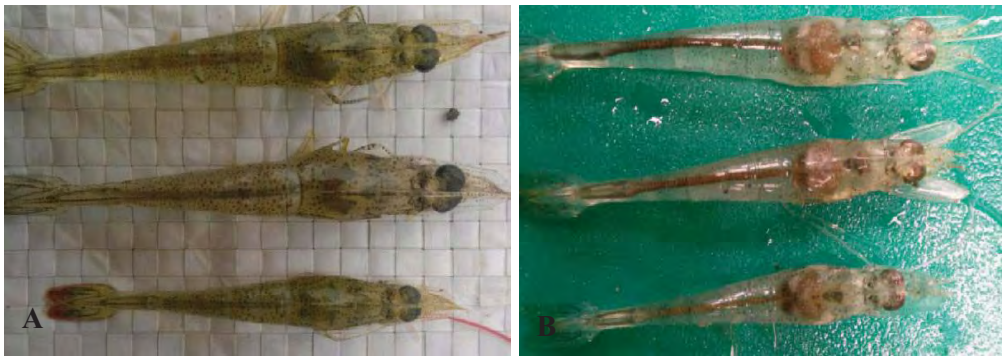

Fig. 7. A. Shrimp collected from experimental hapas. B. Shrimp collected from the same stock kept in the laboratory.

Although the experiment lasted for only ten days, it is long enough to recognize the abnormal growth (which was slower than normal), lethargic swimming, relatively weak, soft shells, and the darkened, atrophied and pale HP of AHPNS-affected shrimp as compared to those control shrimp that were maintained in the laboratory (Fig. 7).

As can be clearly seen in Figure 8, the HP of experimental shrimp displayed clinical signs that were quite similar to the gross signs of AHPNS as described by Lightner et al. (2012).

Fig. 8. Hepatopancreas of infected shrimp at the end of the experiment.

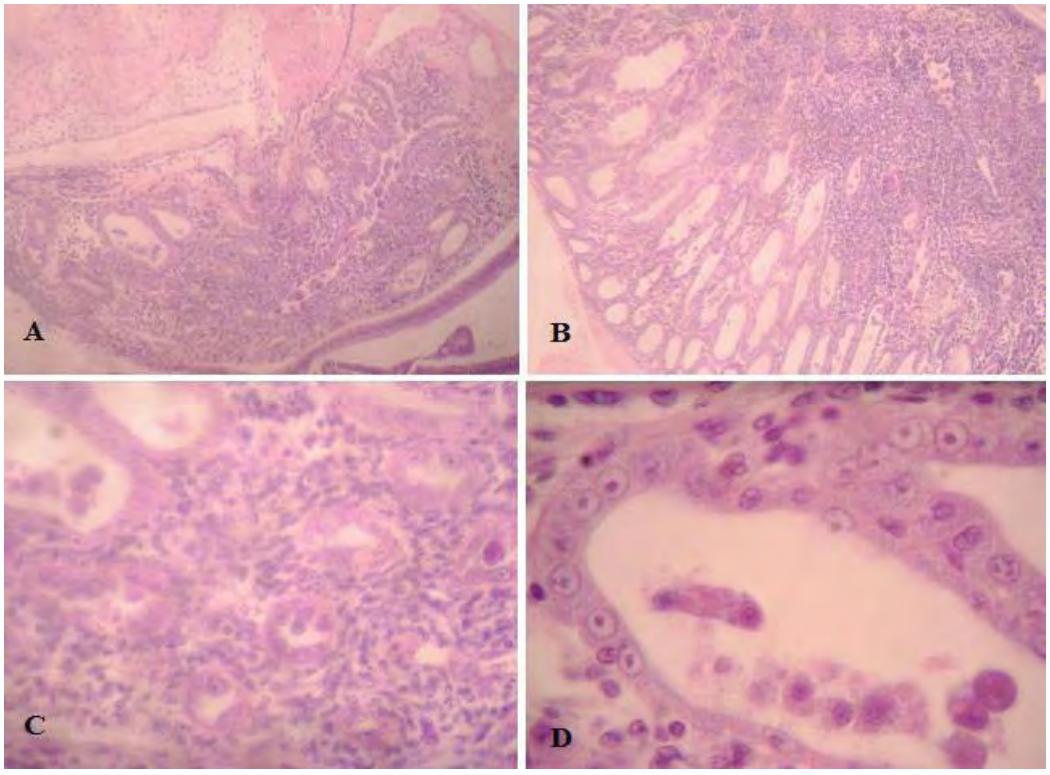

Fig. 9. Histopathological preparations of the hepatopancreas (HP) of infected shrimp collected from experimental hapas. A & B: distruction of HP cells, lack of E, B and R cells (10X & 20X magnification. C&D: HP sloughing of tubule epithelium, significant proximal haemocytic inflammation, some tubules with putative vibriosis (40X & 100X magnification). H&E stain.

Shrimp with gross signs of AHPNS were subjected to histological analysis. The HP of these shrimp displayed histopathological changes typical of AHPNS, including distruction of HP cells, lack of E, B and R cells, sloughing of HP tubule epithelium, significant proximal haemocytic inflammation, and some tubules with putative vibriosis (Fig. 9).

A total of 120 shrimp (10 from each hapa) was randomly collected at the end of the experiment. Among the 60 shrimp sampled from hapas stocked with healthy shrimp, only 18 displayed typical AHPNS pathology, whereas 27 of the shrimp collected from hapas stocked with both healthy and AHPNS-affected shrimp displayed AHPNS pathology.

Mortality

During the experimental period, shrimp were observed daily to check for abnormal signs and to record mortality. The mortality data (Fig. 10) clearly showed the difference in the number of shrimp which survived in the different experimental groups, as well as between the two experimental ponds.

In Pond 1, the lowest mortality of experimental shrimp was noted in hapa 1.4 (19 %), and the highest was in hapa 1.6 (57 %). In Pond 2, the lowest mortality of experimental shrimp was noted in hapa 2.2 (2 %) and the highest was in hapa 2.2 (44 %). Although the mortality rates between hapas with the same treatment, as well as between treatments were different, these differences were not statistically significant ($P > 0.05$).

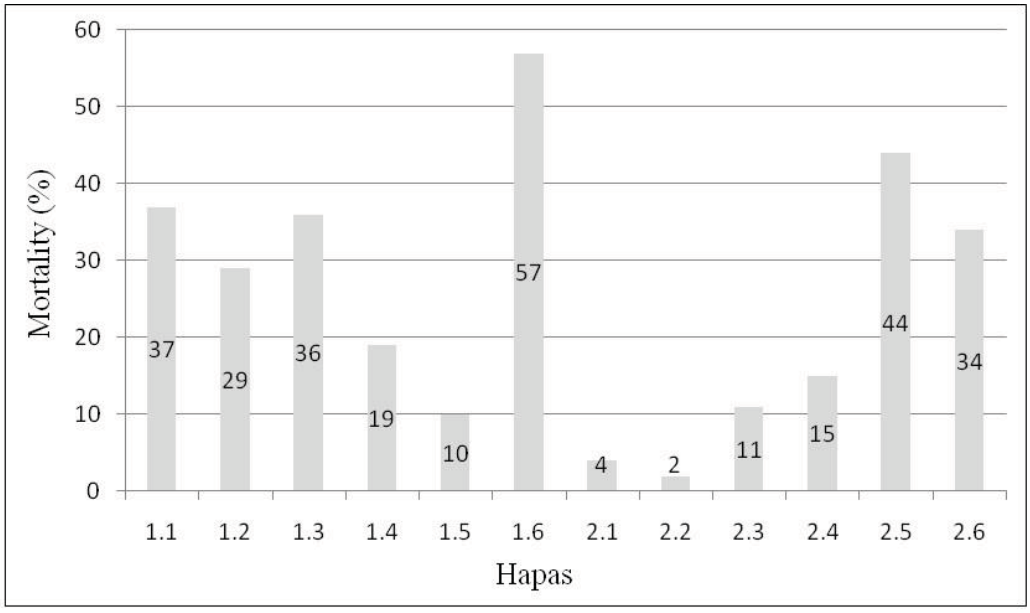

Fig. 10. Mortality of experimental shrimp in hapas in the two ponds. Numbers 1.1–1.6 indicate hapas 1–6 in Pond 1 and numbers 2.1–2.6 indicate hapas 1–6 in Pond 2.

Bacterial isolation and characterization

Seventy-four bacterial isolates (primarily *Vibrio* spp.) were obtained from the HP of shrimp specimens. Of these, 31 isolates were identified as *V. parahaemolyticus* by API 20E. PCR analysis detected the *thl* gene from isolates of *V. parahaemolyticus* (Fig. 11) but not the *thd* or the *trh* genes.

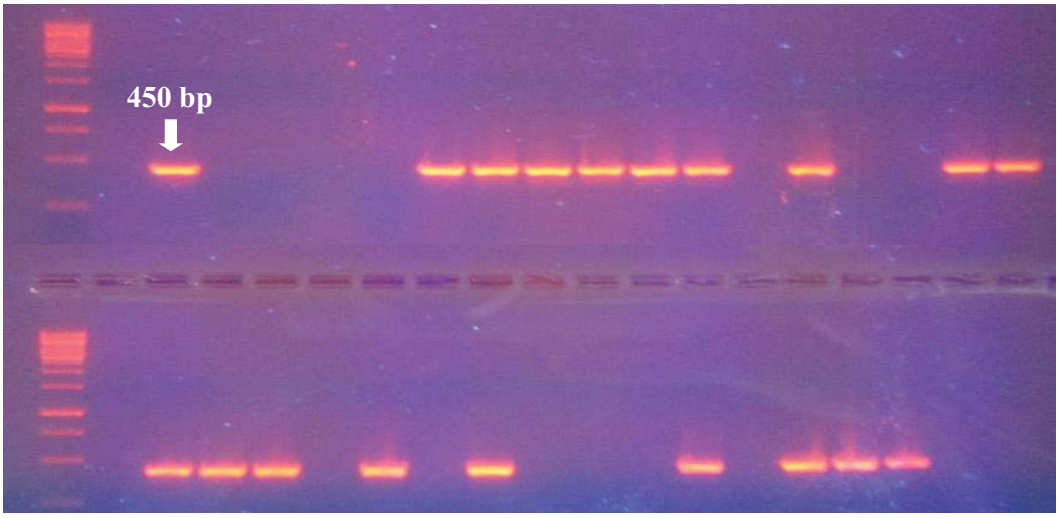

Fig. 11. Polymerase chain reaction (PCR) detection of *tlh* gene from isolates of *Vibrio parahaemolyticus*.

Discussion

This study indicates that AHPNS can be transmitted through infected water and via co-habitation. It also confirms that AHPNS is an infectious disease as reported by Tran et al. (2013). Although not statistically significant, the data obtained from the experiment suggest that the mortality rate in cohabitation was higher than that for water transmission only (average mortality of 26.7 % vs 13.7 %). Higher mortality in cohabitation may be due to AHPNS infections resulting from direct contact between healthy and infected shrimp, via water and/or by cannibalism.

Conclusion

AHPNS is transmissible both via water and by cohabitation of uninfected and infected shrimp in AHPNS-affected ponds. Healthy shrimp experimentally infected with AHPNS displayed pathological and histopathological characteristics typical of AHPNS as seen in natural infections in shrimp ponds. The mortality rate in cohabitation was higher than in water transmission alone. Isolates of *V. parahaemolyticus* from AHPNS-infected shrimp did not carry the *thd* of *trh* toxin genes.

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Actions Taken by Members of the Andean Community against Acute Hepatopancreatic Necrosis Disease (AHPND) in Shrimp Culture and Other Diseases that Affect Aquatic Animals

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Abstract

This document presents the activities carried out by the Member Countries of the Andean Community (CAN) to establish an Andean regional legal framework for the prevention, surveillance, control and eradication of aquatic animal diseases and the Andean Contingency Plan against early mortality syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND) of cultured shrimp.

Keywords: AHPND, Andean Community, contingency planning, disease prevention and control, EMS, legal framework

Introduction

Acute hepatopancreatic necrosis disease (AHPND) is among those diseases and syndromes of penaeid shrimp that have been included within the generalized term "early mortality syndrome" (EMS). It is a disease that causes high mortalities in farmed shrimp, seriously affecting production in affected countries. The disease first appeared in the People's Republic of China around 2009 and was called covert mortality disease. It has since been reported from Viet Nam, Malaysia, Thailand, Mexico and the Philippines, is suspected to be present in India, and is also suspected in, but unreported from other countries in both Asia and Latin American and the Caribbean (LAC).

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The devastation caused by AHPND in Asian shrimp farms awakened the concern of LAC countries and prompted the General Secretariat of the Andean Community (la Comunidad Andina, CAN) (Bolivia, Colombia, Ecuador and Peru) and the Servicios Oficiales de Sanidad de los Animales Acuáticos (SOSAA) to convene meetings and discuss joint work to develop actions to prevent the entry of diseases affecting aquatic animals of interest to the region. During the work carried out in the various meetings and based on the provisions of Article 88 paragraph f) of the Cartagena Agreement, which states that countries should "Establish and execute common provisions and programs on plant and animal health", CAN countries agreed to work on an Andean plan to evaluate joint actions on AHPND affecting shrimp farming, with a view to preventing entry of this disease in cultured penaeid shrimp and other crustaceans in Member Countries.

Decision 808 of 2016 by the Commission of the Andean Community

The result was the issuance of Decision 808 of 2016 by the Commission of the Andean Community, which approved Measures "on the prevention, surveillance, control and eradication of diseases in aquatic animals". The objective is to establish an Andean regional legal framework for the prevention, surveillance, control and eradication of diseases in aquatic animals; approve the Andean Contingency Plan against Acute Hepatopancreatic Necrosis Disease (AHPND) of farmed shrimp; and harmonize measures and national legislation of Member Countries on this matter.

Scope of the Decision

The provisions of the Decision are applicable to all species and products capable of acquiring or transmitting important diseases of aquatic animals in the entire territory of Member Countries.

Objectives of the Decision

The following are the objectives of the Decision:

a) Prevent or mitigate the risk of introduction and spread of aquatic animal diseases;
b) Carry out Andean surveillance plans for early detection of aquatic animal diseases of importance to the region;
c) Develop programmes for the control and eradication of major diseases of aquatic animals;
d) Achieve and maintain the health status of the country or zones free of the diseases of importance to the region;
e) Strengthen cooperation and technical assistance between Member States and with other countries in the field of aquaculture and health management;
f) Contribute to food security;
g) Promote health management programmes throughout the production chain; and
h) Facilitate trade in species of aquatic animals and their products while ensuring compliance with health regulations.

The Decision also provides that the Official Services of Aquatic Animals (SOSAA) of the Member Countries are the competent and internationally recognized authorities for the management, supervision and implementation of activities related to aquatic animal health in Member Countries, and responsible for implementing and complying with the provisions of the Decision. The SOSAA will promote participation and coordination with various public authorities and the private sector to formulate and implement programmes and activities identified in the Decision. Another issue covered by the Decision is the establishment and adoption of the list of diseases of aquatic animals notifiable to the World Organisation for Animal Health (OIE) and those that are considered important to the Andean Region. Thus, CAN countries should incorporate into their procedures the obligation of timely notification in compliance with OIE guidelines and provisions. Provisions should also include systems to mitigate the economic effects arising from the application of sanitary measures.

Structure of the Andean Community

The CAN is composed of a number of organizations and institutions that are articulated in the Andean Integration System, better known as the SAI, a system allowing the CAN to function almost as does a state. Under the SAI, each organization has its role and specific functions, such as:

- The Andean Presidential Council, formed by the presidents of Bolivia, Colombia, Ecuador and Peru, is in charge of the political leadership of the CAN.
- The Andean Council of Foreign Ministers formulates foreign policy of the Andean countries on issues related to integration and, if necessary, coordinates joint positions in international forums and negotiations.
- The Commission, composed of plenipotentiaries or delegates with full powers, is responsible for formulating, implementing and evaluating integration policy on trade and investment and creates standards that are mandatory for the four countries.
- The General Secretariat administers and coordinates the integration process.
- The Andean Court of Justice controls the legality of acts of all the organs and institutions and solves existing disputes between countries, between citizens or between countries and citizens when the agreements reached within the framework of the Andean Community are not met.
- The Andean Parliament, made up of 20 parliamentarians elected by popular vote (five for each Member Country), is the body that represents the people (i.e. Andean citizens in general). It deliberates on Andean integration and proposes regulatory actions to strengthen integration.
- The consultative bodies of civil society are also part of the SAI, such as Indigenous Peoples, Workers and Employers.
- Simon Bolivar Andean University, with several locations in the region, is the educational institution.
- Financial institutions are the Andean Development Corporation and the Latin American Reserve Fund.

Development of the Andean Contingency Plan Against AHPND

The Andean Contingency Plan Against AHPND (the Plan) will be the reference document in the Andean region to develop a *Technical Manual for the Implementation of the Andean Contingency Plan Against AHPND* (the Technical Manual) containing recommended sanitary actions according to the recommendations of the OIE and the Food and Agriculture Organization of the United Nations (FAO). The document will guide the actions of the SOSAA and producers to prevent the entry of diseases or their control and eradication. The Plan includes the following:

- The Introduction, which presents the current state of aquaculture at the international and regional level
- History of the disease, the aetiological agent, the affected species and impact of the disease
- Laws in each country to implement the Andean Contingency Plan
- Responsibilities of SOSAA in monitoring compliance with the Plan
- Guidelines in preparing the Technical Manual
- Preparation of subregional regulations
- Establishment of the list of notifiable diseases
- Raising funds to implement the Plan and its accompanying Technical Manual

Under the Andean Contingency Plan, there is also a need to strengthen the technical capacity of SOSAA, which includes capacity-building in disease diagnosis (i.e. histopathology and molecular techniques) and control, and risk analysis; cooperation with international laboratories; participation in conferences, practical exercises, forums; support for scientific research; and development of public and private programmes on better management practices (BMPs).

Another aspect of the Plan is the establishment and operation of a surveillance system for disease control, including reporting of results. This requires each Member Country to establish protocols for surveillance in seaports, airports and at border crossings; conduct surveillance and control campaigns in production areas, with focus on BMPs; and establish a early-warning system and conduct continuous monitoring until the case is resolved.

Finally, it is recognized that there is a need for training programmes, dissemination of good aquaculture practices (GAPs) suited to the needs of each country, and the conducting of awareness campaigns for all stakeholders in the production chain about the transmission of infectious diseases in aquatic animals in order to prevent disease introduction and outbreaks.

Technical Manual for the Implementation of the Andean Contingency Plan Against AHPND

The Technical Manual is envisaged as the document containing the strategic actions for prevention of disease introduction. The plan for the document was approved through the Resolution Secretaría General de la CAN – SGCAN 1851 of 2016.

The prevention strategy focuses on the preservation of the health and sanitary status of the region as being free of AHPND. It includes strengthening of the technical capabilities of the SOSAA of each Member Country through training programmes for the prevention and detection of disease, as well as programmes for GAPs and biosecurity measures. It also includes building awareness among producers and civil society in order to improve the health status of aquaculture establishments and prevent the entry of diseases. Both producers and consumers need to know the disease risk in shrimp farming and how it negatively affects the socio-economic development of the shrimp culture sector and its trade.

The Technical Manual will also include intervention strategies to develop actions required to control, eradicate and report any confirmed case of AHPND should this important disease occur in CAN countries.

The following presents the general outline that will be used for the Technical Manual:

I. GENERALITIES

1. Introduction

2. Objective

2.1 Specific objectives

3. Legal framework

4. Competent Authorities

4.1 Responsibilities

4.2 Functions

- Functions of those responsible for the Plan
- Functions of the Epidemiological Surveillance Team
- Functions of the Education Health and Communication Team
- Functions of the Control and Eradication Team
- Functions of the Diagnostic Team
- Functions of the Administrative Support Team

5. Characterization of the disease

6. Identification of risk factors

7. Detection of the disease

7.1 Clinical signs

7.2 Detection techniques

- Histopathology
- Polymerase chain reaction (PCR)

II. PREVENTION STRATEGIES

1. Actions for surveillance

1.1 Case definition

1.2 Disease notification

1.3 Location of areas and establishments at risk

1.4 Sampling

1.5 Surveillance of borders

2. Strengthening of biosecurity

2.1 Awareness and training in good practices

2.2 Biosecurity measures recommendations

- Operational staff and farm personnel
- Farms and culture establishments
- Visitors

III. INTERVENTION STRATEGY

1. Suspected report

2. Diagnosis of disease

2.1 National laboratories

2.2 International Reference Laboratory

3. Control

4. Eradication

4.1 Depopulation and disinfection

4.2 Sanitary disposal

5. Sentinelization and repopulation

6. Indemnification and compensation

GLOSSARY

FORMS

REFERENCES