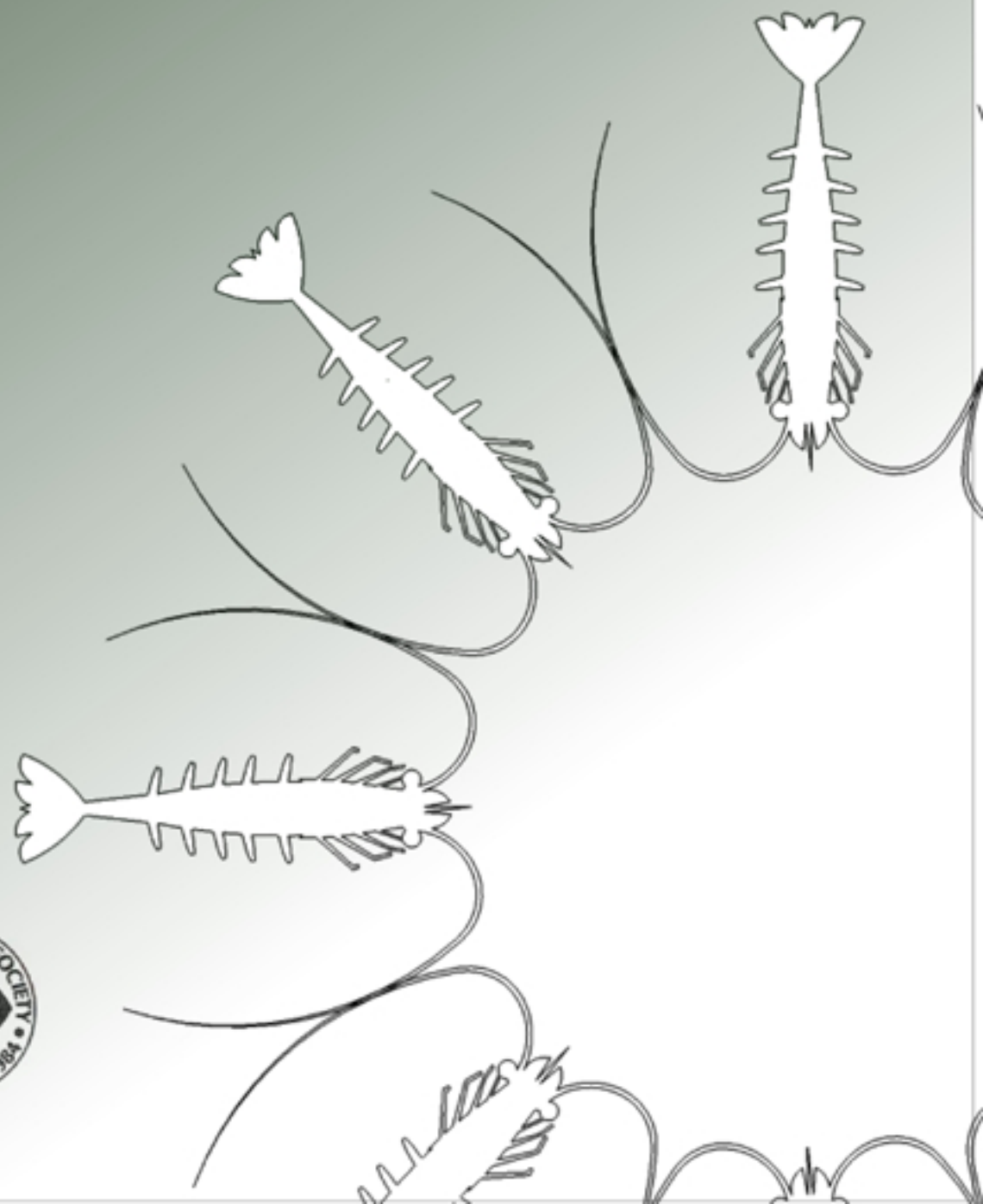
# Integrated Technologies for Advanced Shrimp Production



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# Integrated Technologies for Advanced Shrimp Production

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# PREFACE

This special issue of the Asian Fisheries Science consists of 12 papers presented at the Symposium on Integrated Technologies for Advanced Shrimp Production which was held in October 13-15, 2009 in the East-West Centre, University of Hawaii and sponsored by a grant from the National Oceanic and Atmospheric Administration (NOAA) to the Oceanic Institute (grant # NA07NMF4440354).

The special issue includes contributions from renowned scientists who are no strangers to the shrimp farming world. Together, they present the latest in shrimp farming, dealing with cutting edge technologies on topics ranging from genetics, to environmental and health management.

I would acknowledge Dr Cheng-Sheng Lee, Executive Director of Center for Tropical and Subtropical Aquaculture of Oceanic Institute, for initiating this collaborative effort with the Asian Fisheries Society. Dr Shi-Yen Shiau the immediate past Editor of the Asian Fisheries Science for his initial role in framing the collaboration. My deepest appreciation goes to NOAA and CPF Malaysia for sponsoring the publication of this special issue.

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# The global status of significant infectious diseases of farmed shrimp

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## Abstract

A discussion of the global status of shrimp diseases might best begin with a review of those diseases that are listed by the International Office des Épizooties (OIE). The OIE is also known as the World Animal Health Organization. The OIE is an international organization formed in 1924 in Europe by 28 countries in an effort to more effectively manage certain diseases of livestock. As of June 2010, the OIE consists of 176 member countries. The OIE was designated in 1995 by the newly formed World Trade Organization as the scientific reference body for animal health as it relates to international trade issues. The OIE has, among its functions, the listing of diseases (terrestrial and aquatic) which may pose risks of being transferred to new regions or nations as a consequence of global trade. Because of their economic importance and their potential for transfer with live or dead crustacean commodities, the OIE listed nine crustacean diseases in 2009. Of these nine listed diseases, six are diseases of penaeid shrimp (five viral and one bacterial); the seventh is a viral disease of *Macrobrachium rosenbergii*, the eighth is a disease of farmed spiny (*Panulirus* spp.) lobsters and it is due to infection by a rickettsial-like bacterium and the ninth listed disease affects freshwater crayfish and it is due to infection by a phycomycetous fungus. Two of the nine diseases were listed by the OIE as "under study" in the 12th Edition of the OIE Aquatic Animal Health Code. These diseases were necrotizing hepatopancreatitis (NHP), a rickettsia-like bacterial disease of penaeid shrimp which was approved for full listing in May 2010 and milky hemolymph disease (MHD) of spiny lobsters, which also has as its etiological agent a rickettsial-like bacterium and which may remain "under study" pending further consideration for full listing or removal from the list. While OIE listing gives these diseases global recognition, especially in relation to trade of crustacean commodities (e.g. live, dead or commodity products made from crustacean hosts for one or more OIE listed disease agents), there are other emerging diseases that are not listed by OIE that are also important locally and in some cases globally, to the global shrimp farming industry. Included in this review are the current OIE listed diseases of penaeid shrimp and several examples of emerging diseases which are of potential importance globally.

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## Introduction

Penaeid shrimp aquaculture is an important industry in Asia and the Americas that employs millions of people and provides valuable foreign exchange to many developing nations. While the industry is now dominated by the culture of the Pacific white shrimp, *Litopenaeus vannamei* (the shrimp taxonomy used in this paper is according to Perez Farfante and Kensley 1997), there is also significant culture of black tiger (*Penaeus monodon*) and Indian white shrimp (*Fenneropenaeus indicus*), which contribute significant quantities of shrimp to the global market (FAO 2006, 2009). Since shrimp aquaculture became a significant commercial entity in the 1970s, disease has had a major impact on the industry nearly everywhere it has been developed (Lightner 1996a, 1999; Flegel and Alday-Sanz 1998; Flegel 1997, 2006; Bondad-Reantaso et al. 2001; OIE 2009a, 2009b). Diseases due to viruses, rickettsial-like bacteria, true bacteria, protozoa and fungi have emerged as significant diseases of farmed shrimp (Lightner 1996a). Many of the bacterial, fungal and protozoan which caused diseases are managed using improved culture practices, routine sanitation, chemotherapeutics and, recently, the use of probiotics. Some bacterial diseases of farmed shrimp are increasingly difficult to manage, especially with the restriction of antibiotic use in farmed shrimp intended for certain markets.

The virus diseases have been far more problematic to manage and they have been responsible for the most costly epizootics. Examples of those with the most important socioeconomic impacts include the Taura syndrome and yellow head disease pandemics that began in 1991-92 when these diseases emerged in Ecuador, Thailand, respectively and the white spot disease pandemic that emerged in East Asia at about the same time. The most important diseases of cultured penaeid shrimp, in terms of economic impact, in the Americas (and in Asia) have viral agents as their cause (Flegel and Alday-Sanz 1998; Flegel 1997, 2006; Lightner 1999; Walker and Mohan 2009; OIE 2009a, 2009b). Of significance is that some of the most important diseases (and their etiological agents) were once limited in distribution to either the Western or Eastern Hemisphere (Lightner 1996a, 1996b, 2003a, 2003b; Flegel and Alday-Sanz 1998; OIE 2009b; Walker and Mohan 2009). However, the international movement of live (for aquaculture) and dead (commodity shrimp for reprocessing, direct retail commerce and for use as bait by sport fishermen) shrimp have been implicated or suspected as being responsible for the transfer and establishment of certain pathogens from Asia to the Americas (Lightner 1996b; Durand et al. 2000; AQUIS 2000; Hasson et al. 2006; Walker and Mohan 2009). While frozen commodity shrimp have been implicated as the route by whichwhite spot syndrome virus (WSSV) was moved from Asia to the Americas, TSV was moved in the opposite direction with infected live broodstock from Central America (Nunan et al. 1998a; Tu et al. 1999; Yu and Song

2000; Durand et al. 2000; Tang and Lightner 2005; Walker and Mohan 2009). Because of their socioeconomic importance and their significance to shrimp farming, five of the seven crustacean diseases listed by the World Animal Organization (OIE) in 2009 were virus diseases of shrimp (Fig. 1). Two additional diseases of crustacean, necrotizing hepatopancreatitis (NHP) of shrimp and milky hemolymph disease (MHD) of spiny lobsters were listed in the 12th Edition of the OIE Aquatic Animal Health Code (OIE 2009a) as "under study" and were being considered for full listing by the OIE. Both NHP and MHD have bacterial etiologies. NHP was approved for full listing by the OIE in May 2010 while MHD may remain listed as "under" study pending further consideration for full listing or removal from the list (OIE 2009a; OIE 2010). MHD (also called milky hemolymph syndrome - MHS) is also a disease affecting farmed penaeids and captive-wild crabs. In farmed *P. monodon* in East Asia and Madagascar, MHD has caused significant epizootics recently (Nunan et al. 2010). Should it continue to emerge in that region and spread to other regions, the possible full listing of MHD in spiny lobsters might be expanded to shrimp.

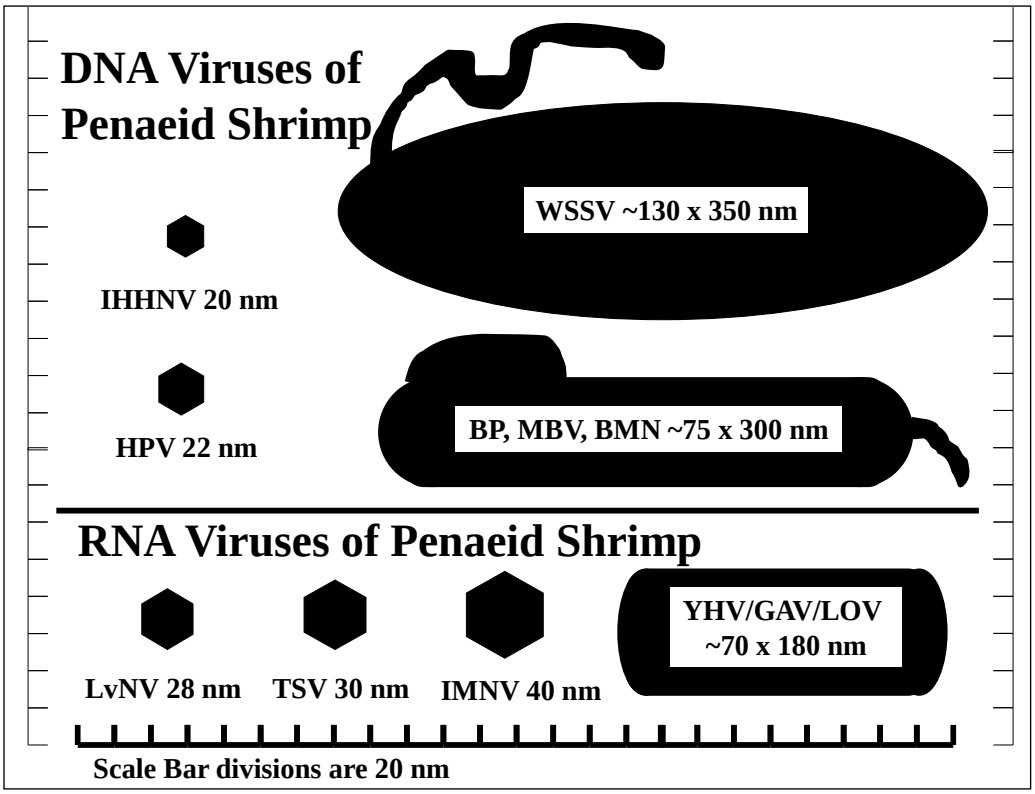


**Fig. 1.** Schematic of the major viruses of penaeid shrimp. The virions are drawn to scale;scale divisions are 20 nm. See text for definition of the acronym shown for each virus.

As a consequence of the rapid growth and development of the penaeid aquaculture industry, many of the most significant shrimp pathogens were moved from the regions where they initially appeared to new regions even before the "new" pathogen had been recognized, named, proven to cause the "new" disease and before reliable diagnostic methods were developed. The diseases due to the shrimp viruses IHHNV, TSV and WSSV were all transferred with live shrimp stocks from country to country and from one continent to another, well before their etiology was understood and diagnostic methods were available. With some diseases, the introduced pathogen encountered totally naive hosts with little or no innate resistance. The pandemics due to the penaeid viruses WSSV and TSV and to a lesser extent to IHHNV, IMNV and YHV have collectively cost the penaeid shrimp industry billions of dollars in lost crops, jobs and export revenue (Lightner 2003a; Walker and Mohan 2009). The social and economic impacts of the pandemics caused by these pathogens have been profound in countries in which shrimp farming constitutes a significant industry and this has led to the listing of several of the virus diseases of penaeid shrimp by the OIE or World Animal Health Organization (OIE 2009a).

## World Animal Health Organization (OIE) and Listed Diseases

The World Animal Health Organization (or Organization des Epizooties or OIE) was founded in 1924 in response to the need to control Rinderpest, which first occurred in Europe as a consequence of transfer of zebus being shipped from India to Brazil through a seaport in Belgium. In 1924, there were 28 member countries. By 2010, OIE was composed of 176 member countries. In 1955, the World Trade Organization (WTO) was created to replace the GATT (General Agreement on Trade & Tariffs) that was established in 1947 to facilitate international trade following World War II. WTO's SPS (Sanitary and Phytosanitary Measures) Agreement recognized the OIE as the leading international standards-setting organization for animal health and animal diseases that are transmissible to humans (zoonosis) (OIE 2009c).

The OIE has three principal functions, which are: 1) to inform members of the occurrence and course of animal diseases throughout the world and of means of controlling these diseases; 2) to coordinate international research devoted to the surveillance and control of animal diseases; and 3) to promote the harmonization of health regulations for trade in animals and animal products among members. Among the components of the OIE are four specialists' commissions that work to help the OIE meet these three functions. The Biological Standards Commission has as part of its mandate the responsibility to establish and approve methods for diagnosing OIE listed terrestrial animal (mammal, bird and bee) diseases and for testing of biologics (vaccines) for disease control purposes. The Aquatic Animal Health Standards

Commission (AAHSC) has similar functions for finfish, mollusks, crustaceans and amphibians. These methods are published as the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals and the Manual of Diagnostic Tests for Aquatic Animals. The diagnostic manuals are published at 3-4 year intervals. Another commission, the Terrestrial Animal Health Standards Commission and the AAHSC, annually produce and publish the Terrestrial Animal Health Code and the Aquatic Animal Health Code, respectively, to ensure that these codes reflect current scientific information on OIE listed diseases. The fourth OIE commission, the Scientific Commission for Animal Diseases, identifies the most appropriate strategies and measures for disease prevention and control. This commission also reviews requests for official OIE "disease-free status" for four major diseases of terrestrial food animals (FMD, Rinderpest, CBPP and BSE) and it reviews and publishes in the OIE Bulletin "self" declarations for "disease-free status" from member countries (OIE 2009d).

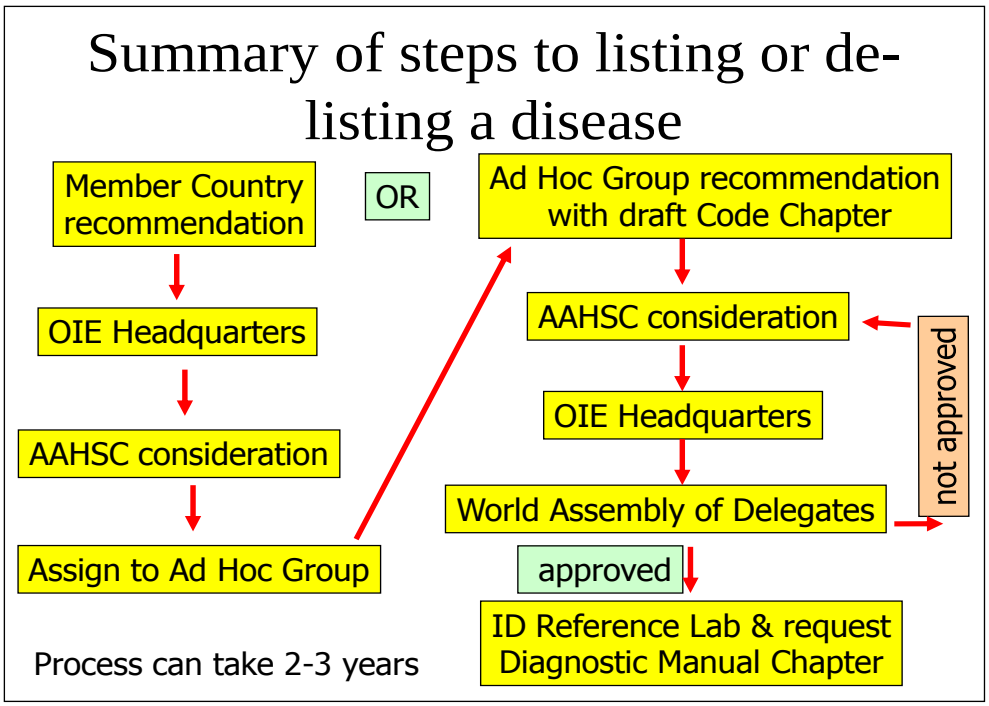


**Fig. 2.** A summary of the steps to listing (or de-listing) a disease by the OIE. The process begins with a recommendation that a disease be listed that may come from a Member Country, from an ad hoc group of experts appointed by the Aquatic Animal Health Standards Commission (AAHSC), from an OIE Reference Laboratory, or from OIE. The process can take 2-3 years and the disease in question becomes listed (or de-listed) when the recommendation is approved by the World Assembly of Delegates.

The OIE specialist commissions also have the mandate of ensuring that listed diseases reflect current scientific information. For the AAHSC, this means that it has the responsibility of either adding or deleting listed diseases depending on the most

current and available scientific information. To do this, the AAHSC forms ad hoc groups (AHGs) of specialists that assess the available information for disease(s) under consideration to determine if the criteria for listing is as defined in Articles 1.2.2.1 or 1.2.2.2 of Chapter 1.2.2 of the Aquatic Animal Health Code (OIE 2009a). The AHGs provide recommendations to the AAHSC for consideration and when the Commission agrees with an AHG's recommendations, it recommends to the OIE Central Bureau and to the International Commission (composed of delegates from all OIE member countries) that a disease be listed (Fig. 2). In the case of those diseases which no longer justify being listed by OIE (e.g. it no longer meets the criteria of Article 1.2.2.1 of the Aquatic Code), the AHG and the AHHSC can recommend to the OIE Central Bureau and to the International Commission that one or more specific diseases be removed from the list. With this background stated about the functions of the OIE and its listed diseases, the list of diseases (and de-listed diseases) of Crustaceans for 2009-2010 is given in Table 1 (OIE 2009d).

**Table 1.** OIE listed crustacean diseases for 2009-2010 and those de-listed in 2009 (OIE 2009a; OIE 2010)

| Disease Name | Agent | Agent Classification & Type |
|---|---|---|
| Taura syndrome (TS) | Taura syndrome virus (TSV) | *Dicistroviridae*; ssRNA |
| White spot disease (WSD) | White spot syndrome virus (WSSV) | *Nimaviridae*; dsDNA |
| Yellowhead disease (YHD) | Yellowhead virus (YHV) & gill associated virus (GAV) | *Roniviridae*; ssRNA |
| Infectious hypodermal & hematopoietic necrosis (IHHN) | IHHN virus (IHHNV) | *Parvoviridae*; ssDNA |
| Infectious myonecrosis (IMN)[1] | IMN virus (IMNV) | *Totiviridae*; dsRNA |
| White Tail Disease (WTD)[1] | WTD virus (MrNV) | *Nodaviridae*; ssRNA |
| Tetrahedral baculovirosis[2] | *Baculovirus penaei* (BP) | *Baculoviridae*; dsDNA |
| Spherical baculovirosis[2] | Monodon baculovirus (MBV) | *Baculoviridae*; dsDNA |
| Necrotizing hepatopancreatitis (NHP)[4] | NHP-bacterial (NHP-B) | Alpha proteobacteria |
| Crayfish plague | *Aphanomyces astaci* | Phycomycete fungus |
| Milky hemolymph disease (MHD) of spiny lobsters[3] | MHD rickettsial-like bacteria | Rickettsial-like bacteria |

[1] Listed by OIE, May 2007; [2] De-listed by OIE in May 2009; [3] Listed as "under study" by OIE; [4] Listed by the OIE in May 2010.

The 2009-2010 list of crustacean diseases listed as notifiable to the OIE consisted of five virus diseases of penaeid shrimp (TS, WSD, YHD, IHHN and IMN), one virus disease of freshwater prawns (WTD) and one fungus disease of freshwater crayfish. Two virus diseases of penaeid shrimp (tetrahedral baculovirosis and spherical baculovirosis) were de-listed by the OIE in May 2009 because control of both diseases

progressed to the point where, for the purposes of international trade, neither disease fully met the listing criteria in Chapter 1.2.2 of the Aquatic Animal Code (OIE 2008a, 2009a). Of the two bacterial diseases (necrotizing hepatopancreatitis – NHP, a bacterial disease of penaeid shrimp and milky hemolymph disease of spiny [*Panulirus* spp.] lobsters) that were listed as "under study" for possible future listing by the OIE, NHP was approved by the OIE for full listing in May 2010 (OIE 2010) (Table 1).

This paper provides a brief review of the current status of the virus diseases due to TSV, WSSV, IHHNV, IMNV and YHV in the Americas in terms of their biology, history, distribution, production impacts and diagnostic methods. There is little new information on baculovirus-caused diseases that is not well covered in other reviews (Lightner 1996a; Bondad-Reantaso et al. 2001; Flegel 2006; OIE 2006b; Walker and Mohan 2009). Hence, not included in this review will be information on the baculovirus caused diseases caused by monodon baculovirus (MBV), *Baculovirus penaei* (BP) and baculoviral midgut gland necrosis (BMN). Also reviewed is some of the current information of the bacterial diseases necrotizing hepatopancreatitis (NHP), milky hemolymph syndrome/disease (MHS or MHD) and Streptococcosis.

## Taura Syndrome and Taura Syndrome Virus (TSV)

### *Biology of the agent*

Taura syndrome is caused by Taura syndrome virus (TSV), a small, simple RNA virus. The virion is a 32 nm diameter, nonenveloped icosahedron with a buoyant density of 1.338 $g \cdot mL^{-1}$ (Table 1; Fig. 1). The genome of TSV consists of a linear, positive-sense single-stranded RNA of 10, 205 nucleotides. The TSV genome contains two large open reading frames (ORFs). ORF 1 contains the sequence motifs for nonstructural proteins, such as helicase, protease and RNA-dependent RNA polymerase. ORF 2 contains the sequences for TSV structural proteins, including the three major capsid proteins CP1, CP2 and CP3 (40, 55 and 24 kDa, respectively) (Bonami et al. 1997; Mari et al. 1998; Mari et al. 2002; Robles-Sikisaka et al. 2001). The virus replicates in the cytoplasm of host cells. Based on its characteristics, TSV has been assigned by the International Committee on Taxonomy of Viruses (ICTV) to the newly created genus *Cripavirus* in the new family *Dicistroviridae* (in the "superfamily" of picornaviruses) (Mayo 2002a, 2000b).

Physicochemical and more recent molecular studies of TSV suggest that a single strain of the virus was present in the initial TSV pandemic in the Americas, but that new strains, which differ in host range and virulence, are emerging (Yu and Song 2000; Zarain-Herzberg and Ascencio-Valle 2001; Erickson et al. 2002; Chang et al.

2004; Tang and Lightner 2005; Nielsen et al. 2005; Wertheim et al. 2009). Curiously, the strain of TSV in the early diagnostic cases from the Americas reacts with the monoclonal antibody MAb1A1, but more recently, emerged genetic variants of TSV from the Americas do not react with the antibody in Western blot transfers or in IHC (immuno-histochemistry) preparations with paraffin embedded TSV infected tissues (Erickson et al. 2002; Erickson et al. 2005). MAb1A1 reacts with CP2, which is the most variable of the three TSV capsid proteins. Genotyping of TSV has been based on variations in the sequence of CP2 (Tang and Lightner 2005; Wertheim et al. 2009). Comparison of the cDNA sequence of TSV CP2 from approximately 80 TSV isolates in the author's collection shows that there are currently four distinct strains (or genetic variants) of the virus (Tang and Lightner 2005; Wertheim et al. 2009).

***History, hosts and geographic distribution of Taura syndrome (TS)***

TSV emerged from an unknown source in Ecuador in 1991. The disease was recognized as a major new disease of farmed *L. vannamei* by early 1992 and it was named 'Taura syndrome' (Jimenez 1992; Lightner 1996a, 2003a, 2003b; Brock et al. 1995, Brock 1997; Lightner et al. 1995). The viral etiology of TS was confirmed in 1994 and the virus was named Taura syndrome virus (TSV) (Hasson et al. 1995). In the interest of supporting litigation brought by a group of Ecuadorian shrimp farmers against several international pesticide companies, whose products had been implicated as the cause of a toxicity syndrome they called 'Taura syndrome', Intriago et al. (1997) and Jimenez et al. (2000) reported on the epizootiology of the disease in Ecuador and suggested that TSV be assigned the synonym 'infectious cuticular epithelial necrosis virus (ICENV)' to distinguish it from the 'Taura syndrome' with a putative toxic etiology (Jimenez et al. 2000).

The principal host for TSV is the Pacific white shrimp, *L. vannamei*, although other species can be infected and present the disease (Aguirre Guzman and Valle 2000; Hasson et al. 1995, 1999a, 1999b; Lightner 1999; Overstreet et al. 1997; Robles-Sikisaka et al. 2001; Srisuvan et al. 2006). Cumulative mortalities due to TSV epizootics have ranged from 40 to >90% in cultured populations of postlarval (PL), juvenile and subadult *L. vannamei*. TS is best known as a disease of nursery- or grow-out-phase in *L. vannamei* that occurs within ~14 to 40 days after stocking postlarvae into grow-out ponds or tanks; hence, shrimp with TS are typically small juveniles of ~0.05 g to <5 g. Larger shrimp may also be affected, especially if they are not exposed to the virus until they are larger juveniles or subadults. Survivors of TSV infections may carry the virus for life (Brock et al. 1995, 1997; Hasson et al. 1999a, 1999b; Lightner 1996a, 1996b; Lotz 1997b). In regions where the virus is enzootic in farmed stocks, the prevalence of chronic phase TSV infections has been found in various

surveys to range from 0 to 100% (Brock 1997; Jimenez et al. 2000; Laramore 1997). TSV can also infect other Western Hemisphere penaeid species (i.e. *L. stylirostris, L. setiferus* and *L. schmitti*), sometimes resulting in disease and mortalities in PL or early juvenile stages, but also in asymptomatic persistent infections (Brock et al. 1997; Overstreet et al. 1997). Other Western Hemisphere penaeids (*Farfantepenaeus aztecus* and *Fa. duorarum*) and Eastern Hemisphere penaeids (*Fenneropenaeus chinensis*, *P. monodon* and *Marsupenaeus japonicus*) have been experimentally infected with TSV, but appear not to develop clinical disease (Brock et al. 1997; Overstreet et al. 1997; Flegel 2006).

Transmission of TSV may be by horizontal or vertical routes. Horizontal transmission by cannibalism, or by contaminated water, has been demonstrated (Brock, 1997; Hasson et al. 1995; Lightner 1996a, 1996b; Lotz 1997b; Overstreet et al. 1997; White et al. 2002). Vertical transmission from infected adult broodstock to their offspring is strongly suspected but has not been experimentally confirmed (Lightner 1996a, 2003a).

By 1994, when the viral etiology of TS had been established, the virus had been moved with live shrimp transfers to many of the shrimp growing countries of the Americas (Brock et al. 1995; Hasson et al. 1995, 1999a; Bonami et al. 1997; Lightner 1996a, 1996b, 2003a). While wild postlarvae with TSV infections were reported found near shrimp farms with ongoing TSV epizootics (Lightner et al. 1995), TSV infections in wild shrimp have not been further documented, suggesting that TSV does not have a discernable impact on wild populations of shrimp (Brock 1997). In 1998, TSV was documented in Taiwan in infected stocks of *L. vannamei*, introduced for aquaculture purposes (Tu et al. 1999; Yu and Song 2000). Within a few years, TSV had been disseminated to most of the shrimp farming countries in SE Asia with trans-boundary movements of TSV infected *L. vannamei* (Phalitakul et al. 2006; OIE 2009b).

In addition to being moved from country to country with live shrimp, there is also evidence that TSV has the potential of being transferred in frozen TSV-infected shrimp products.

TSV has been found in frozen commodity shrimp (*L. vannamei*) products in samples from markets in the USA that originated in Latin America and Southeast Asia. Improper disposal of wastes (liquid and solid, i.e. peeled shells, heads, intestinal tracts, etc.) from value-added reprocessing of TSV-infected shrimp at coastal locations may provide a source of TSV that may contaminate wild or farmed stocks near the point of the waste stream discharge (Lightner 1996b; Nunan et al. 2004; OIE 2009b).

Shrimp eating birds and insects may be important factors in the transmission of TSV within shrimp farms and among shrimp farms within a geographical zone or region (OIE 2009b). TSV has been demonstrated to remain infectious in the feces of sea gulls that have ingested infected shrimp carcasses (Garza et al. 1997; Lightner 1999). The virus was demonstrated to remain infectious for up to 48 h (after ingestion of TSV infected shrimp carcasses) in the feces of wild or captive sea gulls (*Larus atricilla*) and chickens (*Gallus domesticus*, used as a laboratory surrogate for all shrimp-eating birds). These findings implicate birds as being an important mechanical vector for the transmission of the virus within affected farms or farming regions (Vanpatten et al. 2004). There is some evidence that flying aquatic insects, such as the water boatman (*Trichocorixa reticulata* [*Corixidae*]) that feed on shrimp carcasses can also serve as mechanical vectors of TSV (Brock 1997; Lightner et al. 1995, 1996b).

***Taura syndrome gross signs in susceptible host species***

Gross signs of Taura syndrome have been documented in all life stages (i.e. postlarvae, juveniles and adults) of *L. vannamei* except in eggs, zygotes and larvae (Brock and Main 1994; Lightner 1996a). Following experimental or natural infection, Taura syndrome has three distinct phases: acute, transition and chronic (Brock 1997; Hasson et al. 1999b). In disease outbreaks at farms the onset of mortality is often sudden and massive, with moribund shrimp coming to the pond surface or edges where large numbers of shrimp eating birds may be attracted to the dead and dying shrimp. Moribund shrimp in the acute phase of the disease typically present a pink to reddish coloration due to expansion of red cuticular chromatophores (especially in the tail fan), are off feed and with empty guts and they are lethargic. The acute phase is rapid in individual shrimp, probably lasting less than 24 h, but the acute phase in a shrimp farm pond may last for several days in an affected population (Brock 1997; Garza et al. 1997; Hasson et al. 1999b). As implied by its name, the transition (or recovery in some publications) phase of Taura syndrome is that phase of the disease when affected shrimp may resolve the lesions due to TSV infection that developed in the acute phase. Shrimp in the transition phase typically present randomly distributed variably sized melanized lesions in or under the cuticle (exoskeleton). Those shrimp that successfully resolve the acute phase TSV lesions and survive the next molting process typically appear normal. Death due to TSV infection during the three phases most often occurs in the acute phase, probably due to osmotic failure. In the transition phase of the disease, death may also occur due to osmotic failure as a consequence of widespread destruction of the cuticular epithelium and two localized or systemic infections by opportunistic bacteria (Lightner 1996a; Brock 1997). Shrimp in the chronic phase of Taura syndrome may carry the virus for life as a persistent infection (Lotz 1997b; Hasson et al. 1999b). While persistently infected *L. vannamei* may appear and behave

normally, they show slightly less tolerance to low salinity stress than uninfected shrimp (Lotz et al. 2005). Some members of populations of *L. vannamei* or *L. stylirostris* that survive TSV infections and/or epizootics may carry the virus for life and, although not documented, pass the virus on to their progeny by vertical transmission (Hasson et al. 1999a, 1999b; OIE 2009b).

### *Diagnostic methods*

Available methods for diagnosing infection by TSV include routine histology, in situ hybridization (ISH) with specific cDNA probes, antibody-based methods and cDNA amplification methods using standard and real-time PCR after a reverse transcription step (RT-PCR) to convert the viral ssRNA genome to cDNA (OIE 2009b). Of the available methods, RT-PCR is recommended in the OIE Manual of Diagnostic Tests for Aquatic Animals ("Aquatic Manual"; OIE 2009b) for disease surveillance and screening purposes. For TSV, disease surveillance/screening information can be applied to determine presence, absence or prevalence of infection in wild or cultured populations, in commodity products made from susceptible hosts and to support declarations of disease freedom (OIE 2009a, 2009b).

## White Spot Disease and White Spot Syndrome Virus (WSSV)

### *Biology of the agent*

The causative agent of white spot disease (WSD) is white spot syndrome virus (WSSV). WSSV is a very large, enveloped, double-stranded DNA (dsDNA) virus with a density of approximately 1.2 $g \cdot mL^{-1}$. WSSV was recently assigned by the ICTV to its own new genus, *Whispovirus* and family, *Nimaviridae* (Table 1; Fig. 1) (Mayo 2002a, 2002b). Virions are large (80-120 x 250-380 nm), rod-shaped to elliptical and with a trilaminar envelope (Wang et al. 1995; Durand et al. 1997; Inouye et al. 1994, 1996; Kanchanaphum et al. 1998; Nadala et al. 1998; van Hulten et al. 2001; Vlak et al. 2005; Greenwood et al. 2005). Negatively stained virions purified from shrimp hemolymph show unique, tail-like appendages (Wang et al. 1995; Fauquet et al. 2005). The virions are generated in hypertrophied nuclei of infected cells without the production of occlusion bodies (Lo et al. 1997). In initial reports, WSSV was described as a non-occluded baculovirus, but WSSV DNA sequence analysis has shown that it is not related to the baculoviruses (van Hulten et al. 2001; Yang et al. 2001). The size of the WSSV genome has been differently reported for different isolates: 305,107 bp (GenBank Accession No. AF332093), 292,967 bp (GenBank Accession No. AF369029) and 307,287 bp (GenBank Accession No. AF440570) for viruses isolated from the People's Republic of China, Thailand and Taipei, China, respectively. The

sequences of these three isolates are almost identical, with the size differences being due mostly to several small insertions and one large (~12 kbp) deletion. In accordance with a genome size of ~300 kb, a total of 531 putative open reading frames (ORFs) were identified by sequence analysis, among which 181 ORFs are likely to encode functional proteins. Thirty-six of these 181 ORFs have been identified by screening and sequencing a WSSV cDNA library, or have already been reported to encode functional proteins, many of which show little homology to proteins from other viruses (OIE 2009b).

Temperature was found to have a profound effect on the expression of disease in WSSV infected *L. vannamei* (Vidal et al. 2001; Granja et al. 2003). These authors found that at temperatures above 32 °C, WSD did not develop in WSSV infected *L. vannamei*. However, when the same shrimp were cooled to 25 °C, the disease would quickly develop with 100% mortality. Subsequent studies demonstrated that the hyperthermic phenomenon also occurred in other penaeids (Guan et al. 2003). Recent work has shown that replication of WSSV is significantly reduced or stopped under hyperthermic conditions (Du et al. 2006). These findings have helped to explain why WSD epizootics occur most often in the cooler seasons in most shrimp farming regions. In the Americas, that information has helped shrimp farmers manage WSD by avoiding stocking in the cool season and in some countries like Ecuador and Peru, growing shrimp year-round in temperature controlled greenhouses.

***History and geographic distribution of white spot disease***

WSSV has a wide host range among decapod crustaceans (Lo et al. 1996; Lo and Kou 1998; Flegel 1997; Flegel and Alday-Sanz 1998) and is potentially lethal to most of the commercially cultivated penaeid shrimp species (OIE 2009b). WSD, caused by WSSV, emerged in East Asia in 1992-93 and was quickly dispersed with infected seed and broodstock across the Asian continent to Southeast Asia and India where it caused a major pandemic and continues to cause significant losses in some regions. In Japan, WSD outbreaks were first reported from farmed *Marsupenaeus japonicus* in 1993 (Inouye et al. 1994, 1996; Nakano et al. 1994) and the causative agent was named penaeid rod-shaped DNA virus (PRDV) or rod-shaped nuclear virus of *M. japonicus* (RV-PJ). Later, outbreaks of viral disease with similar gross signs caused by similar rod-shaped viruses were reported from elsewhere in Asia and other names were applied: hypodermal and hematopoietic necrosis baculovirus (HHNBV) in the People's Republic of China (Huang et al. 1995a, 1995b); white spot baculovirus (WSBV) and PmNOBIII in Taipei China (Chou et al. 1995, Lo et al. 1996); and systemic ectodermal and mesodermal baculovirus (SEMBV) or PmNOBII in Thailand (Wongteerasupaya et al. 1995). The virus from the People's Republic of China has also been called Chinese

baculovirus (CBV) (Lu et al. 1997). Shrimp exhibiting the gross signs and histopathology of WSD have also been reported from Korea (Kim et al. 1998), India (Karunasagar et al. 1998), the Philippines and the USA (Lightner 1996a, 1996b; Durand et al. 2000). WSSV has even reached shrimp farms in southeastern Europe (1997) and the Middle East (1999) via live shrimp movements and Australia and Spain with introductions of frozen infected shrimp, which were used as fresh food for broodstock (OIE 2006b; Stentiford and Lightner "in press").

Beginning in 1999, WSD had a severe impact on the shrimp farming industries of both Central and South America (GAA 1999a, 1999b; Durand et al. 2000; Vidal et al. 2001; Lightner 2003a, 2003b; OIE 2009b). Despite the absence of evidence of live shrimp introductions from Asia to the Americas, WSSV was diagnosed at several sites between 1995-1997 in captive wild shrimp and crayfish and in cultured domesticated shrimp stocks in the eastern and southeastern U.S. (Nunan et al. 1998b; Durand et al. 2000; Lightner et al. 2001). Early in 1999, WSSV was diagnosed as the cause of serious epizootics in Central American shrimp farms. By mid to late 1999, WSSV was causing major losses in Ecuador (then among the world's top producers of farmed shrimp) and by 2000-2001, export of shrimp from Ecuador was down nearly 70% from pre-WSSV levels (Rosenberry 2001, 2003; Lightner 2003a). Although the documentation is sketchy, WSSV has been found in wild shrimp stocks in the Americas (Nunan et al. 2001; Chapman et al. 2004). In the US, the virus was successfully eradicated from shrimp farms and except for two confirmed outbreaks at an isolated shrimp farm on the Island of Kauai, Hawaii in 2004 and 2008 (CEI 2004; Ostrowski 2004; USMSFP 2008), WSSV has not been reported from farmed shrimp stocks grown in other regions of the USA since 1997. However, its sporadic detection in wild shrimp stocks (Pacific Coast of Panama, Gulf of Mexico and SE Atlantic states) (Nunan et al. 2001; Chapman et al. 2004; Hasson et al. 2006) suggests that WSSV has become established in wild penaeid shrimp stocks in coastal waters of the eastern Pacific and southeastern U.S. and the Gulf of Mexico, or that it continues to be introduced. Introduction is possibly from wastes (peeled shells, etc.) from value-added reprocessing of imported shrimp in coastal packing plants, or from infected shrimp used as bait by sport fishermen. It has been proposed that the introductions of WSSV to the Americas were the result of importation of frozen shrimp products from WSSV-affected areas of Asia and the value-added reprocessing of those frozen shrimp for the US market in coastal processing plants (Nunan et al. 1998b; Durand et al. 2000; Lightner et al. 2001; Lightner 2003a), or are possibly due to the use of imported frozen WSSV-infected shrimp as bait by sport fishermen (Hasson et al. 2006). WSSV also reached Spain and Australia in 2000-2001. In both cases, successful containment and eradication were reported and for both events the importation and use of infected frozen shrimp as a fresh feed for broodstock were implicated as the route of introduction (OIE 2003; Lightner 2003a). Regardless of

where they were obtained, isolates of WSSV have shown little genetic or biological variation, suggesting that the virus emerged and was spread from a single source (OIE 2003, 2006b, 2009b).

### *WSD gross signs and histopathology in Litopenaeus vannamei*

The gross signs, histopathology and diagnostic procedures (antibody-based and molecular) for WSSV infections have been thoroughly reviewed since the disease emerged (Lightner 1996a; Flegel 1997, 2006; Lightner and Redman 1998a, 1998b; Lightner 1999; Loh et al. 1997; Lo and Kou 1998; Greenwood et al. 2005; OIE 2006b, 2009b). The reader is referred to these reviews for additional details on the disease in Asia and elsewhere that are not included in the present review.

*Litopanaeus vannamei* acutely affected with WSD are reported to show a rapid reduction in food consumption, become lethargic and have a loose cuticle with some showing characteristic white spots 0.5 to 2.0 mm in diameter, which are most apparent on the inside surface of the carapace but may be present anywhere on the inner surface of the exoskeleton. The white spots represent abnormal deposits of calcium salts by the WSSV-infected cuticular epithelium. In many cases, moribund shrimp with WSD display a pink to reddish-brown coloration, due to expansion of the cuticular chromatophores and few, if any, white spots. Populations of shrimp showing these signs display high mortality rates with cumulative mortalities reaching 100% within 3 to 10 days of the onset of clinical signs (Lightner 1996a; OIE 2006b, 2009b).

### *Diagnostic methods*

Available methods for diagnosing infection by WSSV include routine histology, in situ hybridization (ISH) with specific DNA probes, antibody-based methods and DNA amplification methods using standard PCR and real-time PCR (OIE 2009b). Of the available methods, a nested PCR is recommended in the OIE Manual of Diagnostic Tests for Aquatic Animals (“Aquatic Manual”; OIE 2009b) for disease surveillance and screening purposes. For WSSV, disease surveillance/screening information can be applied to determining presence, absence or prevalence of infection in wild or cultured populations, in commodity products made from susceptible hosts and to support declarations of disease freedom (OIE 2009a, 2009b).

# Infectious Hypodermal and Hematopoietic Necrosis and IHHNVirus

### *Biology of the agent*

Infectious hypodermal and hematopoietic necrosis disease (IHHN) is caused by IHHN virus, which is the smallest of the known penaeid shrimp viruses. The IHHN virion is a 22 nm diameter, nonenveloped icosahedron (Table 1; Fig. 1) with a density of 1.40 $g \cdot mL^{-1}$ in CsCl. Its genome is linear single-stranded DNA of 4.1 kb in length. Because of its characteristics, IHHNV has been classified as a member of the *Parvoviridae* and a probable member genus *Brevidensovirus* (Bonami et al. 1990; Bonami and Lightner 1991; Mari et al. 1993; Nunan et al. 2000; Shike et al. 2000).

### *Hosts, history and geographic distribution of IHHN disease*

The disease IHHN and later its causative agent, IHHNV, was first described as the cause of acute epizootics and mass mortalities (> 90%) in juvenile and subadult *L. stylirostris* farmed in super-intensive raceway systems in Hawaii (Brock et al. 1983; Lightner 1983, 1988; Lightner et al. 1983a, 1983b; Brock and Lightner 1990). Shortly after its discovery in *L. stylirostris*, the virus was found in *L. vannamei* being cultured at the same facility in Hawaii and these *L. vannamei* were also shown to be generally asymptomatic carriers of the virus (Lightner et al. 1983b; Bell and Lightner 1984). Some members of populations of *L. stylirostris* and *L. vannamei* that survive IHHNV infections and/or epizootics may carry the virus for life and pass the virus on to their progeny and other populations by horizontal and vertical transmission (Bell and Lightner 1984; Lightner 1996a; Morales-Covarrubias et al. 1999, Morales-Covarrubias and Chavez-Sanchez 1999; Motte et al. 2003). IHHNV has been demonstrated by PCR (Motte et al. 2003) and by in situ hybridization with IHHNV specific probes to be vertically transmitted in the oocytes (Lightner 2011).

A few years after it was reported that *L. vannamei* can be infected with IHHNV and not cause significant mortalities (Lightner et al. 1983b; Bell and Lightner 1984), IHHNV was shown to be the cause of 'runt deformity syndrome' (RDS) in *L. vannamei* (Kalagayan et al. 1991). With RDS, affected shrimp present irregular, reduced growth, cuticular deformities and generally no remarkable elevation in mortality (Kalagayan et al. 1991; Browdy et al. 1993; Bray et al. 1994; Brock and Main 1994; Lightner 1996a). Hence, the economic and production impacts of IHHNV infection in *L. vannamei* are due to reduced and irregular growth and small count size shrimp at harvest, not to elevated mortality (OIE 2006b, 2009b; Lightner 2011). To mitigate this effect, several strategies have been used. With one strategy, selected lines of *L. stylirostris* were

developed that were not only resistant to IHHN disease but also refractory to infection (Tang et al. 2000; Dhar et al. 2001). IHHNV-free lines of *L. vannamei* were also developed as SPF (specific pathogen-free) lines and these stocks were the first developed in the SPF stock development program (Wyban et al. 1992; Pruder et al. 1995; Moss et al. 2002; Lightner et al. 2009a; Moss and Moss 2009).

After its initial discovery in cultured shrimp in Hawaii in 1981, IHHNV was subsequently found to be widely distributed in cultured shrimp in the Americas and in wild shrimp collected along the Pacific coast from Peru to Mexico. As of 2006, the only country in the Americas, which can claim to have IHHNV-free zones or compartments (OIE 2009a; Lightner et al. 2009a) is the United States. This was achieved with the development and use of SPF shrimp stocks (Pruder et al. 1995; Lightner et al. 2009a; Moss and Moss 2009). The introduction of IHHNV into shrimp farms in northwestern Mexico and wild shrimp stocks in Mexico's Gulf of California during the late 1980's and early 1990's resulted not only in significant losses in farmed *L. stylirostris*, but also contributed to the collapse of the northern Gulf of California'swild fishery for *L. stylirostris* that began in 1990 (Lightner et al. 1992a; Martinez-Cordova 1992; Lightner 1996b; Pantoja et al. 1999; Morales-Covarrubias and Chavez-Sanchez 1999; Morales-Covarrubias et al. 1999; Walker and Mohan 2009). A decade later, the *L. stylirostris* fishery of the northern Gulf of California had recovered sufficiently to support commercial fishing, but the prevalence of IHHNV infection in adult *L. stylirostris* collected from the northern Gulf fishery has remained high (80% to 100% females and 60% in males)(Morales-Covarrubias et al. 1999; Morales-Covarrubias and Chavez-Sanchez 1999). *L. stylirostris* collected from the Gulf remain unsuitable for aquaculture because they carry IHHNV and consequently, these stocks do not survive well in culture tanks or ponds.

IHHNV has been found to be widely distributed in wild and cultured *P.monodon* in East and Southeast Asia where it does not seem to cause production losses (Flegel 1997, 2006; Primavera and Quinitio 2000; Tang et al. 2003; Chayanburakul et al. 2005; Withyachumnarnkul et al. 2006). Molecular studies show considerable variation among Asian isolates of the virus (Tang et al. 2003; Krabsetsve et al. 2004; Tang and Lightner 2006), while little variation was found in isolates from the Americas (Tang and Lightner 2002). All isolates of IHHNV from the Americas are nearly identical with IHHNV from the Philippines. This finding, along with other aspects of its history and epidemiology of IHHN in the Americas, suggests that IHHNV was introduced from the Philippines, perhaps with live *P. monodon* that were imported in the early 1970's as a candidate aquaculture species during the very early development of shrimp farming in the Americas (Lightner 1996b; Tang and Lightner 2002; Tang et al. 2003).

In addition to the Americas/Philippines genotype of IHHNV, three other genetic variants of the virus have been documented. For the purposes of this review, the IHHNV genotype from the Americas/Philippines genotype will be designated as IHHNV-I; the variant from Southeast Asia will be designated IHHNV-II; and the IHHNV variant from East Africa, Madagascar and Mauritius and Australia will be referred to as IHHNV-III. The first two genotypes (IHHNV-I and IHHNV-II) are infectious to the representative penaeids, *L. vannamei* and *P. monodon*, while the latter genetic variant has been demonstrated to be not infectious to these species (Tang et al. 2003; Krabsetsve et al. 2004; Tang and Lightner 2006). The apparent reason for the lack of infectivity of the IHHNV-III genotype was recently explained by the discovery that the DNA fragment represented by IHHNV-III is incorporated into the genome of some genetically distinct populations of *P. monodon* in the Indo-Pacific region (Duda and Palumbi 1999; Tang and Lightner 2006; Tang et al. 2007).

***IHHN gross signs in L. stylirostris***

IHHNV often causes an acute disease with very high mortalities in juveniles of this species. Vertically infected larvae and early PL do not become diseased, but in approximately 35-day-old or older juveniles, gross signs of the disease may be observed, followed by mass mortalities. In horizontally infected juveniles, the incubation period and severity of the disease is somewhat size and/or age dependent, with young juveniles always being the most severely affected. Infected adults seldom show signs of the disease or mortalities (Bell and Lightner 1984, 1987; Brock and Lightner 1990; Lightner 1996a). Gross signs are not IHHN specific, but juvenile *L. stylirostris* with acute IHHN show a marked reduction in food consumption, followed by changes in behavior and appearance. Juvenile shrimp at this stage of infection often have white or buff-colored spots (which differ in appearance and location from the white spots that sometimes occur in shrimp with WSSV infections) in the cuticular epidermis, especially at the junction of the tergal plates of the abdomen, giving such shrimp a mottled appearance. This mottling later fades in moribund *L. stylirostris* as such individuals become more bluish. In *L. stylirostris* and in *P. monodon* with terminal phase IHHNV infections, moribund shrimp are often distinctly bluish in color, with opaque abdominal musculature (Lightner et al. 1983; Brock and Lightner 1990; Lightner 1996a).

***IHHN gross signs in L. vannamei***

The chronic disease, RDS, occurs in this species as a result of IHHNV infection. The severity and prevalence of RDS in infected populations of juvenile or older *L. vannamei* may be related to infection during the larval or early PL stages (Kalagayan et al. 1991; Motte et al. 2003). RDS has also been reported in cultured

stocks of *L. stylirostris* and *P. monodon* (Lightner 1996a; Primavera and Quinitio 2000). Juvenile shrimp with RDS may display a bent (45° to 90° bend to left or right) or otherwise deformed rostrum, a deformed 6$^{th}$ abdominal segment, wrinkled antennal flagella, cuticular roughness, 'bubble-heads' and other cuticular deformities. Populations of juvenile shrimp with RDS display disparate growth with a wide distribution of sizes and many smaller than expected ('runted') shrimp. The coefficient of variation (CV = the standard deviation divided by the mean of different size groups within a population) for populations with RDS is typically greater than 30% and may approach 90%, while IHHNV-free (and thus RDS-free) populations of juvenile *L. vannamei* and *L. stylirostris* usually show CVs of 10–30% (Kalagayan et al. 1991; Browdy et al. 1993; Brock and Main 1994; Carr et al. 1996; Lightner 1996a; Motte et al. 2003).

### *Diagnostic methods*

The available methods for diagnosing infection by IHHNV include routine histology, in situ hybridization (ISH) with specific DNA probes and DNA amplification methods using standard PCR and real-time PCR (Nunan et al. 2000; Tang and Lightner 2001; OIE 2009b). Of the available methods, PCR is recommended in the OIE Manual of Diagnostic Tests for Aquatic Animals ("Aquatic Manual"; OIE 2009b) for disease surveillance and screening purposes. For IHHNV, disease surveillance/screening information can be applied to determining presence, absence or prevalence of infection in wild or cultured populations, in commodity products made from susceptible hosts and to support declarations of disease freedom (OIE 2009a, 2009b).

IHHN disease was recently found to be unique among the shrimp virus diseases when IHHNV related sequences were found in the genome of some stocks of *P. monodon* from the Indian Ocean (Tang and Lightner 2006). ISH and qPCR (real-time) studies of PCR positive *P. monodon* with this genome integrated form of IHHNV showed no signs of virus replication in the normal target tissues for the virus. Further, shrimp to shrimp transmission studies showed no evidence of this IHHN Type III agent being transmissible. In order to distinguish infectious forms of IHHNV (Types I and II) from the genome integrated form of IHHNV (Types III) in *P. monodon*, a PCR method was developed that produces a PCR product that bridges and hence includes, portions of the IHHNV sequence and a segment of the adjacent *P. monodon* genome (Tang et al. 2007; OIE 2009b). The lessons learned from applying PCR detection methods to IHHNV and the resulting false positive tests for the virus in some stocks of *P. monodon* underscore the importance of confirming unexpected positive and/or negative PCR results for IHHNV with a second primer set, or by the use of another diagnostic method (i.e. real time PCR, bioassay, ISH).

# Yellow Head Disease (YHD) and Yellow Head Virus (YHV)

### *Biology of the agent*

The causative agent of yellow head disease (YHD) is yellow head virus (YHV). Six closely related strains of YHV, including gill associated virus (GAV) have been reported which vary greatly in their ability to cause disease (Table 1) (OIE 2006b, 2009b; de la Rosa-Vélez et al. 2006; Cedano-Thomas et al. 2009). YHV is an enveloped, rod-shaped, single stranded RNA virus in the family *Roniviridae* of the order Nidovirales. The density of virions is approximately 1.20 $g \cdot mL^{-1}$ (OIE 2009b; Walker and Mohan 2009). Transmission electron microscopy (TEM) of YHV-infected tissues shows enveloped bacilliform virions. The virions range from approximately 150 nm to 200 nm in length and from 40 nm to 50 nm in diameter and are located within vesicles in the cytoplasm of infected cells and in intercellular spaces. The virions arise from longer, filamentous nucleocapsids (approximately 15 nm x 130-800 nm), which accumulate in the cytoplasm and obtain an envelope by budding at the endoplasmic reticulum into intracellular vesicles. Negatively stained YHV virions show regular arrays of short spikes on the viral envelope (Boonyaratpalin et al. 1993; Chantanachookin et al. 1993; Lightner 1996a).

YHV was originally described mistakenly as a granulosis-like virus (Boonyaratpalin et al. 1993; Chantanachookin et al. 1993), but it was later found to be a single-stranded, positive sense RNA (ssRNA) virus (Tang and Lightner 1999) related to nidoviruses in the Coronaviridae and Arteriviridae (Sittidilokratna et al. 2002). GAV, the Australian strain of YHV, has been recognized as the type species for the new virus genus *Okavirus* in the new family *Roniviridae* (Mayo 2002a, 2002b; OIE 2003, 2009b).

### *History and geographic distribution of yellow head disease*:

Yellow head disease (YHD) was first described in 1991 as an epizootic in Thai shrimp farms (Limuswan 1991) and subsequent outbreaks have been reported from other shrimp farming countries in Asia (OIE 2003, 2009b). A closely related strain of YHV, named gill associated virus (GAV), has been reported from Australian shrimp farms (Walker et al. 2001) and at least six genetic variant of the virus are now recognized (OIE 2006b; OIE 2009b). Laboratory trials have shown that YHV can cause high mortality in representative cultured and wild penaeid species from the Americas (Lu et al. 1994, 1997; Lightner 1999; Pantoja and Lightner 2003). YHD in *P. monodon* is characterized by high and rapid mortality that is sometimes accompanied by the gross signs of yellowing of the cephalothorax and general bleaching of body color from which the disease got its name. Although in laboratory studies American

penaeids challenged with YHV did not develop yellow heads or signs of marked discoloration (Lightner and Redman 1998a, 1998b), infection by YHV is potentially lethal to most of the commercially cultivated penaeid shrimp species (OIE 2009a, 2009b).

***YHD in the Americas?***

While there are no confirmed reports of YHD outbreaks in the Americas, YHV has been reported from frozen imported commodity shrimp in the United States (Nunan et al. 1998b; Durand et al. 2000) and in some early reports it was incorrectly reported in farmed shrimp from the Americas. The errant reports were based on the presentation of severe necrosis of the lymphoid organ, a lesion once thought to be pathognomonic for YHD (Limuswan 1991; Lightner 1996a; Lightner and Redman 1998a; Pantoja and Lightner 2003). However, the diagnosis of YHV infection in these cases was not confirmed with a second diagnostic method until after the errant reports were published. More recent work has shown that the presumptive histological diagnoses were due to severe infections with white spot virus, which can cause histopathology in the lymphoid organ to mimic that occurring in severe acute YHD (Pantoja and Lightner 2003).

More recently, several well documented studies have documented that that an apparently avirulent genotype of YHV is present in farmed and wild penaeid shrimp in northwest Mexico (de la Rosa-Veléz et al. 2006; Castro-Longoria et al. 2008; Cedano-Thomas et al. 2009; Sánchez-Barajas et al. 2009). In the first of these reports, de la Rosa-Veléz et al. (2006) reported the discovery of suspect cases of YHV infections from shrimp farms along the Pacific coast of Mexico. From 39 samples from 26 randomly chosen shrimp farms, 11 YHV positive samples of *L. vannamei* were found by RT-PCR using primers specific for the virus (Tang and Lightner 1999). Further analysis of selected isolates using primers that amplify other regions of the YHV genome also gave positive results (de la Rosa-Veléz et al. 2006). Of considerable interest in the de la Rosa-Veléz et al. (2006) report was the general absence of notable mortalities in farms with the YHV positive shrimp. However, a laboratory challenge study with the putative YHV agent resulted in 50% mortality in 14 days in the indicator *L. vannamei* used to assay for the presence of YHV (de la Rosa-Veléz et al. 2006).

In subsequent studies on YHV in Mexico, Castro-Longoria (2008) surveyed wild blue shrimp, *Litopenaeus stylirostris* and *L. vannamei* collected from the Gulf of California near areas with significant shrimp farms. The samples of wild *L. vannamei* were negative for YHV, but a few YHV positive specimens were found in the wild *L. stylirostris*. YHV was confirmed to be present by bioassay with healthy *L. vannamei*. The presence of YHV in the challenged shrimp was confirmed by RT-PCR, by

sequencing the amplicons and by sequence alignment with YHV and GAV sequences in GenBank.

Another recent study demonstrated the presence of YHV in inland freshwater aquaculture systems growing *L. vannamei* in Colima in west-central Mexico (Sánchez-Barajas et al. 2009). In this study the prevalence of YHV in the affected farms was 13%, but as was reported in earlier reports of YHV in Mexico, significant mortalities were not observed in the YHV infected shrimp stocks.

In another study run to further confirm that the agent found in affected farms in Mexico was YHV, Cedano-Thomas et al. (2009) has amplified replicative and structural protein encoding regions of the several Mexican YHV isolates and compared the sequences obtained with homologous virus sequences from YHV, GAV and other coronaviruses. The authors found that the Mexican YHV isolates differed slightly from YHV and GAV, but nonetheless were closely related to Asian YHV and GAV (Cedano-Thomas et al. 2009).

The finding of a strain of YHV in Mexico (de la Rosa-Veléz et al. 2006; Castro-Longoria et al. 2008; Cedano-Thomas et al. 2009; Sánchez-Barajas et al. 2009), reflects the ongoing risk of additional introductions of YHV into the Western Hemisphere with imported frozen commodity shrimp from Asia (Nunan et al. 1998a, Durand et al. 2000). In addition, because of the possibility that concurrent WSSV/YHV infections may occur, all YHV suspect samples should be further analyzed by another method (i.e. RT-PCR or ISH with a YHV specific probe) to confirm or rule out the presence of YHV.

Of interest is the observation that YHD has not emerged as a major disease in cultured stocks of *L. vannamei* in East and Southeast Asia where YHV is enzootic and highly prevalent in wild and farmed stocks of *P. monodon* (OIE 2009b; Flegel 2006). According to FAO statistics, the shrimp farming industry in Asia began to switch from culturing *P. monodon* to *L. vannamei* in 1999 and by 2005, more than half of the ~2 million metric tons of production from the region was *L. vannamei* (FAO 2006).Despite the predominance of monocultures of *L. vannamei*, a presumably highly susceptible species to YHV, Flegel (2006) did not report the occurrence of any significant outbreaks of yellow head disease in this species in the Southeast Asian region.

***Diagnostic methods***

The available methods for diagnosing infection by YHV include routine histology, in situ hybridization (ISH) with specific cDNA probes and DNA amplification after reverse transcription using standard RT-PCR and real-time RT-PCR

(OIE 2009b). Of the available methods, a RT-PCR is recommended in the OIE Aquatic Manual (OIE 2009b) for disease surveillance and screening purposes. For YHV, disease surveillance/screening information can be applied to determining presence, absence, or prevalence of infection in wild or cultured populations, in commodity products made from susceptible hosts and to support declarations of disease freedom (OIE 2009a, 2009b).

## Infectious Myonecrosis Disease (IMN) and IMN Virus (IMNV)

### *Biology of the agent*

Infectious myonecrosis disease (IMN) is caused by infectious myonecrosis virus (IMNV). IMNV particles are icosahedral in shape and 40 nm in diameter, with a buoyant density of 1.366 $g \cdot mL^{-1}$ in CsCl (Fig. 1). The genome consists of a single, double-stranded (dsRNA) molecule of 7560 bp. Sequencing of the viral genome reveals two non-overlapping open reading frames (ORFs). ORF 1 encodes a RNA-binding protein and a capsid protein. The coding region of the RNA-binding protein is located in the first half of ORF 1 and contains a dsRNA-binding motif. The second half of ORF 1 encodes a capsid protein with a molecular mass of 106 kDa. ORF 2 encodes a RNA-dependent RNA polymerase (RdRp). Based on these characteristics, IMNV is most similar to members of the *Totiviridae* (Poulos et al. 2006).

### *History and geographic distribution of IMN disease*

Infectious myonecrosis was listed by OIE in 2007 (Table 1) (OIE 2006a). The disease was first described in cultured *L. vannamei* in Northeast Brazil (Lightner 2003a; Lightner et al. 2004). IMN causes significant disease and mortalities in juvenile and subadult pond-reared stocks of *L. vannamei*. In 2003, IMN was reported to have been responsible for millions of dollars in losses in Northeast Brazil and by 2004 losses due to IMN in the affected regions of Brazil were estimated at $20 million (Nunes et al. 2004). More recent estimates for IMN losses from 2002 to 2006 in Brazil exceed $100 million (Brazilian Shrimp Farmers Association, unpublished). In Brazil, outbreaks of the disease seemed to be associated with certain types of environment and physical stresses (i.e. extremes in salinity and temperature, collection by cast net, etc.) and possibly with the use of low quality feeds (Lightner 2003a).

Although IMN seemed confined to the Northeast of Brazil, the disease spread to Southeast Asia and was reported from Indonesia in May 2006 (Wilkinson 2006). Because of the ever increasing importance of *L. vannamei* in the Asia-Pacific and the large scale trans-boundary movement and culture of the species, IMNV was considered

important for the region and it was added in January 2006 to the NACA/FAO/OIE (Asian Region) Quarterly Aquatic Animal Disease Report list for the purpose of surveillance and reporting.

### *Gross signs of IMNV*

IMN presents as a disease in *L. vannamei* with an acute onset of gross signs and elevated mortalities, but it progresses with a more chronic course accompanied by persistent moderate mortalities. Affected shrimp present focal to extensive white necrotic areas in the striated muscle, especially of the distal abdominal segments and tail fan. These may become necrotic and reddened in some individual shrimp. These signs may have a sudden onset following stresses (e.g. capture by cast-net, feeding, sudden changes in temperature or salinity, etc.). Severely affected moribund shrimp may have been feeding just before the onset of stress and will have a full gut. Such severely affected shrimp become moribund and mortalities can be instantaneously high and continue for several days. Exposure of the paired lymphoid organs (LO) by simple dissection will show that the paired LO are hypertrophic to twice or more its normal size (Poulos et al. 2006; OIE 2009b).

### *Diagnostic methods*

The available methods for diagnosing infection by IMNV include routine histology, in situ hybridization (ISH) with specific cDNA probes and DNA amplification after reverse transcription using standard RT-PCR and real-time RT-PCR (Tang et al. 2005; Poulos et al. 2006; Poulos and Lightner 2006; Andrade et al. 2007; OIE 2009b). Of the available methods, a RT-PCR is recommended in the OIE Aquatic Manual (OIE 2009b) for disease surveillance and screening purposes. For IMNV, disease surveillance/screening information can be applied to determining presence, absence, or prevalence of infection in wild or cultured populations, in commodity products made from susceptible hosts and to support declarations of disease freedom (OIE 2009a, 2009b).

## Necrotizing Hepatopancreatitis (NHP)

### *Biology of the agent and gross signs of the disease*

NHP disease is caused by an as yet uncharacterized, obligate intracellular rickettsial-like bacteria, which for the purpose of this review, will be referred to as NHP-B (Loy et al. 1996a, 1996b). The NHP organism is classified as an α-Proteobacterium based on sequence of analysis of the cloned 16S rDNA. Phylogenetic

analyses also indicate this bacterium is closely related to bacterial endosymbionts of the protozoa, *Caedibacter caryophila* and *Holospora obtusa*. NHP-B is a gram-negative, dimorphic, intracellular rickettsial-like organism that occurs free within the cytoplasm of infected hepatopancreatic cells. The predominant form is a rod-shaped rickettsial-like organism (0.25 x 0.9 µm), whereas the helical form (0.25x 2-3.5µm) possesses eight flagella at the basal apex. The target tissue is the hepatopancreas with NHP-B infection reported in all hepatopancreatic cell types (Krol et al. 1991; Frelier et al. 1992; Lightner et al. 1992b; Lightner and Redman 1994; Lightner 1996a). NHP-B is transmitted by the horizontal route via contaminated water (shed in feces) and/or per os (cannibalism) (Frelier et al. 1993; Vincent et al. 2004; Vincent and Lotz 2005). An intermediate vector/reservoir is suspected, but not demonstrated (OIE 2006c).

***History and geographic distribution of NHP disease***

NHP disease was first reported from Texas (Johnson 1990; Krol et al. 1991; Frelier et al. 1992), but it was subsequently found in most Western Hemisphere countries where penaeid shrimp are farmed (Lightner 1996a; OIE 2006c). The disease has been reported in the late postlarval, juvenile and adult stages of various penaeid species (including *L. vannamei, L. stylirostris, L. setiferus, Farfantepenaeus aztecus* and *F. californiensus*). NHP disease tends to occur during the dry season when both water temperature and salinity are elevated. Lengthy periods of elevated water temperature (29-35 °C) and salinity (20-40%) are associated with overt clinical disease (Lightner 1996a; OIE 2006c). Countries with regions having seasonal dry periods when both salinity and water temperature are high and where NHP disease has been documented include the United States (Texas), Mexico (Sonora and Sinaloa), Panama, Belize, Guatemala, Columbia, Ecuador, Nicaragua, Costa Rica, Brazil, Peru, Venezuela (OIE 2006c) and Eritrea (Lightner, unpublished data).

Despite the introduction of *L. vannamei* from the Americas to Southeast Asia and the consequent development of that species as the dominant shrimp species farmed there (FAO 2006), NHP disease has not emerged as a significant disease in the region. Other than an unconfirmed, but official, report from Vietnam (AGDAFF-NACA 2007), NHP has not been reported from Southeast Asian farms where *L. vannamei* is cultured. Perhaps, the necessary environmental conditions (e.g. an extended dry season in which temperature and salinity are elevated (>30 ºC and >30%) for NHP infections to produce disease are not present in Southeast Asia. However, such conditions are more typical in East Africa, Madagascar, the Middle East and West India, where *L. vannamei* is being introduced.

An outbreak of NHP occurred in the East African nation of Eritrea in 2004 in an introduced stock of *L. vannamei* imported from Mexico, which was co-infected with TSV (Wertheim et al. 2009). In Eritrea, the environmental conditions of high water temperature and high salinity were ideal for NHP disease to be expressed and it (with TSV) contributed to high mortalities in the *L. vannamei* and *P. monodon* stocks being cultured at the importing facility (Lightner, unpublished data). Because of the potential for NHP to be introduced with NHP-B infected stocks of *L. vannamei* into the semiarid regions bordering the Western Indian Ocean, the disease is listed as "under study" by the OIE in 2009 and it was fully listed by the OIE in May 2010 (Table 1; OIE 2008a, 2009a).

***Gross signs, pathology and diagnostic methods***

The clinical signs displayed by NHP-B infected shrimp are nonspecific in nature and characterized by lethargy, reduced feed intake, decreased growth rate, softened shell and an atrophied hepatopancreas. On pond-side examination, infected shrimp display empty midguts with increased superficial epicommensal cuticular fouling and/or opportunistic infections (i.e. black spots) that are also often present. Pond mortality rates of up to 95% have been reported in untreated shrimp populations (OIE 2006c).

Microscopic examination of unstained tissue squashes prepared from suspect hepatopancreata may show reduced lipid and dark melanized necrotic tubules. Histologically, NHP-B infected tubular epithelial cells will be hypertrophied with a generalized basophilic intracytoplasmic granularity due to the presence of numerous pleomorphic intracytoplasmic rickettsial-like organisms (=NHP-B). Three stages of infection have been described and defined in histological studies. In early NHP-B infections, scattered tubules with cytoplasmic rickettsial-like organisms can be detected free within the cytoplasm of the tubule epithelial cells. In affected tubules, resorptive (R), fibrillar (F) and/or secretory (B) cells may be infected (Bell and Lightner 1988). In more advanced infections the cytoplasm of most tubule epithelial cells is filled with NHP-B and affected tubules show increasing levels of tubular epithelial cell hypertrophy and desquamation of NHP-B laden cells, as well as marked lipid depletion, tubular necrosis, interstitial hemocytic infiltrates and melanization of hemocyte encapsulated tubules. Some shrimp that survive the first two stages of NHP-B infection present marked atrophy of the hepatopancreas. Most tubules show reduced height of the epithelial cells and some atrophied tubule epithelia appearing squamous. In such shrimp with such chronic NHP-B infections, intertubular hemocytic inflammation is also reduced and there are little or no stored lipid droplets (Frelier et al. 1992; Lightner 1996a; Morales-Covarrubias et al. 2006; OIE 2006c).

Three levels of examination procedures can be used for NHP diagnosis or surveillance/screening. During periods of elevated water temperature and/or salinity, pond-side examinations of statistically significant numbers of shrimp can be conducted to detect and select suspect shrimp (e.g. demonstrating elevated mortality, reduced feed intake, empty midguts, soft shells and atrophy of the hepatopancreas) for further diagnostic testing. When NHP disease is suspected, histopathologic examination of routine H&E stained paraffin sections may be used to demonstrate pathogonmonic lesions in NHP-B infected shrimp. Varying degrees of hepatopancreatic inflammation/necrosis will be present in affected shrimp with the presence of intracytoplasmic organisms that may be confirmed with special stains (e.g. Steiner's & Steiner's stain), ISH with DNA probes, or IHC with specific antibodies to NHP-B (Frelier et al. 1992; Lightner 1996a; Loy and Frelier 1996c; Morales-Covarrubias et al. 2006; OIE 2006c; Bradley-Dunlop et al. 2004). PCR methods are available and may be most suitable for surveillance/screening purposes (Loy and Frelier 1996c; Loy et al. 1996a, 1996b). A rapid, pond-side, antibody-based diagnostic test is being developed that may be useful in early detection and management of NHP disease (Houghton et al. 2009).

## Milky Hemolymph Syndrome (MHS)

### *Biology of the agent and gross signs of the disease*

Milky hemolymph disease (MHD) was recently assigned as the name for a serious disease of farmed spiny (*Panilurus* spp.) lobsters in Vietnam (Lightner et al. 2008; OIE 2008a, 2008b), which is caused by overwhelming systemic infections by an unclassified rickettsial-like bacteria (RLB). While MHD is a descriptive name for this disease in spiny lobsters, the name milky hemolymph syndrome (MHS) has been adopted to include MHD of spiny lobsters and similar RLB-caused diseases in penaeid shrimp and crabs (Nunan et al. 2010). In MHS the hemolymph of these affected decapods becomes increasingly turbid and turns "milky" in appearance in severely affected individuals. The turbidity of the hemolymph is due to the massive numbers of RLB circulating in the hemolymph of affected individuals. Generally, the more moribund an affected animal is, the more turbid ("milky" appearing) its hemolymph. MHD of farmed spiny lobsters is currently listed as "under study" while it is being considered for full listing as a notifiable disease by the OIE (OIE 2008a; OIE 2009a).

### *History and geographic distribution of MHD*

In addition to its occurrence in cultured spiny lobsters in Vietnam, MHS (inclusive of MHD of spiny lobsters) may have been an overlooked disease of shrimp

for many years. Although not called MHS in the earlier literature (Anderson et al. 1987; Brock 1988; Brock and Lightner 1990), MHS in shrimp may have been reported in farmed *P. monodon* from Malaysia more than 20 years ago. Even earlier, Bonami and Pappalardo (1980) described a very similar systemic RLB-caused disease in wild crabs, *Carcinus mediterraneus*, collected from the Sète region of France. Hence, MHS may be an "old" disease that has been overlooked, or so well controlled with medicated feeds intended for treating other bacterial diseases that it was not rediscovered as a significant disease until recently. More recent reports of MHS have also been from *P. monodon* farmed in east Africa and Madagascar (Nunan et al. 2003a, 2003b) and in wild crabs, *C. maenas*, held in shedding tanks in England (Eddy et al. 2007). Laboratory challenge studies with the RLB from *P. monodon* from Madagascar suggest that the disease had the potential to infect *L. vannamei* (Nunan et al. 2003b).

By histology, MHS is characterized by massive accumulation of very small RLB in cytoplasmic inclusions, primarily in connective tissues, hemocytes and fixed phagocytes. In severely affected individuals, masses of palely basophilic (with H&E stains) RLB fill the hemocoel spaces among and within the tissues. Gram staining of infected tissues, or Gram staining of milky hemolymph smears, shows the RLB agents of MHS to be very small (~0.5 x 1.0 µm) gram negative rods (Lightner et al. 2009b).

***Diagnostic methods***

MHD of spiny lobsters is considered to be an emerging disease by OIE (OIE 2009a). Hence, there are no OIE recommended methods for its diagnosis or for surveillance and screening. Nonetheless, gross signs (e.g. turbid or milky hemolymph) combined with histological demonstrations of the characteristic lesions of MHS provide a definitive diagnosis for the disease in spiny lobsters, shrimp and crabs.

Molecular methods for MHS are also available. Nunan et al. (2003a) reported on the development of a PCR test for the Madagascar RLB from *P. monodon* using the 16S rDNA gene of the RLB agent. The same strategy was used to develop PCR test methods for the RLB from spiny lobsters (OIE 2008b) and from the European crab (Eddy et al. 2007). From comparisons of the sequence and alignment of the 16 S rDNA PCR products from each of the RLBs that cause MHS in shrimp, crabs and spiny lobsters, it is presumed that the RLB agents of these diseases are related, but not likely to represent a single species (Fig. 3; Nunan et al. 2010).

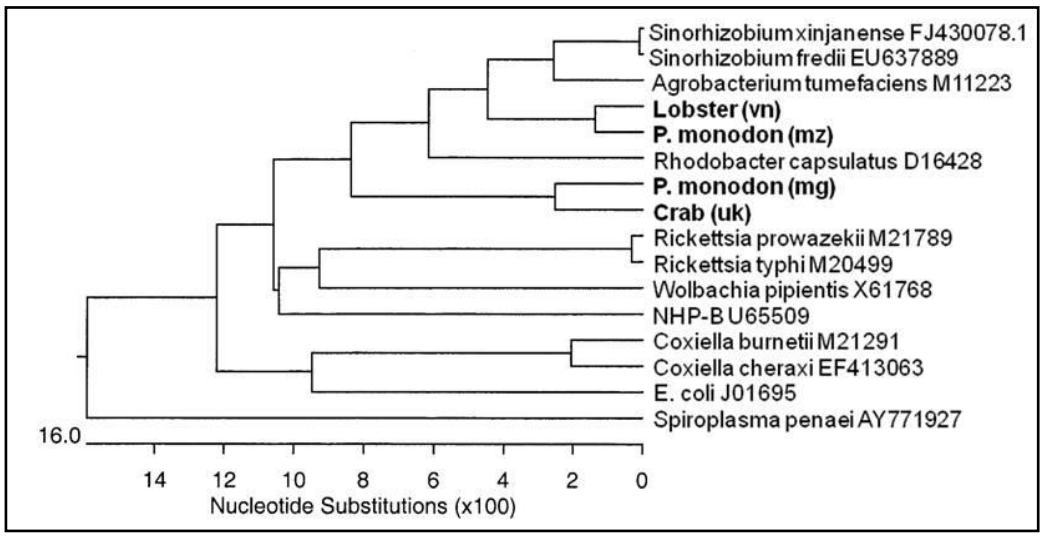


**Fig.3.** A relatedness plot of the 16S rDNA gene sequence of MHD agents from *P. monodon*, *Panulirus* spp. (spiny lobsters) and *C. maenas* (European crab) compared to the most closely related organisms in GenBank. Included for comparison are NHP-B and *Spiroplasma penaei* from *L. vannamei*.The numbers with most of the isolates are GenBank accession numbers.

## Micrococci and Streptococci Diseases

### *Biology of the agent and gross signs of the disease*

Another emerging bacterial disease of shrimp is caused by micrococci, which are probably streptococci based on morphological, biochemical and molecular data. Severe mortalities due toputative *Streptococcus* spp. have occurred in *P. monodon* farmed in the Indo-Pacific (Madagascar and East Africa) and in *L. vannamei* farmed in Central and South Americas and Middle East. *Litopenaeus vannamei* from two significant epizootics due to streptococcus have been processed by the University of Arizona Aquaculture Pathology Laboratory in the past several years. The first was from French Guiana, South America and the second occurred in mid 2008 in several farms culturing *L. vannamei* in Central America (Hasson et al. 2009; Lightner et al. 2009b). Streptococcal disease in shrimp is likely to be due to infections by more than a single species of *Streptococcus*, as the disease has been documented to occur in farms during extended periods of near zero salinity, as well as in farms using seawater of oceanic salinity (Hasson et al. 2009; Lightner et al. 2009b).

The *Streptococcus* spp. that are the putative agents of this disease cause systemic infections in their hosts. The gross signs presented are non-specific and are similar to those induced by both viral and other bacterial diseases. Affected shrimp

become lethargic, anorectic and accumulate at the pond edges, near its surface, or near aerators where dissolved oxygen levels are higher and die there (Lightner et al. 2009b). Histopathological lesions are often remarkable. The most typical presentation of streptococcal infection is a severe acute generalized diffuse necrosis of the lymphoid organ (LO) marked by generalized nuclear pyknosis affecting virtually all LO tubule parenchymal cells, connective tissue cells and circulating hemocytes. Hemocyte accumulations or nodules are sometimes present. Similar, less conspicuous lesions may be present in the heart lumen, gills and diffusely distributed systemically in the connective tissues (Hasson et al. 2009; Lightner et al. 2009b).

Another remarkable feature of these lesions is that while the micrococci may be present in very large numbers in the LO, heart, hemolymph and other tissues, their presence is not easily demonstrated with routine H&E staining. Besides being very small cocci (~1 µm in diameter), these micrococci stain palely basophilic. Their small size and poor staining characteristics with H&E stains make them difficult to demonstrate in low level or developing infections. However, with tissue Gram stains, the putative streptococci stain Gram positive and are prominent in the affected tissues (Hasson et al. 2009; Lightner et al. 2009b).

## Disease Management

Until the WSSV pandemic, the penaeid shrimp farming industry in Asia and the Americas remained largely dependent on wild shrimp for stocking its farms and biosecurity was not part of the shrimp farming industry's vocabulary. The farming of essentially all wild shrimp stocks was accomplished by the practice of collection and use of wild seed (postlarvae) for stocking of farms directly, or by the use of captive wild broodstock for the production of seed stock in hatcheries. This dependence has fostered the intensification and spread of the viral diseases in shrimp aquaculture and in wild populations. The shrimp farming industry as a whole has recognized this fact and it has begun to change its farming practices in order to continue to develop, if not survive. While many of the shrimp stocks currently used to stock farms are produced from captive wild broodstock, only those that test negative for WSSV in Asia and WSSV and TSV in the Americas are used to stock biosecure farms. Biosecure production systems (that are designed to exclude potentially infected wild shrimp seed) stocked with shrimp stocks known to be free of the major shrimp pathogens have become a common practice in many shrimp growing regions. A further sign of a maturing industry is its movement towards the expanded development and use of specific pathogen-free (SPF) domesticated shrimp stocks of the most important shrimp species (Pruder et al. 1995; Lotz 1997a, 1997b; Moss et al. 2002; Lightner 2003b, 2005; Lightner et al. 2009a; Lee and O'Bryen 2003). Domesticated lines of *L. vannamei* are

now the dominant shrimp farmed in Asia. The rapid change over of the industry to *L. vannamei* from *P. monodon* was due in large part to disease (FAO 2006). Domesticated stocks made it possible to better manage the health status of the farmed stocks and with specific pathogenfree (SPF) *L. vannamei*, domesticated lines were readily available (Lightner 2005; FAO 2006; Lightner et al. 2009a). These advances have enhanced the technologies used to farm shrimp and have made the industry far more sustainable than it was before the emergence of the virus-caused diseases discussed in the present paper.

The emergence of "new" bacterial diseases, with MHS and streptococcus-caused diseases as examples may be, in part, a consequence of increasing market pressures on the shrimp aquaculture industry to not use antibiotics. However, some of the outbreaks of these diseases occurred for the first time in regions where there was little or no use of antibiotics, or in a new aquacultured species (e.g. *Panulirus* spp. - spiny lobsters).

The dubious history of intercontinental transfer and introduction of the currently OIE listed shrimp virus diseases and the emergence, or re-emergence, of a number of "new" diseases, illustrate the importance to the industry of qualified disease diagnostic laboratories that can do routine diagnostic and surveillance functions.

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# Emerging diseases in shrimp culture: overview of viral and bacterial diseases in the Americas

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## Abstract

The expansion of shrimp culture in the Americas has been historically limited by disease epidemics that caused severe production crashes. As intensive culture systems provide almost ideal conditions for the propagation of infectious diseases, the producers are constantly looking for means to manage diseases to minimize losses in production and quality. The most attractive options are exclusion or eradication, breeding for disease resistance and various sanitary measures. On the other hand, low market prices limits the resources invested in shrimp diagnostics and prevention. In this scenario, identification of new pathogens could be restricted to outbreaks with high mortalities, thus making shrimp producers more susceptible to the effect of shrimp pathogens. In this review, we analyzed the main shrimp diseases in the Americas.

## Introduction

Due to the fact that shrimps have only recently been cultivated and only since the last three decades have cultivated populations been isolated from wild populations, most populations of cultivated shrimp have only had a relatively short period to evolve and adapt to intensive cultivated production systems. Diseases that remain at a low level of incidence in natural populations may reach epidemic levels in intensive cultivation systems. Modern intensive shrimp systems (with animals confined in tanks or ponds, with limited water exchange and with the cannibalistic behaviour of shrimp) provide almost ideal conditions for the outbreak of diseases. Also, other crustacean species, such as crabs or copepods, may be alternate hosts of shrimp diseases. All these conditions favour epidemics and the appearance of apparently new diseases in intensive shrimp production systems (Cock et al. 2009).

In any population, disease emergence can occur by different mechanisms such as: i) evolution of pathogens; ii) introduction of existing pathogens or hosts to a new

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location with establishment and spread; and iii) simultaneous infection with two or more pathogens that affect the immune mechanisms of the host and produce an outbreak with a pathogen that otherwise would not have been so pathogenic.

In aquatic ecosystems, which are very complex, a clear distinction between health and disease in shrimp culture is not clear; survival is difficult to calculate during the cycle and low chronic mortalities are often not identified. Even during disease outbreaks, the underlying cause is often difficult to ascertain and in most cases, is usually the end result of a series of events involving environmental factors, health condition of the post-larvae, presence of an infectious agent and/or poor management practices.

Diagnostic information and procedures for shrimp diseases must include epidemiological and risk-analysis studies to identify, not only the pathogen and/or pathogens present, but also the conditions that lead to its prevalence and/or pathogenicity. However, many farmers are still only considering one major cause of disease; i.e. every event of mortality in the regions affected by white spot syndrome virus (WSSV) is attributed to the virus while other diseases are missed or underestimated. Effective management of the health of shrimps requires consideration of the fact that there is a balance between the host, pathogen and environment. Many shrimp pathogens are present in association with shrimp, or are present in their environment. With most pathogens of this type, the shrimp are apparently healthy and show normal growth. Often, conditions such as high stocking density, poor water quality, or under optimal oxygen levels induce stress in the shrimp, which can facilitate the outbreak of disease due to parasites or bacteria normally associated with shrimp.

## Shrimp Diseases in America

The shrimp farming industry in the Americas has developed and emerged as one of the major aquaculture industries in the region. It has evolved from the initial stages, where shrimp producers relied almost entirely on the capture of wild post-larvae (PL) in estuaries and coastal areas, to the development of reproductive closed cycle and the establishment of several shrimp breeding programs. This has been done in an attempt to stabilize seed availability and improve disease resistance and growth rates of shrimp stocks.

Most countries in the region are concentrating on the production of specific pathogen resistant (SPR) or specific pathogen tolerant (SPT) shrimp, selecting the best surviving (but not necessarily disease-free) animals from pond grow-out facilities and growing them further in various facilities before transferring them to maturation

systems. Due to the culture systems employed, with extensive non-lined ponds and no water treatment, the use of specific pathogen free (SPF) animals is not the preferred seed for pond stocking.

As in most of the producing regions in the Americas, diseases are still one of the major constraints for the growth of the shrimp industry. Diseases such as those caused by Taura syndrome virus (TSV) and WSSV have been significant threats and new viral and bacterial pathogens associated with management practices or environmental factors are emerging as new causes of concern for shrimp producers.

***White Spot Syndrome Virus (WSSV)***

One decade after its initial identification in the Americas, infection by WSSV is still considered to be the main cause of mortalities in shrimp farming along the Pacific coast of America, from Peru to Mexico and in the south of the Atlantic coast in Brazil. Average shrimp survival in WSSV affected regions is 35-50%. During the past year, several outbreaks of WSSV have been reported in Mexico, (Camara Nacional de Acuacultura: http://www.cna-ec.com/index.php?option=com_content&task=view&id=815&Itemid=31 accessed June 24 2009) Guatemala, Ecuador and the states of Santa Catarina and Bahia in Brazil (Muller et al. 2010). These outbreaks have been related to a decrease in the water temperature in the ponds, or are associated with the presence of other opportunistic pathogens such as *Vibrio* sp. WSSV has never been reported in the Atlantic Coast of Colombia and Venezuela, probably due to the higher water temperature in the Caribbean coast that impairs its replication (Reyes et al. 2007).

Breeding for resistance against WSSV has proven to be an elusive task, although there are reports of increased resistance in selected stocks from endemic areas such as Panama and Ecuador (Perez et al. 2001, Chamarro and Mialhe 2004). However, at present, a shrimp line resistant to WSSV is not yet identified. In addition, the negative correlation between shrimp growth and resistance to WSSV infection (Gitterle et al. 2005) makes the selection of animals even more difficult. For disease control, farmers rely on management practices such as low stocking densities, drying out the ponds during the cold season and in some cases, emergency harvest if mortalities are high.

***Taura Syndrome Virus (TSV)***

Taura syndrome virus (TSV), although considered to be a manageable disease responsive to breeding selection for resistance, has caused major losses to the shrimp industry in the Americas since its initial description in 1992. As with other RNA viruses, TSV exhibits high genetic variability. Variation in the nucleotide structure of

the genes found in ORF2, especially of the genes coding for the nucleocapside protein CP2 or VP1, have been described in TSV isolates from different geographical regions: Mexico (Robles-Sikisaka et al. 2002), Belize (Erickson et al. 2005), Venezuela (Côté et al. 2008) and Colombia (Caraballo et al. 2007). It has also been demonstrated that some strains, such as the Belize strain, show higher degree of pathogenicity (Tang and Lightner 2005). At present, TSV is still prevalent in Belize, Colombia, Nicaragua, Panama and Venezuela.

***Other Viruses***

Infectious myonecrosis virus (IMNV) was initially identified in the State of Piaui (Brazil) in 2002 and by 2004 it had extended to almost all of the shrimp farms in the northern states (Piaui, north of Ceara and Rio Grande del Norte). At that time, mortality rates ranged from 35 to 55% in 12 g shrimp and the economic loss was estimated to be about US$20 million in 2003 (Nunes et al. 2004). In 2007, a study of IMNV prevalence identified 10% of samples as RT-PCR positive and 9 out of 11 evaluated farms had at least one animal positive for IMNV. However, in spite of the high prevalence of IMNV, shrimp survival has improved and is now at rates present before IMNV first appeared in Brazil. Brazilian farmers attribute the increase in survival to a higher resistance of the shrimps to the virus and to stress control in the shrimps and stability in the environmental conditions. In 2004, another virus that causes muscle necrosis, similar to IMNV, was identified in *Litopenaeus vannamei* in Belize in affected ponds that had a 50% reduction in production. Cloning and sequencing showed similarity with the *Macrobrachium rosenbergii* nodavirus (Tang et al. 2007). It was denominated *PvNv*. At the present, there are no confirmed reports of *PvNv* in other countries in Central or South America.

***Bacterial Diseases***

Bacterial diseases are one of the emerging disease threats in shrimp ponds in America. Problems ranging from mass mortalities to growth retardation and sporadic mortalities have been identified in almost every country and new species that affect *L. vannamei* have been described. While *Vibrio* spp. continue to be the most important bacterial pathogens of shrimp, with the increasing cases of septic diseases, new bacteria will be identified as potential shrimp pathogens.

*Spiroplasma penaei:* This intracellular bacterium was identified in 2003 in one farm in the Atlantic Coast of Colombia (Nunan et al. 2004). During the outbreak, mortalities in shrimp ponds ranged from 10-90%. Biosecurity measures applied since the identification of the pathogen prevented its spread to other farms, regions or

countries. Management practices such as increasing the dry out time in ponds, managing soil bottom and decreasing stocking densities have been successful and since 2007, *S. penaei* has not been identified in any of the Colombian shrimp farms.

Necrotizing hepatopancreatitis (NHP) is still prevalent in America and has been reported in many countries (Peru, Colombia, Ecuador, Venezuela, Brazil, Panama, Mexico) with variable rates of mortalities in grow-out ponds and in maturation facilities. Outbreaks of pathogenic bacteria have been identified at increased frequency, especially in shrimp intensive ponds. In Venezuela, enteric and systemic bacterial infections are causing high mortalities in shrimp farms located in the Gulf of Maracaibo. In Colombia, *Vibrio harveyi* and other bacteria are responsible for a decrease in production in intensive shrimp ponds. Filamentous bacteria are highly prevalent in ponds stocked at high densities (45-70 animals·$m^{-2}$) and in some cases, high levels of infestation can affect shrimp growth and survival.

***Parasitic Diseases***

Several endo and exo-parasites also affect shrimps at different stages of development. Protozoan genera, such as *Zoothamnium, Epistylis, Vorticella, Anophrys, Acineta, Lagenophrys* and *Ephelota,* are present in shrimp ponds throughout the region and are associated with low water quality. Although their pathogenicity is very low, at high levels of infection, these protozoa may induce gill obstruction (brown gill) leading to anorexia, retarded growth, reduced locomotion and increased susceptibility to infection by other viral or bacterial pathogens. Gregarines, endoparasitic protozoans, are found with different levels of severity, but are not usually associated with a decrease in productivity or survival.

Less known parasites, such as a haplosporidian described in Cuba in 1988, were found again a decade later in some ponds in Belize, where they were causing hepatopancreatic infections in cultured *L. vannamei* (Nunan et al. 2007). Other pathogenic organisms, such as microsporidia (*Agmasoma* sp. and *Ameson* sp.), are also endemic around the region but as in the case with endoparasitic protozoans are not usually associated with high mortalities or production losses.

## Conclusion

Shrimp aquaculture, especially in extensive non-lined ponds, provides a suitable environment for the emergence, establishment and transmission of new pathogens. Diagnostic laboratories, preventive biosecurity measures and risk analyses are all needed to mitigate the risk of disease emergence, establishment and spread.

Studies of disease prevalence at different stages in shrimp culture are useful to identify the pathogens that are, or could be, a potential cause of disease out-breaks. Shrimp farmers need to be aware of the effect of non-optimal conditions in the shrimp susceptibility to pathogens and need to manage the ponds accordingly to reduce the risk of diseases.

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# Immunomodulation by DNA vaccination against white spot syndrome virus (WSSV)

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## Abstract

Vaccines (subunit and DNA) targeting major envelope proteins VP19 and/or VP28 of white spot syndrome virus (WSSV) in penaeid shrimp were developed and elicited good protection against white spot disease (WSD). However, the immune responses in shrimp after administration of these vaccines are not well understood. In this study, we developed a DNA vaccine encoding the VP28 envelope protein in kuruma shrimp (*Marsupenaeus japonicus*) and confirmed the potentiality of protection against WSSV infection. The efficacy of the DNA vaccine against WSSV infection was confirmed by WSSV artificial challenge at 7 days post vaccination in kuruma shrimp. However, the efficacy of the vaccine did not last 30 days post vaccination. The transcript of VP28 gene derived from expression vector in tissues of vaccinated shrimp was analyzed by RT-PCR. The transcript of VP28 gene was detected in various tissues including muscle, hemolymph, gill, intestine, stomach, heart, hepatopancreas and lymphoid organ tested at 1, 3 and 7 days post vaccination. Subsequently, the expression of innate immune-related genes in intestine and lymphoid organ was analyzed at 1, 3 and 7 days post vaccination. The expression of innate immune-related genes such as Rab7, penaeidin, lysozyme, and crustin was up-regulated upon DNA vaccination. These results suggest that DNA vaccination induces significant protection against WSSV by stimulating innate immune responses in kuruma shrimp.

## Introduction

Viruses are among the most crucial pathogens affecting crustaceans, especially shrimp. Among various viruses affecting shrimp, white spot syndrome virus (WSSV) is currently the most serious viral pathogen of cultured shrimp worldwide. The rapid onset

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and lethality of white spot disease (WSD) is remarkable (Chou et al. 1995). It causes up to 100% mortality within 3 to 10 days of infection, resulting in major economic losses to the shrimp farming industry (Chou et al. 1995; Inoue et al. 1994; Karunasagar et al. 1997; Takahashi et al. 1994; Wang et al. 1995). WSSV infects a wide range of aquatic crustaceans, including crabs, lobsters, and freshwater crayfish (Lo et al. 1996). WSSV is extremely virulent, possesses a wide range of host specificity and targets various tissues. The virus is pathogenic to several species of shrimp, such as black tiger shrimp, *Penaeus monodon* and kuruma shrimp, *M. japonicus.* The first major WSSV outbreak, reported in 1993, resulted in a 70% reduction in shrimp production in China (Cen, 1998; Zhan and Wang, 1998), and this virus has remained a major concern for shrimp aquaculture throughout the world since. The presence of WSSV has been reported in both wild and hatchery reared postlarvae (Hao et al. 1999; Lo et al. 1997; Tsai et al. 1999). WSSV has become an epizootic disease and is not only a major threat to shrimp aquaculture, but also to marine ecology (Flegel et al. 1996).

WSSV is a large DNA virus with five major proteins with expected sizes of 28 kDa (VP28), 26 kDa (VP26), 24 kDa (VP24), 19 kDa (VP19) and 15 kDa (VP15). VP28 and VP19 are associated with the virion envelope and the others are associated with the nucleocapsid (van Hulten et al. 2000). Moreover, it has been reported that the VP28 envelope protein located on the surface of the virus particle plays an important role in the initial steps of WSSV infection in shrimp (van Hulten et al. 2001). To date, subunit vaccines targeting envelope proteins VP28 and/or VP19 expressed in *E. coli* have been studied and their protective ability against WSSV infection by oral administration (Witteveldt et al. 2004b) or intramuscular injection (Witteveldt et al. 2004a) has been reported. More recently, DNA vaccines encoding envelope proteins VP15, VP28, VP35 and VP281 of WSSV were developed and tested in black tiger shrimp (Rout et al. 2007). The report suggested that DNA vaccination using expression vectors encoding VP28 and VP281 has potential to increase protection against WSSV infection. However, the immune responses stimulated in shrimp by DNA vaccination have not been studied thoroughly to date.

In this study, we developed a DNA vaccine (recombinant plasmid DNA driven by CMV-promoter) against WSSV and investigated its efficacy by artificial WSSV challenge. Post vaccination, the expression of several innate immune-related genes was analyzed to investigate the immune responses of shrimp.

# Materials and Methods

### *Plasmid DNA construction for vaccine*

Viral DNA was extracted from WSSV infected shrimp using a DNeasy Tissue Kit according to the manufacturer's instructions (Qiagen). PCR was performed using a WSSV VP28 F1 (5'-ATGGATCTTTCTTTCAC-3') and R1 (5'-TTACTCGG TCTCAGTGC-3') primer set. The cycle conditions were: one cycle of 94 ℃ for 3 min, 30 cycles of 94 ℃ for 30 sec, 50 ℃ for 30 sec and 72 ℃ for 45 sec, followed by one cycle of 72 ℃ for 5 min. The amplified product of the VP28 gene was ligated into an expression vector that contained the human CMV-promoter (pTARGET Mammalian Expression Vector, Promega, USA). The ligated product (pCMV-VP28) and the plasmid vector without VP28 gene (pCMV) were transfected into TAM competent *E. coli* (ActiveMotif, Belgium) and recombinants were identified through red-white color selection on MacConkey agar (Sigma-Aldrich). Plasmid DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced using a CEQ8000 Automated Sequencer (Beckman Coulter).

### *Vaccination and artificial WSSV challenge*

WSSV-free kuruma shrimp, approximately 15 g body weight were injected intramuscularly with 10 $\mu$g of pCMV-VP28 plasmid DNA dissolved in 100 $\mu$L of phosphate buffered saline (PBS). The control group shrimp were either injected with 100 $\mu$L of PBS or 10 $\mu$g of the pCMV plasmid DNA dissolved in 100 $\mu$L of PBS.

WSSV artificial challenge was carried out by immersion at 7 and 30 days post vaccination. Heart and hepatopancreas were collected under sterile conditions from WSSV-infected shrimp. Pooled tissues were homogenized with PBS. DNA was extracted from homogenates using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions and copy number of WSSV challenge stock (homogenate) was determined by quantitative real-time PCR. Shrimp (n = 25. group) were immersed in 4 L of artificial sea water which contained 5 mL of homogenate ($1 \times 10^{10}$ copies. $mL^{-1}$) for 2 h at 20 $^0$C. The survival rate of each experiment: 7 and 30 days post vaccination was recorded for 12 and 20 days, respectively. Assessment of statistical significance was analyzed by the Chi-square test. Relative percent survival (RPS) was calculated according to the method described by Amend (1981).

***Tissue distribution of VP28 transcript post vaccination***

Hemolymph, muscle (injected part), gill, stomach, heart, hepatopancreas, lymphoid organ and intestine were isolated from three individual shrimp injected with PBS and pCMV-VP28 at 1, 3 and 7 days post injection. Prior to the isolation of RNA, all post-injection tissues were pooled for the PBS injected group. Total RNA was isolated using ISOGEN (Nippon Gene) following the manufacturer's instructions and any contaminating DNA was digested by the treatment with DNase I (Takara Bio, Shiga Japan) at 37 $^{o}$C for 30 min. cDNA was synthesized from 2 $\mu$g of total RNA using ReverTra Ace qPCR Kit (Toyobo).

Nested PCR was performed with WSSV VP28 F1 and R1 (1$^{st}$ PCR), WSSV VP28 F2 (5'-TGGATCAGGCTACTTCAAGAT-3') and R2 (5'-AAAGGTGGTACCACACACAAA-3') (2$^{nd}$ PCR) primer sets using the previously described conditions. Shrimp ß-actin gene (F 5'- ATGACACAGATCATGTTCGA-3'; R 5'- GTAGCACAGCTTCTCCTTGA-3') was used as the internal control for RT-PCR. PCR products were separated on 2.0% agarose gels and visualized by staining the gels in tris-borate-EDTA (TBE) buffer containing 100 ng.mL$^{-1}$ ethidium bromide (Sigma-Aldrich).

***Expression analysis of innate immune-related genes by semi-quantitative RT-PCR analysis***

The intestine and lymphoid organ were isolated from shrimp at 1, 3 and 7 days post injection with PBS, pCMV or pCMV-VP28. Each tissue was extracted from three individual shrimp in each group and pooled together prior to the RNA extraction. RNA extraction and cDNA synthesis was carried out using the kits described above. PCR was conducted with primer combinations; Mj (kuruma shrimp) Rab7 F (5'-CTCGCAAGAAGATTCTCCTG-3') and R (5'- CTTCGTTGATACCGCCCTAT-3'), Mj lysozyme F (5'- TCCTAATCTAGTCTGCAGGGA-3') and R (5'-CTAGAATGGGTAGATGGA-3') (Hikima et al. 2003), Mj crustin F (5'-CACCTTCAGGGACCTTGAA-3') and R (5'- GTAGTCGTTGGAGCAGGTTA-3'), Mj penaeidin F (5'-GCTGCACCCACTATAGTCTTT-3') and R (5'-CTACCATGGTGATGAAACAAA-3'), Mj β-actin F and R (primer sequences provided above). To conduct a semi-quantitative approach of gene expression, both kuruma shrimp innate immune-related and β-actin genes were amplified using a series of cycle numbers (21-35) following the conditions described above. After specific PCR was conducted using the optimal cycle number, the expression ratio of innate immune-related (35 cycles) / β-actin (25 cycles) was determined by densitometry using Science Lab99 Image Gauge software (Fujifilm). The expression analysis was conducted in

triplicate. Assessment of statistical significance was analyzed by one-way ANOVA, followed by a Tukey's test.

## Results

### *The efficiency of DNA vaccine*

The survival rate of the vaccinated group was 78.5% at 12 days post challenge (Fig. 1-A). In contrast, the survival rates of control groups PBS and pCMV were 28.5 and 42.8%, respectively. The RPS value between vaccinated and control groups (PBS and pCMV) was 70.0 and 62.4%, respectively. A significant increase of protection against WSSV was observed in the vaccinated groups compared to the control groups (7 days post vaccination). However, its efficacy did not last 30 days post vaccination (Fig. 1-B). The survival rate of the vaccinated group was low 26.2%.

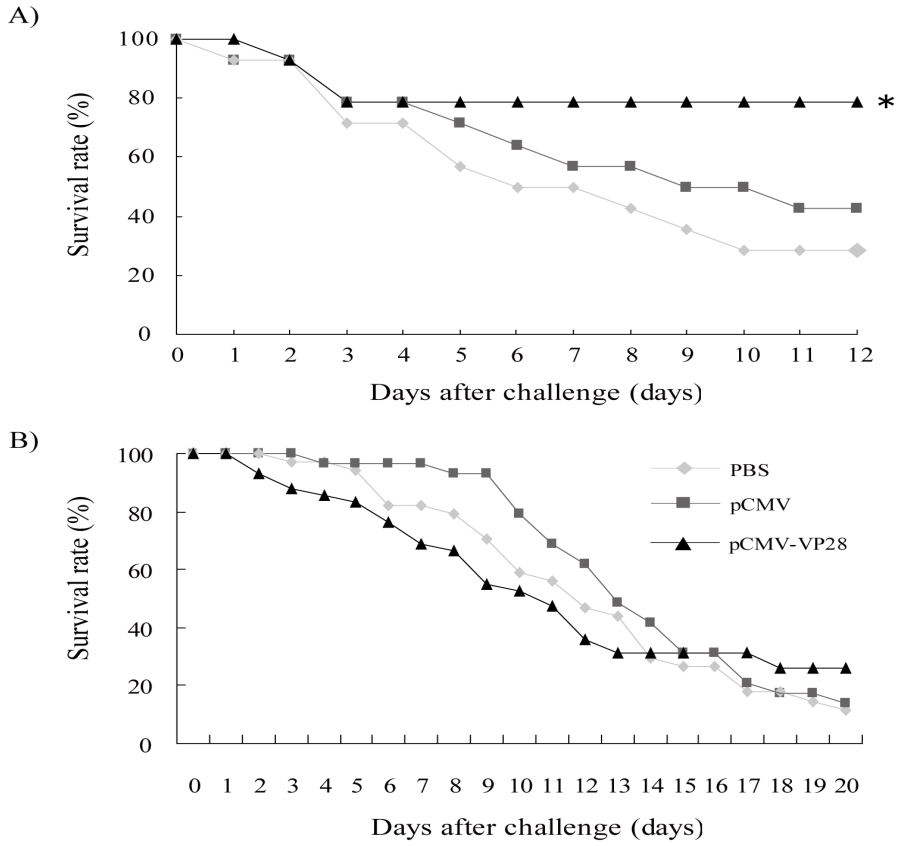


**Fig. 1**. Survival rates (%) of shrimp from the experimental groups vaccinated with pCMV-VP28 (▲), pCMV (empty vector; ■) and PBS (♦) are plotted against the time after 7 days (A) and 30 days (B) of vaccination. Asterisk indicates the significant difference ($P < 0.01$) from the pCMV and PBS.

### *Tissue distribution of VP28 transcript*

The VP28 transcript was detected in hemolymph, muscle, gill, intestine, stomach, heart, hepatopancreas and lymphoid organ when analyzed by RT-PCR at all time points (1, 3 and 7 days post vaccination). However, the expression of the transcripts was uneven in each individual shrimp (Table 1).

**Table 1.** Analysis of the relative tissue expression of VP28 mRNA in vaccinated shrimp (3 individuals).

| | days after vaccination | | | |
|---|---|---|---|---|
| organ* | 0 | 1 | 3 | 7 (days) |
| HL | - | +++** | +++ | +++ |
| Mus | - | +++ | ++ | ++ |
| Gl | - | +++ | ++ | ++ |
| Int | - | ++ | ++ | +++ |
| Stm | - | +++ | +++ | +++ |
| Ht | - | +++ | +++ | +++ |
| Hp | - | +++ | +++ | +++ |
| Lo | - | + | + | ++ |

*: HL: hemolymph, Mus: muscle, Gl: gill, Int: intestine, Stm: stomach, Ht: heart, Hp: hepatopancreas, Lo: lymphoid organ

**: number of shrimp expressing VP28 mRNA; -(-ve / 3 individuals), + (1 +ve / 3 individuals), ++ (2 +ve / 3 individuals), +++ (3 +ve / 3 individuals)

### *Expression analysis of innate immune-related genes in shrimp post vaccination*

Expression of the Rab7 gene was significantly increased in the intestine of vaccinated shrimp compared with the control shrimp at all time points after vaccination. The expression of lysozyme, penaeidin and crustin genes was significantly increased in the intestines and lymphoid organ after vaccination. The most conspicuous increased expression of these genes was confirmed in lymphoid organ at 7 days post vaccination. In the pCMV injected group, up-regulation of penaeidin (intestine and lymphoid organ) and lysozyme (lymphoid organ) genes was confirmed compared to the control shrimp (Fig. 2).

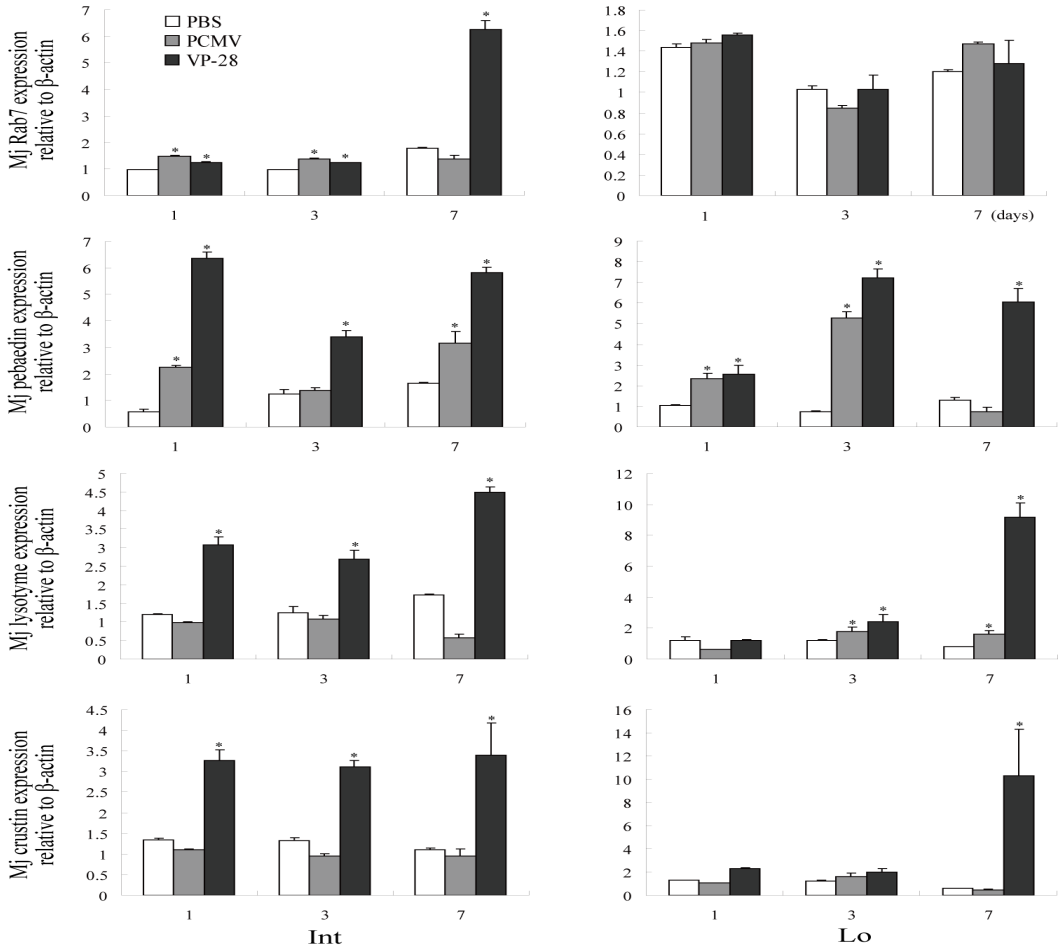


**Fig. 2.** Effects of DNA vaccination on the expression of kuruma shrimp (*M. japonicus*: Mj) innate immune-related genes in intestine (left side) and lymphoid organ (right side) at 7 days post vaccination. Semi-quantitative RT-PCR to shrimp innate immune-related genes was performed with cDNA synthesized from intestine and lymphoid organ of DNA vaccinated shrimp. Data are presented as shrimp innate immune-related gene PCR products after normalizing against products β-actin gene. The X-axis indicates the tissues tested (Int, intestine; Lo, lymphoid organ) and relative expression of the shrimp innate immune-related gene is on the Y-axis. Data are presented as mean ± S.D. of triplicate samples. Asterisks indicate the significant difference ($P < 0.05$) compared to the control (healthy shrimp). Graphs indicate the expression pattern of innate immune-related genes; A) Rab7 (Acc. No. AB379643), B) penaeidin (AU175636), C) lysozyme (AB080238) and D) crustin (AB121740).

## Discussion

The WSSV VP28 envelope protein plays an important role as an attachment protein for the infection of shrimp and directs WSSV into the cytoplasm (van Hulten et al. 2001; Yi et al. 2004). Thus, VP28 envelope protein was selected as a target antigen

for a DNA vaccine for the present study. Recently, plasmid DNA vaccines using VP28 envelope protein and other envelope proteins as an antigen were injected in black tiger shrimp, resulting in resistance that was effective for at least 1 month post vaccination (Kumar et al. 2008; Rout et al. 2007). However, at present, few works have been conducted on DNA vaccines of shrimp and the resulting immune responses in vaccinated shrimp have not been well researched. In this study, we report the efficacy of the DNA vaccine in kuruma shrimp and analyze the expression of innate immune-related genes in these shrimp after vaccination.

Prior to the construction of the DNA vaccine, we considered which kind of promoter inserted in the expression vector should be selected for the study. In the commercial protein expression system, insect cells, p10 or polyhedron promoters etc. derived from baculovirus are generally used to express / synthesize the protein of interest. It has been confirmed that the CMV-promoter derived from human cytomegalovirus functions in insect (Fall armyworm, *Spodoptera frugiperda*) cells (Lo et al. 2002) and black tiger shrimp (Sulaiman et al. 1999) by reporter assay using luciferase and β-galactosidase genes, respectively. More recently, the expression of VP28 protein in the muscle of black tiger shrimp injected with a CMV-promoter driven expression vector inserted with VP28 was confirmed by immunohistochemistry (Kumar et al. 2008). Therefore, our choice was to use a plasmid expression vector containing the CMV-promoter for the construction of the DNA vaccine.

Protective immunity against WSSV was increased by immunization with the DNA vaccine at 7 days post vaccination in kuruma shrimp. However, this protection was not long lasting after vaccination (i.e., not at 30 days post vaccination). Recently, it was reported that primary vaccination with WSSV recombinant VP26 and VP28 showed recovery of the resistance against WSSV infection (Satoh et al. 2009). This result suggests that the prime boost immunization with DNA vaccine will extend the duration period of DNA vaccine. At the same time, these results may suggest that the immune responses of vaccinated shrimp against WSSV are weak and not lasting for long period because shrimp does not have memory cells like mammals. However, it is difficult to draw firm conclusion from the present data. For further study, it is necessary to clarify the existence of molecules or cells related to immune responses. The injection of empty vector slightly increased the protection against WSSV infection compared to the control. To date, it is known that DNA vaccines possess their own adjuvant activity in vertebrates due to the presence of unmethylated cytosine-guanine dinucleotide (CpG) motifs in particular base contents (Sasaki et al. 2003; van Drunen Littel-van den Hurk et al. 2000). It has also been reported that CpG oligodeoxynucleotides activate shrimp innate immune responses such as phenoloxidase activity (Chuo et al. 2005) and respiratory burst (Sung et al. 2008). Therefore, this increased protection will depend on

the CpG motif included in expression vector used in this study. However, the survival rate of the vaccinated group was significantly higher than that of the control groups. Therefore, the constructed vaccine may be considered as an effective tool to combat WSSV.

The transcript of the WSSV VP28 gene derived from the expression vector was confirmed in hemolymph, injected part of muscle, gill, intestine, stomach, heart, hapatopancreas and lymphoid organ of shrimp at 1, 3 and 7 days post vaccination. Although the transcript of VP28 gene was not detected by the 1$^{st}$ PCR (data not shown), it was detected in various tissues at 1, 3 and 7 days post vaccination by the nested PCR. We conducted immunohistochemistry and Western blotting to detect the expressed VP28 protein from the internal organs of shrimp but the protein was not detected in the organs tested (data not shown). This suggests that transcription level of the VP28 gene derived from expression vector in shrimp was low. Previous reports of DNA vaccination in black tiger shrimp showed transcripts of WSSV VP28 gene in muscle tissue after vaccination, and the expression lasted for 30 to 50 days (Kumar et al. 2008; Rout et al. 2007). The transcription level of constructed vaccine in the penaeid shrimp body may depend on the kind of vector, the size of vaccinated shrimp and the condition to maintain shrimp after vaccination.

The expression of innate immune-related genes was analyzed at 1, 3 and 7 days post vaccination in order to investigate the immune status of vaccinated shrimp. The expression of Rab7 gene involved in WSSV infection (Sritunyalucksana et al. 2006) was significantly increased in the intestine compared with that of control shrimp at all the time periods after vaccination. Previous reports have shown an increase of the protection against WSSV infection by the injection of recombinant Rab7 protein and this molecule plays an important role for the attachment of WSSV at the early infection stage (Sritunyalucksana et al. 2006). Therefore, the increase of Rab7 might be related to the increased protection against WSSV. However, the difference of induction mechanism of Rab7 gene activation in WSSV infection or DNA vaccination is not clear.

In other innate immune-related genes such as lysozyme, penaeidin and crustin, expression was also significantly increased in intestine and lymphoid organ after vaccination. These genes are known as antimicrobial peptides; the antimicrobial activity against bacteria and fungi has been well defined for lysozyme (Hikima et al. 2003), penaeidin (Destoumieux et al. 1997), and crustin (Relf et al. 1999) but their potential involvement in antiviral responses are still unclear. However, the up-regulation of antimicrobial peptides as a response to viral infection has been reported in shrimp (Robalino et al. 2007) and *Drosophila* (Zambon et al. 2005). This suggests the overlap of the response may be induced with viral or bacterial infection (Robalino et al.

2007). To date, factors directly related to the antiviral responses (like Mx, 2-5A, PKR known in vertebrates) are still unknown in shrimp. Thus, the expression of specific genes was analyzed to understand the immune response in shrimp after vaccination. The results suggest the transcriptional activation of antimicrobial peptide genes might be involved in the protective immunity to WSSV infection.

This DNA vaccination will not only be a combat tool against specific pathogens but also an activator of innate immune responses in shrimp. As such, the use of some innate immune-related genes as biomarkers may enable us to know whether vaccines will be an effective tool to combat against pathogens.

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# Promoting health management of shrimp aquaculture on Guam and Commonwealth of Northern Mariana Islands

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## Abstract

Shrimp disease outbreaks remain the most profound threat to the fast growing shrimp aquaculture industry, and they have caused billions of dollars in economic losses worldwide. Biosecurity has been applied in aquaculture as a preventive practice for the exclusion of specific pathogens from cultured aquatic species at various levels from facility/farm level to regional and country levels. Isolated in the Western Pacific, the Mariana Islands have a unique geographic advantage and great potential in playing a more significant role in shrimp aquaculture locally, regionally and even globally. Yet, some biosecurity measurements are quite relaxed and the whole region is lacking health monitoring and regulatory control programs. There is a need to increase awareness of biosecurity measurements on the individual farm level, as well as to establish systematic health management to protect the whole region from the introduction of viral pathogens. This will avoid major disease outbreak, reinforce the clean zone image of the region, and eventually lead to long-term sustainable shrimp aquaculture development on Guam and the Commonwealth of the Northern Mariana Islands (CNMI).

This project aimed at promoting the health management of shrimp aquaculture on Guam and CNMI, with three specific objectives: 1) to evaluate current shrimp health management practices in the region by conducting biosecurity audits of all existing shrimp farms and identify the key risk factors; 2) to set up farm-specific bimonthly surveillance program in two major shrimp facilities; 3) to promote the awareness of biosecurity in the region via various means of education, and to prepare and distribute a comprehensive summary report to aquaculture stakeholders and the corresponding government agencies.

Biosecurity audits were conducted for seven shrimp farms/facilities in the region. The strengths and risks of each shrimp facility were evaluated and suggestions were also provided. During the biosecurity audit, shrimp samples were taken and analyzed to identify the presence of any infectious shrimp disease. Based on the information collected from this project, four sessions are covered in this manuscript: 1) introduction and principles of health management; 2) current status of shrimp health management on Guam and CNMI; 3) challenges to improve biosecurity in Guam and CNMI; 4) future outlook and direction.

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The research effort serves as a useful tool for shrimp farmers in the region in terms of improving the health management of shrimp aquaculture.

## Introduction and Principles of Health Management

Since the 1970s, shrimp aquaculture has grown rapidly from 0 to 71% of the total world supply of shrimp, in the past three decades, with a global annual production level that exceeded 3.2 million metric tons in 2006 (Josupeit 2008). Epizootics remain the most catastrophic threat to the industry of shrimp aquaculture, especially viral disease outbreaks. Major viral diseases include white spot syndrome virus (WSSV), yellow head virus (YHV), Taura syndrome virus (TSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), infectious myonecrosis virus (IMNV) etc (OIE 2009). One good example is that China, the largest shrimp producer in the world, was hit by YHV in 1988, and then WSSV in 1992. As a result, its total production level dropped two thirds, and it took 8 years to recover. Similarly, shrimp aquaculture in Thailand and Ecuador, another two major producers, suffered greatly due to WSSV outbreak as well. It was estimated that the economic losses due to the shrimp disease outbreak have easily surpassed 10 billion US dollars (Lightner 2005). The infectious pathogens have been transferred from facility to facility, country to country and even from one continent to another, and the widespread pandemics could be prevented if proper health management is in place.

The guiding principles for proper health management include:

- ✓ Start with stocks of premium health status
- ✓ Implement biosecurity to protect unit from disease introduction
- ✓ Monitor health/conduct surveillance
- ✓ Have contingency plans in place

Specific pathogens free (SPF) concept was introduced to exclude the pathogens of concern in shrimp stock, with successful development in two peneaid species, *Penaeus stylirostris* and *Penaeus vannamei* (Wyban 1992; Wyban et al. 1992; Pruder et al. 1995; Lightner 2005). *P. vannamei* has become the dominant shrimp species cultured globally because of the availability of SPF stocks and genetic improved stocks through selective breeding.

The application of biosecurity, a concept that is widely used in livestock industries, has been introduced to aquaculture as a preventive practice for exclusion of the specific pathogens from cultured aquatic species at various levels from facility/farm level to regional and country levels (Lee and O'Bryen 2003). Biosecurity may be

defined as the sum of procedures used to protect living organisms from contracting, carrying and spreading diseases. In other words, biosecurity is the implementation of measures to avoid the entrance of infection into a unit, control the dissemination of an infection within the unit, and avoid spreading of an infection to other units. Implementation of biosecurity measurements will help to protect the facility from disease introduction, and the biosecurity audit is a useful tool to examine how well those measurements are executed. The priorities of biosecurity audit include categories such as:

- Site location
- Water intake
- Fresh/frozen feed sources
- Introduction of new shrimp sources or genetic materials
- Dispatch & loading procedure
- Personnel and visitors
- Others

The details for each category are specified below.

Other than the biosecurity audit, shrimp health monitoring/surveillance is the essential component of health management program at various levels, such as national, regional, and farm levels. In the current project, we focused on the farm level health monitoring of the shrimp stocks. Both the observations on shrimp clinical signs and disease diagnostics are important elements to assess the health status of the shrimp stocks. The basis of good surveillance programs is observant and skilled people with appropriate support resources, who understand what is normal, are alert to changes and can describe the abnormalities they see (Baldock et al. 2006). Only two of the seven facilities have submitted shrimp samples on their own for disease diagnosis and monitoring regularly in the past.

It is also essential for the aquaculturists to understand that the proper sampling size, tissue and fixation method should be followed so that the diagnosis can be correctly performed. For example, hemolymph or pleopod would be ideal samples for detecting the presence of IHHNV, WSSV, TSV, YHV or IMNV by PCR, but will not be suitable for NHP diagnosis.

Lastly, having the contingency plan in place are also critical so that prompt action can be carried out in controlling disease outbreak and preventing further spreading of disease problem.

# Current Status of Shrimp Health Management on Guam and CNMI

***Current Status***

Guam and Commonwealth of the Northern Mariana Islands (CNMI) have all year round warm climate, the natural seawater range from 27 ºC to 29 ºC, which is suitable for warm-water shrimp aquaculture. Currently, *P. vannamei* is the sole penaeid species cultured in Guam. Isolated in Western Pacific, yet within 4h flight time to major cities in Asia, where the center of world shrimp aquaculture production is located, the Mariana Islands have a unique geographic advantage and great potential in playing a more significant role in shrimp aquaculture locally, regionally and even globally. In addition to the steadily growing consumer's needs of local shrimp meat production, there is increasing demands of specific pathogen-free shrimp broodstocks from the Asian countries. Despite the multiple advantages favoring shrimp aquaculture development in the region, biosecurity is absent in general and the whole region is lacking health monitoring and regulatory control programs. There is a need to increase awareness of biosecurity measures at individual farm level, as well as to establish a systematic health management to protect the whole region from introduction of viral pathogens and avoid major disease outbreak, to reinforce the clean zone image of the region, and to eventually lead to long-term sustainable shrimp aquaculture development on Guam and CNMI.

In the past, a biosecurity audit or similar efforts to collect the information across all the shrimp farms in the region and evaluate the current health status of shrimp facilities in the region has not been done. There is a great need to increase the awareness of biosecurity measures at the farm level, as well as to establish a systematic health management plan to protect the whole region from the introduction of viral pathogens so as to avoid major disease outbreak, to reinforce the clean zone image of the region, and to eventually lead to long-term sustainable shrimp aquaculture development on Guam and CNMI.

Funded by USDA Center of Tropical and Sub-tropical Aquaculture (CTSA), University of Guam (UOG) took the initiatives in conducting the biosecurity audits for the seven current shrimp facilities (which were in operation at the time of biosecurity audits being conducted) across the region, four of which are in Guam, two in Saipan and one in Tinian. All the four facilities in Guam are locatedin the eastern coast of the island, one in Mangilao, one in Talafofo and two in Inajaha. The other three facilities are situated inland of Saipan and Tinian. In addition, shrimp samples were collected on

site and then submitted to the Aquaculture Pathology laboratory at the University of Arizona for diagnosis.

***Biosecurity Priorities***

Detailed illustration of the biosecurity priorities involved in the evaluation process of the biosecurity audits are documented as follows:

**a. Site Location**

Location is the most important factor for biosecurity consideration. Compared to a location close to an estuary, an inland location is considered more biosecure in terms of preventing the aquatic species from intruding to or escaping from the perimeter. But the cost of drilling the wells, pumping the water from a deep well and/or transporting water from the ocean may be significantly higher for the inland site than a coastal location. The total numbers and distribution of shrimp aquaculture facilities in the regions should be an important index for the evaluation of site location.

Guam and CNMI could be categorized as a low density region in terms of shrimp aquaculture activities, with only seven shrimp facilities in operation throughout the region. Among them, there are four coastal shrimp facilities in Guam, two inland facilities in Saipan and one inland facility in Tinian. According to the functionality, there is one hatchery and the rest are mainly for production.

The shrimp compound should be clearly defined and fences are necessary to provide a biosecurity barrier. Fences and gates are essential to control outside traffic of people, vehicles and animals. Gates must remain closed and locked at all times except when in use. “Keep Out” or “No Entry” signs should be placed at the active areas of the perimeter fence. Most of the shrimp facilities in the region are fenced, or partially fenced around the gate at the entrance. Signs were usually present nearby the gate to discourage unauthorized visits. However, not all the facilities were clearly defined and/or locked and tighter control of access should be enforced.

**b. Water Intake**

Water is the medium where shrimp live and grow. It is also the medium for some water borne shrimp pathogens to be transmitted for infecting the shrimp. Water free of shrimp pathogens is critical for the exclusion of shrimp pathogens being in contact withthe shrimp.

Currently, only two shrimp facilities were drawing water from the deep well through gravel filtration. Reservoir tanks were equipped to stock the intake water before putting into the culture system in two facilities. The rest were getting their water from the ocean or nearby estuary without further water treatments, except that primary bag filtration was used by a couple of the facilities. In addition, the intake water was close to the discharge site in three facilities situated nearby the estuary. Four facilities were using flow through or low water exchange during the culture period. Recirculation systems wereused in varying degrees at three facilities.

**c. Shrimp Feed**

Feed comprises approximately 50% of the overall operational cost in shrimp farming. Rising fuel costs have driven shrimp feed to reach record high price, especially in Guam and CNMI where almost all goods are imported. Control of feed ingredients and fresh feed must be exercised to prevent the introduction of disease from the use of contaminated components.

Except for the facilitythat functions as a hatchery, all the rest of the farms used dry pellets.In the hatchery, two kinds of fresh/frozen feeds produced from lower risk areas were used: artemia (cysts) and squid. The term lower risk areas refer to a specific pathogen negative zone. *Artemia franciscana* were harvested from the Great Salt Lake and squid were from clean water region (such as northern California, northern Europe or southern Australia). However, there was no procedure in place to log the date received, type of feed, quantity, source, results of any diagnostic testing (when performed).

Shrimp pellet diets came from various manufacturer sources in US, China, Taiwan and the Philippines. A regular monitoring program of shrimp feed sources, which was lacking, should be implemented before starting its usage in any facility. Feed suppliers have not been evaluated and a feed source audit is recommended to be conducted once a year. Feed delivery was not regulated so that feed trucks did enter the secure perimeter. Trucks that haul shrimp and then feed were often the case.

**d. Introduction of New Shrimp Sources or Genetic Materials**

Currently, *P. vannamei*, the Pacific white shrimp, is the sole marine shrimp species cultured in the region. Only SPF shrimp postlarvae from a reliable hatchery source should be introduced to any aquaculture facilities. In other words, the hatchery should have at least a 3- year history of clean broodstocks free of OIE listed shrimp pathogens and the postlarvae production should be conducted in a biosecure facility where the biosecurity protocols are carefully followed. In addition, the shrimp stocks

need to be closely monitored for clinical signs, regularly sampled and diagnosed. The hatchery had a 5-year clean history, free of OIE or USMSF listed shrimp pathogen. The biosecurity audit found that various sources of *P. vannamei* postlarvae had been introduced to the farms in the region, without strict background check, health monitoring and proper quarantine procedures. Most of the imported seed stocks were from Taiwan and Thailand, high density shrimp aquaculture areas, where shrimp disease is much more problematic than Guam and CNMI. There were several reasons for the farmers to choose sources other than the local hatchery, such as lower costs, larger volumes for stocking and business strategies, etc. Regardless of the reasons, this practice posesgreat biosecurity risk to the importing facilities, especially when the proper quarantine procedures are not in place. Recommendations were prepared to be distributed to the farm managers in this respect.

Quarantine/isolation and acclimatization play an important role in preventing any pathogenic introduction to the facilities through the new shrimp stocks.

The role of proper quarantine and disease diagnostics has been greatly overlooked in the region when importing foreign sources of shrimp postlarvae. All movements of shrimp in the region have not been conducted properly through a quarantine/isolation procedure using a quarantine station preferably inspected/run by government agency. A quarantine/isolation period would allow sufficient time to detect the presence of possible significant pathogens in the source unit. Diagnostic sampling should be done during the quarantine. Ideally, the quarantine/isolation should be a minimum of 30 days or up to the time that laboratory results from sampling are back. Before releasing the shrimp from quarantine/isolation, the facility manager should obtain information from the source facility that there was no new disease outbreak and the laboratory reports confirmed that with negative test results. Afterwards, all shrimp should follow a farm-specific acclimatization program. The acclimatization period allows the newly introduced shrimp to adapt to a new environment in the receiving facility.

Actions need to be taken at both farm and regional level for increasing the public awareness of this biosecurity aspect.

**e. Dispatch and Loading**

The most common incoming shipments to the shrimp farms were postlarvae, feed and certain equipments/materials. The outgoing shipments were mostly shrimp harvested for salein the local market. A clear distinction between the clean and dirty side for people, vehicle and shrimp flow should be established and maintained in the

facility.Almost all the facilities overlooked this aspect in biosecurity. The postlarvae receiving facility should discard the exterior packing materials or thoroughly disinfect the styrofoam boxes outside the perimeter if keeping them for reuse so that the possibility of the transmission of shrimp pathogenic agents during the transportation can be minimized. Holding tanks and ramps must be power-washed and disinfected after each use. Effluents must be prevented from flowing back into the compound. Once deliveries are made, the facility truck is cleaned and disinfected at a truck wash before the next trip. However, this is not the case in the farms throughout the region. There were cases that some equipments and supplies were loaned to one facility by another without proper disinfection procedures. Thorough disinfection procedures for the equipments and supplies from other farms should be established prior to bringing them into a farm in order to eliminate health risk.

**f. Personnel and Visitors**

Production personnel mostly worked and lived routinely in the same facility. Staff contact with other aquaculture species outside the facility should be minimized by the staff. No clothes, footwear, etc. that are used around the other aquaculture farms should be worn in the facility. Depending on the risk tolerance, proper downtime requirement should be established if production staff go to any seafood market, go fishing, visit shrimp laboratories or other facilities and are in contact with fresh/frozen shrimp or crustaceans outside a shrimp facility. In addition to the downtime, shower, changing clothes and footwear should be done prior to reporting to work.

Each shrimp facility should have its own practical visitor policy to avoid the possibility of disease transmission through visitors. Visitors should submit visiting request and meet the requirements set by the farm management. The visiting schedule should be determined by the management. Upon approval of the request, all persons entering any shrimp aquaculture facility should observe the downtime regulations. Overnight means a minimum of 8 h. One more night should be added on the downtime requirement wherever a shower is not available. Each aquaculture facility should have its own sign-in book. All visitors must sign the visitors' book before entering a unit. The sign-in book should include: date, name, reason for visit, date and place of last contact with shrimp/aquaculture species or related materials.

Training on biosecurity was officially provided to the personnel before employment in one of the facilities in the region. No visitor was allowed to visit the facility. Not all the managers educated their employees to understand that they could pass on shrimp pathogens if they do not follow the proper preventive measures. Although sign-in book for visitors were used in two facilities, neither emphasized the

importance of downtime nor used a questionnaire about the aquaculture related activities prior to the visit on site.

**g. Others**

Few facilities audited have policies for routine pest and crustacean (crabs) control procedures. Feed spillage needs to be minimized and proper disposal of waste should be practiced to discourage pests. The local environment may be favorable to insect nesting. Therefore, practices to keep the insect population at a low level (inside and around the facility) whenever possible were recommended. Two facilities in Saipan and Tinian were bird proofed, while some ponds in one facility in Saipan were not. Birds were seldom a problem for the aquaculture farms in Guam, as a result of wide predation of birds by the browntree snakes. On the other hand, crabs could be easily found in the shrimp facilities near the ocean. Those farms need to build barriers to block crabs from entering the perimeter.

Water discharge should be in line with the local permits and regulations. It would be better to have a settling pond to separate the suspended particles from the water column before the discharge. This would be an effective way to control eutrophication caused by the nutrient loads in the discharge water, and minimize adverse environmental impacts.

Although there are no related regulations for dead shrimp and molts in Guam and CNMI, their disposal should be done properly. Certain protocols need to be developed based on the farm specific condition. Methods of disposing the dead shrimp include incineration, compost pits, and burial. If a burial system is used, the area must be completely fenced and covered. Proper sanitation procedures for the equipment and materials used in disposal are required. However, there were no rules on shrimp carcass and molt disposal in most facilities, and the general sanitation was poor in a couple of the facilities visited.

***Summary of evaluation***

A site-specific executive report was generated for each individual facility. A brief summary of key components in biosecurity is listed in Table 1.

During the biosecurity audit, shrimp samples were collected from the seven facilities in Guam and the CNMI. Both PCR and histopathogicaldiagnoses were performed by the shrimp pathology laboratory at the University of Arizona. The pathogens tested for are listed in Table 2.

**Table 1**. Summary of key components in biosecurity audits among the facilities.

| Facility | Location | Water treatment | Seed source | Personnel/ visitor | Diagnostic results of the pathogens for SPF stocks | Facility Impact |
|---|---|---|---|---|---|---|
| #1 | Coastal | No | Asia | Some restriction | One pooled sample weak positive IHHNV by PCR, but not confirmed by histopathology | Medium |
| #2 | Coastal | No | Asia | Some restriction | Microsporidian infection detected by histopathology | Medium |
| #3 | Coastal | No | Asia | Little restriction | IHHNV positive by both PCR and histopathology | Medium |
| #4 | Inland | No | UOG | Some restriction | Not detected | Medium |
| #5 | Inland | No | UOG | Little restriction | Not detected | Low |
| #6 | Inland | Gravel filtration | Asia | Highly restricted | Not detected | High |
| #7 | Coastal | Gravel filtration | UOG | Highly restricted | Not detected | Very strong |

**Table 2**. USMSFP Working List of Specific Pathogens for SPF Penaeids in the United States (Lightner 2005 and UAZ lab report).

| Pathogen Type | Pathogen | Pathogen Group | Pathogen Category[1] |
|---|---|---|---|
| Viruses | TSV | dicistrovirus (n.f.) | C-1* |
| | WSSV | nimavirus (n.f.) | C-1* |
| | YHV/GAV/LOV | ronivirus (n.f.) | C-1,2* |
| | IHHNV | parvovirus | C-2* |
| | BP | occluded baculovirus | C-2* |
| | MBV | occluded baculovirus | C-2* |
| | BMN | unclassified nonoccludedbaculovirus | C-2 |
| | HPV | parvovirus | C-2 |
| | IMNV | totivirus | C1,2* |
| Procaryote | NHP | alpha proteobacteria | C-2 |
| Protozoa | Microsporidians | Microsporidia | C-2 |
| | Haplosporidians | Haplosporidia | C-2 |
| | Gregarines | Apicomplexa | C-3 |

* OIE listed diseases as of year 2007. Any of these diseases may be notifiable to OIE (within 48 h of a confirmed diagnosis) if it fulfills any of the following criteria: First occurrence or re-occurrence in a country or zone of a country, if the country or zone of the country was previously considered free of that particular disease; or occurrence in a new host species; or new pathogen strain or new disease manifestation; or potential to spread the disease globally; orzoonotic potential.

**[1]** Pathogen category (modified from Lotz et al. 1995) with C-1 pathogens defined as excludable pathogens that can potentially cause catastrophic losses in one or more American penaeid species; C-2 pathogens cause economically significant disease and are excludable; and C-3 pathogens cause less serious disease, but should be excluded from breeding centers, hatcheries, and some types of farms.For more information on some of these pathogens and the most appropriate diagnostic methods see: OIE 2006, Diagnostic Manual for Aquatic Animal Diseases, Fifth Edition. Available at:http://www.oie.int/eng/normes/fmanual/A_summry.htm

From the health management point of view, the strengths of having shrimp aquaculture in Guam and CNMI are: 1) the region is in the category of low density shrimp aquaculture area; 2) good isolation and far enough from the high density area of shrimp aquaculture; 3) relatively "clean" area with good source of water; 4) some of the farms have certain appropriate elements of infrastructure which could be utilized to improve the biosecurity measurements; 5) most farm managers have years of experience in commercial shrimp production, which is an asset for practical solution in biosecurity implementation; 6) no major shrimp disease outbreak has been reported in the past 5 years.

Despite the multiple advantages favoring shrimp aquaculture development in the region, the concept of biosecurity is vague and the practice is sporadic. The whole region, Guam in particular, is lacking health monitoring and regulatory control programs. Five major weaknesses with detail illustrations are listed below.

**1) High health risk seedstock imported from Taiwan and Thailand**

There is a hatchery in the region, UOG hatchery, which supplies the local shrimp farms in Guam, two farms in Saipan and one farm in Tinian on a limited basis. Currently, UOG hatchery has limited resources to meet all the postlarvae requirements of the regional shrimp farms in terms of the quantity and price. Several farmers still source some postlarvae from abroad, such as Taiwan and Thailand, which could introduce shrimp diseases into Guam. More reliable and larger scale local SPF postlarvae production is needed to sustain and expand shrimp farming in Guam and CNMI.

**2) No proper quarantine facility and protocols**

As a territory of the United States, the importation and exportation of aquaculture products is jointly regulated by both Guam (or CNMI) and Federal authorities.

The Government of Guam Department of Agriculture requires that the importation of live aquatic animals and plants be permitted and inspected upon arrival. An import permit must accompany aquatic animals from the US, while those from a foreign country require a certificate of origin. In addition, a certificate of health must accompany the animals from a certified agent in the country of origin verifying that the animals are disease free.

Similarly, a declaration for importation or exportation of fish or wildlife must be filed ahead of the shipping and cleared by US Fish and Wildlife Service, Department of Interior, at the port-of-entry or exit.

However for the importation of shrimp postlarvae, a proper quarantine procedure should be included in addition to the appropriate paperwork stated above in order to effectively prevent the intrusion of significant shrimp pathogens to the region. During the quarantine stage, the shrimp from the shipment should be closely monitored and samples need to be collected for diagnosis to confirm the absence of the OIE listed shrimp pathogens. The quarantine could be run either in a government operated facility or a farmer owned facility with sufficient time so that any clinical signs can be observed and diagnostic results become available. In addition, the facility needs to be a closed system isolated from the other shrimp operations and water intake and discharge need to be disinfected and tightly controlled. Meeting these requirements will require the investment in terms of infrastructure, equipments, and personnel, etc. How to fund such a practice is an issue to be resolved.

**3) No proper water treatment for either intake or discharge.**

Although the risk tolerance of most regional shrimp farms is relatively high since their products are for food consumption in the local market, the proper disinfection and/or treatment is necessary to exclude the pathogenic agents and increase the shrimp crop's performance and reduce the adverse environment impact. For example, settling pond would be a practical way to reduce suspended matter from the discharge water. Combined with other treatments, the water can be reused in large scale farms. As for the medium and small scale facility, recirculation systems are more environmentally sustainable. However, it will require substantial effort and investment to make a change in the direction of the long term sustainability.

**4) Relaxed attitude toward biosecurity issues.**

Compared to the other regions conducting aquaculture activities, the regional farmers have been fortunate because no major shrimp disease outbreak has occurred in the past 5 years. Most of the farmers do not want to invest in biosecurity because of a lack of immediate return. They do not understand the biosecurity concept in depth, as well as the importance of its role. But in reality, Guam and CNMI are not immune to shrimp viruses if they are brought in. Therefore, there is a need for being more cautious as new shrimp viruses are emerging and there is no cost effective cure for infections caused by these known pathogens. It will be much more costly or too late to correct the

problem if catastrophic disease outbreak occurs. It requires vision and action to protect the investment and regional shrimp health status.

**5) No PCR diagnostic capacity in the region for accurate and fast diagnostic result**

In the past, all the shrimp samples were collected and sent to Dr. Lightner's laboratory at the University of Arizona for diagnosis. It usually takes 2 weeks up to 1 month from sample collection to the delivery of the results. Besides the diagnostic cost, the long turnaround time and high shipping cost make it impractical to conduct the diagnosis during quarantine stage, and make it excusable to exclude the diagnosis as the routine monitoring tool.

University of Guam has recently established a molecular biology laboratory with PCR capability. The laboratory has the potential to be used for shrimp disease diagnosis after the validation by an OIE authorized laboratory.

***Setting up Health Surveillance for Two Facilities***

Based on the geographic location and the levels of impact of the facilities on the aquaculture development in the region, two facilities were selected for health surveillance: one is in Guam and one is in Saipan. Sampling schemes have been tailored to fit the sources and numbers of shrimp stock in the facility. On-site training for sampling shrimp tissues for specific diagnostics was also provided during the health surveillance visit.

## Challenges to Improve Biosecurity in Guam and CNMI

From the information collected by the biosecurity audit and health surveillance, there are three major challenges to improving the biosecurity control measures in Guam and CNMI: the awareness, the resources and the cost related to implementation of biosecurity management. Actions at both farm and regulatory government agencies levels are required for the improvement.

It is fundamental to continuously increase the awareness of biosecurity for shrimp farming in the region. Proper training should be provided to not only the farmers, but also to regulatory government officials. The latter group needs to understand the importance of biosecurity, both principals and practices. Without the correct mindset, it is impossible to do the job properly.

Secondly, it seems that generation of resources to improve the regional biosecurity level for shrimp aquaculture has been low in priority. It would require a well-structured plan, collaborative efforts and legislative support. Government agencies, such as the Department of Agriculture and US Fish and Wildlife Service, should take a leading role in this, especially in the development of a quarantine station and procedure, regional diagnostic laboratory, shrimp health surveillance service, preparation of the contingency plans, etc.

Thirdly, cost is inevitable in implementing the biosecurity measurements. The cost includes manpower costs, investment in infrastructure, equipments and supplies, diagnostics costs, etc. Compare to the potential losses due to disease outbreak, it would be much less expensive to take the preventive approach. It requires both farmers and government to work together in solving this cost issue.

Overall, the greatest risk for some facilities is the seedstocks imported from Asian countries, and no proper quarantine procedure is in place to minimize the disease risk. Fortunately, there has not been any major shrimp disease outbreak in the region in the past decade even though there are indications of the presence of IHHNV in a couple of locations. If such health issues are not being scrutinized and proper solutions being sought at both the facility and regional levels, the adverse effects may magnify and could evolve to a much more serious situation.

## Future Outlook and Direction

Information generated from this health project provided baseline information on health status of shrimp farming in Guam and CNMI. In addition, the project also served as a useful tool in increasing awareness of the need for biosecurity among the regional shrimp aquaculture society and improving the health management of shrimp aquaculture in Guam and CNMI.

In the end, the establishment of a systematic health management regime requiresthe collaborative efforts from all the stakeholders in order to improve the health status of the regional shrimp industry, which will eventually lead to the development of a long-term, sustainable shrimp aquaculture for Guam and the CNMI.

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# Environmental management of shrimp farms in Asia to promote healthy shrimp and reduce negative impacts

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## Abstract

Shrimp production in Asia ranges from extensive culture of *Penaeus monodon* to super-intensive culture of *Litopenaeus vannamei*. Farms often are small but there also are large farms, a few of which produce more shrimp than some shrimp producing countries in the Western Hemisphere. Production technology varies from primitive techniques to the most modern methods. There is a trend towards greater biosecurity by using disease-free broodstock, disinfection of source water and reduction in water exchange. Mechanical aeration allows for great increases in production, but most aerators used in Asia are low in efficiency compared to aerators used in the United States. Aerators cause much erosion in earthen ponds. Lined ponds allow for greater aeration and two to three times more shrimp production per hectare than in unlined ponds. Aeration requirements should be based on feed conversion efficiency, feed BOD, target DO concentration and aerator efficiency. Energy use in aeration is a major expense, often amounting to US$0.40 or more per kilogram shrimp. Aerator efficiency could be improved through changes in aerator design. Moreover, a reduction in daytime aerator use when water usually contains plenty of dissolved oxygen could reduce electricity costs. At some farms, organic sediment is removed from lined ponds during the production cycle to lessen oxygen demand and increase the proportion of dissolved oxygen added by aerators available to shrimp. Biological oxidation of ammonia nitrogen in lined ponds causes total alkalinity concentration to decline and lime applications should be made at frequent intervals in proportion to feed input. The usual amount of lime needed is about 0.3 to 0.5 kg $CaCO_3$ equivalent per kilogram feed. There is no evidence that water quality in ponds can be improved through additions of zeolite, coagulants, microbial products (sometimes called probiotics) and other amendments. There is increasing attention on implementation of better management practices to lessen negative environmental impacts of shrimp farming and comply with eco-label certification programs.

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# Introduction

Shrimp farmers in Asia rely upon a variety of production strategies. The major species traditionally was the black tiger prawn, *P. monodon* but several countries, particularly Thailand, Indonesia and China are currently producing large quantities of Pacific white shrimp, *L. vannamei*. Culture techniques range from semi-intensive with production of only a few hundred kilograms per hectare, to super-intensive with production of more than 10,000 $kg \cdot ha^{-1}$. Biosecurity measures are frequently installed to lower disease risk and more attention is given to reducing the potential for water pollution and other negative environmental impacts.

The purpose of this report is to discuss how shrimp ponds are managed in Asia and to present suggestions for improvements that could enhance culture conditions and lessen negative environmental impacts. The discussion will focus on intensive and semi-intensive shrimp farming because little management is applied in extensive shrimp culture.

### *The Culture Animals*

There is much debate about the wisdom of introducing non-native species, but *L. vannamei* has already been widely introduced in Asia. A lower crude protein concentration and less fish meal can be used in feed for *L. vannamei* than in feed for *P. monodon* (Fegan 2002a). Moreover, it is apparently easier to obtain disease-free broodstock of *L. vannamei* than *P. monodon* (Fegan 2002b). The introduction of *L. vannamei* has allowed farmers to realize greater survival, higher yields and lower production costs than are possible with *P. monodon* (Fegan 2007). Of course, the large production of *L. vannamei* in Asia has been a factor in the declining price of shrimp and escapes of *L. vannamei* from farms may eventually have negative impacts on native shrimp populations.

Widespread problems with viral diseases have caused farmers to seek specific-pathogen-free postlarvae for stocking in ponds. Such postlarvae usually are acquired from hatcheries using disease-free broodstock and PCR tests often are used to confirm that postlarvae are free of specific pathogens.

### *Pond Preparation*

Ponds are drained completely for harvest and there are several steps to prepare ponds to receive postlarvae for the next cycle (Suresh et al. 2006).Aerators generate currents that erode pond bottoms and the suspended particles settle in areas of the pond

where water currents are weaker. Most farmers remove this sediment from pond bottoms between crops and almost all farmers allow pond bottoms to dry. It also is common practice to apply 500 to 2,000 $kg \cdot ha^{-1}$ of liming material over bottoms of empty ponds. Tilling is sometimes done to enhance dry-out, but this practice favors erosion by aerators during the next production cycle. The best procedure would be to compact bottoms of ponds following tilling but this is seldom done.

Free viral particles apparently do not survive for more than a few days in water, so they can be eliminated by holding water for a few days before stocking postlarvae. However, various species of small shrimp, planktonic microcrustaceans and other aquatic animals can be vectors of viral disease. They survive longer than free viral particles and can serve as sources of viral diseases to infect newly-stocked, disease-free postlarvae.

Shrimp farmers often apply treatments to remove or kill disease vectors. Several methods are used to include liming, filtration, chlorination and dichlorvos treatment. Source water may be treated in reservoirs before adding it to ponds or water in ponds may be treated before stocking shrimp.

Fine mesh filters are necessary for removing microcrustaceans from water. Large volumes of water are needed to fill and maintain water levels in shrimp ponds. Filters for removing small crustaceans from water quickly become clogged and the fabric often rips. Filtration is usually not practical or effective for treating source water at large shrimp farms.

Liming is done with burnt lime (calcium oxide) or hydrated lime (calcium hydroxide). When added to a reservoir or pond at a rate of 1,500 to 2,000 $kg \cdot ha^{-1}$ (0.15 to 0.2 $kg \cdot m^{-3}$), lime can increase pH above 10 and kill aquatic organisms that are vectors of shrimp disease (Boyd and Tucker 1998). Lime is not highly soluble in water and is difficult to apply uniformly. It is virtually impossible to achieve a high pH throughout the water volume following lime treatment. Thus, liming is not highly effective in eliminating vectors of viral diseases.

Chlorination with calcium hypochlorite, commonly called high-test hypochlorite (HTH) can be effective in killing vectors of shrimp diseases (Baticados and Pitogo 1990). Chlorine residuals resulting from HTH application quickly are degraded to non-toxic form by sunlight and postlarvae can be safely stocked within 1 week following treatment. However, chlorine residuals also are rendered non-toxic by reaction with organic matter and other reduced substances in water and their toxicity declines with increasing pH (White, 1992). Source water for shrimp ponds usually has a

pH around 8 and contains several milligrams per liter of dissolved and particulate organic matter. The effective treatment rate for HTH is normally 20.0 to 30.0 $mg \cdot L^{-1}$ (Potts and Boyd 1998; Boyd and Massaut 1999). HTH is expensive and most producers cannot afford to apply it at such a high concentration. Unfortunately, the effectiveness of treatment with lower concentrations is highly variable.

Dichlorvos, dimethyl 2,2-dichlorovinyl phosphate, often abbreviated as DDVP, is an organophosphate pesticide highly toxic to shrimp, zooplankton and other aquatic organisms (Johnson and Finley 1980). It has a short residual life in water and does not accumulate in shrimp. For example, data collected on one shrimp farm showed the following residues at different times after treatment with 3.0 $mg \cdot L^{-1}$ of DDVP: 6 hr, 0.28 $\mu g \cdot L^{-1}$; 24 hr, 0.21 $\mu g \cdot L^{-1}$; 2 days, 0.1 $\mu g \cdot L^{-1}$; 3 days, 0.05 $\mu g \cdot L^{-1}$; 4 days, 0.03 $\mu g \cdot L^{-1}$; 5 days, 0.01 $\mu g \cdot L^{-1}$; 6 and 7 days, not detectable. Moreover, DDVP residue was not detected in shrimp tissue when shrimp were harvested 4 months later. Treatment of source water with DDVP at 2 to 3 $mg \cdot L^{-1}$ active ingredient is an effective and economical technique for killing vectors of shrimp disease.

Dichlorvos must be handled carefully to avoid spills and possible toxicity to aquatic animals in the vicinity of farms. It also is toxic to humans and workers should wear protective gear to avoid breathing vapors or making direct skin contact with this compound. Nevertheless, it is no more environmentally hazardous or dangerous to workers than HTH. Even lime can be hazardous to workers because of its caustic properties. The main issue with DDVP is that environmentalists and the public tend to have a negative perception of most pesticides. This is unfortunate in the case of DDVP because treatment with this compound is probably the most reliable and safest way to destroy vectors of shrimp diseases in farm water supplies.

### *Fate of Feeds in Ponds*

Natural productivity will not support over a few hundred $kg \cdot ha^{-1}$ shrimp (Boyd and Tucker 1998). Feed can be applied to increase shrimp production, but dissolved oxygen from natural sources usually will not support more than 1,000 $kg \cdot ha^{-1}$ of shrimp. Feeding is a common practice for increasing shrimp production.

The fate of feed applied to aquaculture systems is illustrated in Fig. 1. Shrimp nibble feed pellets, and pellets disintegrate before they are completely ingested. In some cases, nearly half the organic carbon in feed pellets is not ingested (Ruttanagosrigit 1997); however, shrimp usually consume 85 to 90% of their feed. Shrimp feeds are of high quality, and up to 80 to 90% of dry matter that is eaten is absorbed across the intestine. The remainder is expelled as feces. Organic carbon

absorbed across the intestine of shrimp is either oxidized in respiration or converted to biomass. Nitrogen not incorporated into shrimp biomass is excreted into the water as ammonia. Organic carbon and nitrogen in biomass are either catabolized and excreted as carbon dioxide and ammonia, or removed from the culture system at harvest. Uneaten feed and feces are decomposed by bacteria with organic carbon oxidized to carbon dioxide, and organic nitrogen mineralized to ammonia.

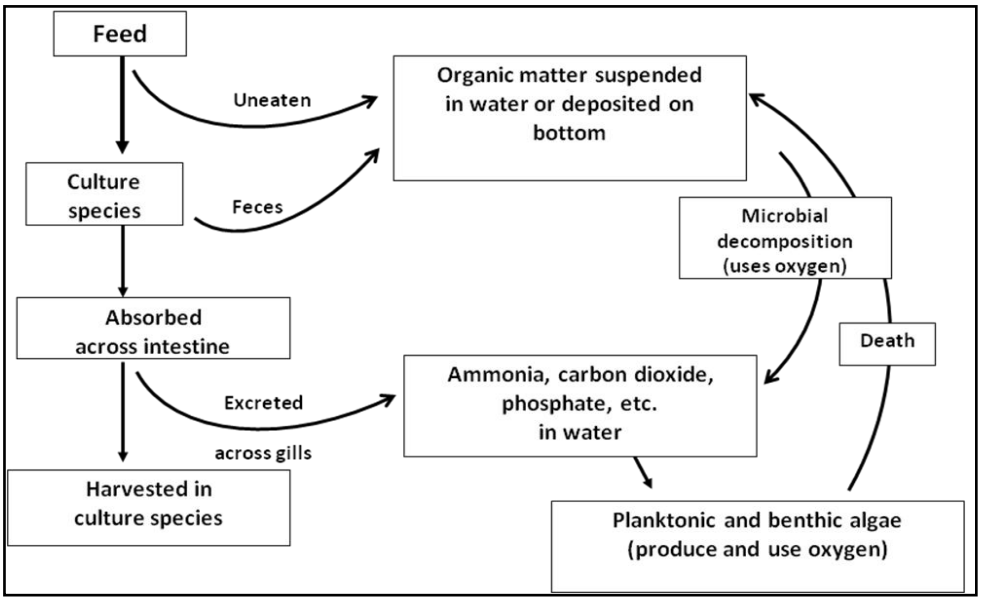


**Fig. 1**. Fate of feeds in aquaculture ponds.

In summary, some of the organic carbon and nitrogen added to shrimp ponds in feed becomes biomass and is harvested. The rest of it is oxidized to carbon dioxide, or mineralized to ammonia by shrimp and bacteria. The majority of uneaten feed and feces will be oxidized and mineralized, and nitrifying bacteria in pond water will oxidize ammonia to nitrate.

Phosphorus also enters pond water as a waste from feed. Thus, feeding increases the availability of carbon dioxide, ammonia, nitrate and phosphate, resulting in greater phytoplankton productivity. Accumulation of organic carbon in biomass, detritus, and sediment in un-aerated ponds is irrefutable evidence that more oxygen is produced in photosynthesis than used by respiration of the biota in the short term. Nevertheless, much of the oxygen produced by phytoplankton is used in their respiration or in respiration of heterotrophic microorganisms that degrade dead phytoplankton. Aeration usually must be applied in ponds with feeding rates over 30 $kg \cdot ha^{-1}$ per day (Boyd and Tucker 1998).

***BOD of Feed***

The BOD of feed can be defined as the amount of oxygen required to oxidize organic carbon and nitrogen applied to ponds in feed but not recovered in shrimp at harvest. Organic carbon oxidation has the same stoichiometry regardless of whether it is done by bacteria or by shrimp:

$$\text{Organic C} + O_2 \rightarrow CO_2 \quad (1)$$

Oxidation of 12 units of organic carbon requires 32 units oxygen – 2.67 kg $O_2 \cdot kg^{-1}$ organic C. The summary equation for oxidation of ammonia nitrogen by nitrifying bacteria is:

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \quad (2)$$

Oxidation of 14 units of ammonia nitrogen requires 64 units of molecular oxygen – 4.57 kg $O_2 \cdot kg^{-1}$ ammonia N.

Feed for *L. vannamei* usually contains about 45% organic carbon and 5.6% nitrogen. A feed conversion ratio (FCR) of 1.8 often is achieved and live, harvestable-sized *L. vannamei* contain about 11% carbon and 2.86% nitrogen (Boyd and Teichert-Coddington, 1995). One kilogram of feed contains 0.45 kg organic carbon and 0.056 kg organic nitrogen. It produces 0.556 kg live shrimp containing 0.061 kg organic carbon and 0.016 kg organic nitrogen. Thus, 0.389 kg organic carbon and 0.04 kg ammonia nitrogen must be oxidized for each kilogram of feed applied. Oxidation of organic carbon will require 1.039 kg $O_2 \cdot kg^{-1}$ feed and oxidation of ammonia will require an additional 0.183 kg $O_2 \cdot kg^{-1}$ feed – the BOD of the feed in this example is 1.22 kg $O_2 \cdot kg^{-1}$ feed.

Calculations for the BOD of feed can be arranged into an equation (Boyd, 2009) as follows:

$$BOD_f = [C_f - (FCE \times C_s)]2.67 + [N_f - (FCE \times N_s)]4.57 \quad (3)$$

where $BOD_f$ = feed BOD (kg $O_2 \cdot kg^{-1}$ feed); $C_f$, $N_f$, $C_s$, $N_s$ = decimal fractions, %/100, of carbon and nitrogen in feed and shrimp, respectively; FCE = feed conversion efficiency. The FCE is the inverse of the feed conversion ratio (FCR).

The BOD of a given feed will vary with the feed conversion efficiency. If FCE in the example above is changed to 0.455 (FCR = 2.2), $BOD_f$ becomes 1.26 kg $O_2 \cdot kg^{-1}$.

Improving FCE to 0.667 (FCR = 1.5) would lower $BOD_f$ to 1.17 kg $O_2 \cdot kg^{-1}$ feed. Percentages of carbon and nitrogen in feed vary with differences in percentages of major ingredients contained in feed. For example, average percentages of carbon in carbohydrates, proteins, and fats are 40, 53 and 77.2%, respectively. Increasing the proportion of ingredients with high protein content or with high fat content will increase the carbon content of feed. Percentage nitrogen will vary with the amount of protein and can be estimated as crude protein divided by 6.25.

The proposed method for estimating BOD of feed obviously would be facilitated by more data on composition of shrimp feeds and shrimp. However, until such data are available, it will suffice to use a $BOD_f$ value of 1.2 kg $O_2 \cdot kg^{-1}$ feed.

## Mechanical Aeration

### *Aerators*

Several types and sizes of aerators are used in aquaculture to include vertical pumps, pump sprayers, diffused-air systems, propeller-aspirator pumps and paddlewheels ranging in size from 0.5 to 10 horsepower (hp): 1 hp = 0.746 kW (kilowatt). Paddlewheel aerators are used almost exclusively in Asian shrimp farming, but propeller-aspirator-pump aerators such as the Aire-$O_2$ (Aeration Industries, Chaska, Minnesota) are popular in some areas. Paddlewheel aerators used in Asian shrimp farming are manufactured by several companies in Asia and the paddlewheels of these devices tend to have a similar design (Fig. 2). There are two basic styles of Asian paddlewheel aerators: 1 and 2 hp units have an electric motor and paddlewheels mounted on floats (Fig. 3). Larger units have 5- to 10-hp electric motors or internal combustion engines on the pond bank which are attached to the paddlewheels mounted on floats (Fig. 3).

The oxygenation efficiency of aerators has been determined in standard oxygen transfer tests and reported in kilograms of oxygen transferred from air to water per kilowatt·hour (kW·hr) of electricity used by the aerator motor (Boyd 1998). Paddlewheel aerators used in channel catfish farming in the United States (Fig. 4) have standard oxygen transfer efficiencies (SAEs) of 2.0 to 2.8 kg $O_2 \cdot kW^{-1} \cdot hr^{-1}$ (Boyd 1998). Recent tests of paddlewheel aerators used in Asia shrimp farming revealed SAEs of 0.8 to 1.5 kg $O_2 \cdot kW^{-1} \cdot hr^{-1}$ (C. E. Boyd, unpublished data). The Aire-$O_2$ aerators are more efficient in transferring oxygen to water than Asian-style paddlewheel aerators (Boyd and Tucker 1998), but they are more expensive per horsepower. There appears to be

great opportunity for improving the performance of Asian-made paddlewheel aerators by incorporating some of the design features of more efficient aerators.

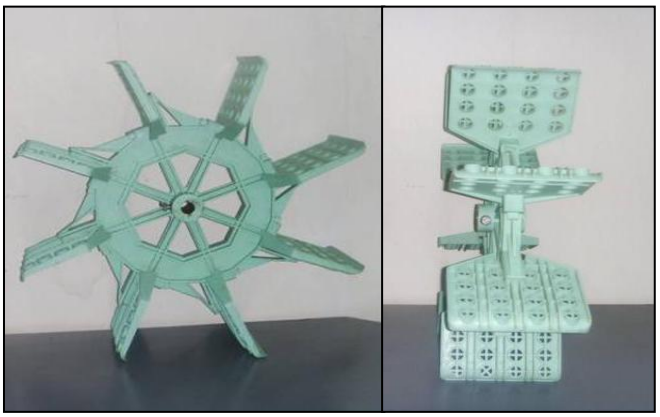

**Fig. 2.** Typical paddle for Asian-style paddlewheel aerators.

**Fig. 3.** Asian-style paddlewheel aerators. Left: motor attached to floating frame. Right: motor positioned on the bank and connected to the floating paddlewheel by drive shaft.

Data from an intensive shrimp farm with production of 6,000 $kg \cdot ha^{-1}$ per crop and a feed conversion ratio of 1.75 were obtained and used to estimate the cost of aeration per tonne of shrimp (Table 1). The cost of aeration was also computed for the same amount of aeration and shrimp production assuming that ponds were aerated with more efficient aerators such as those used in channel catfish farming in the United States (Table 1). The results show that the cost of aeration would decline from US\$0.536$\cdot kg^{-1}$ to US\$0.345$\cdot kg^{-1}$.

**Fig. 4.** Floating electric paddlewheel aerator used in channel catfish farming in the United States.

The US-style paddlewheel aerators are fabricated of mild steel and would corrode badly in saline-water ponds. However, preliminary studies have shown that the design, which is described in detail elsewhere (Boyd, 1998), can be modified to fabricate more efficient plastic paddlewheels for retrofitting Asian-style aerators currently in use. Alternatively, the aerator design used in the United States is not protected by international patents and could be adopted in its entirety and used by Asian aerator manufacturers.

**Table 1**. Estimation of total aeration cost in United States dollars for producing 6,000 kg·ha$^{-1}$ shrimp at a feed conversion ratio of 1.75 using aerators of different standard aeration efficiencies (SAE).

| **Variable** | SAE (kg $O_2$·kW$^{-1}$·h$^{-1}$) | |
|---|---|---|
| | 1.1 | 1.9 |
| Aeration rate (kW·ha$^{-1}$) | 9 (≈12 hp·ha$^{-1}$) | 5.2 (≈7 hp·ha$^{-1}$) |
| Aerator cost per unit ($·kW$^{-1}$) | 402 | 603 |
| Aerator purchase cost ($·ha$^{-1}$) | 3,618 | 3,136 |
| Aerator service life (crops) | 10 | 10 |
| Aerator purchase cost ($·ha$^{-1}$·yr$^{-1}$) | 363 | 314 |
| Aerator maintenance cost ($·kW$^{-1}$·crop$^{-1}$) | 40 | 60 |
| Aerator maintenance cost ($·ha$^{-1}$·crop$^{-1}$) | 360 | 312 |
| Total aerator cost ($·ha$^{-1}$·crop$^{-1}$) | 722 | 626 |
| Electricity use (kW·hr$^{-1}$·crop$^{-1}$) | 20,790 | 12,036 |
| Electricity rate ($·kW$^{-1}$·hr$^{-1}$) | $0.12 | $0.12 |
| Electricity cost ($·ha$^{-1}$·crop$^{-1}$) | 2,495 | 1,444 |
| Total cost of aeration ($·ha$^{-1}$·crop$^{-1}$) | 3,217 | 2,070 |
| Total cost of aeration ($·kg$^{-1}$·shrimp$^{-1}$) | 0.536 | 0.345 |

### *Aeration requirements*

The importance of adequate aeration is underscored by a study that showed improved survival, production and feed conversion ratio for *L. vannamei* in ponds with higher daily minimum dissolved oxygen concentration (Table 2). Most shrimp farmers base the amount of aeration on the widely-held opinion that 1 hp of aeration will support 400 to 500 kg of shrimp. Intensive shrimp farmers in Asia apparently use sufficient aeration, because feed conversion ratios are usually below 2.0. The calculation of aeration requirement, however, may be more objectively made from projected maximum daily feeding rate, minimum acceptable daily dissolved oxygen concentration, feed biochemical oxygen demand and aerator SAE (Boyd 2009).

The amount of aeration applied to ponds should supply enough dissolved oxygen to satisfy $BOD_f$ and prevent dissolved oxygen concentration from falling below an acceptable minimum level (3 $mg \cdot L^{-1}$ often is considered an acceptable minimum concentration in shrimp ponds). The feeding rate in a pond with a final harvest biomass of 6,000 $kg \cdot ha^{-1}$ of shrimp would be around 150 $kg \cdot ha^{-1} \cdot day^{-1}$ (Wickins and Lee 2002). Assuming a $BOD_f$ of 1.2 kg $O_2 \cdot kg^{-1}$, the daily $BOD_f$ load would be 180 $kg \cdot ha^{-1} \cdot day^{-1}$. Although the daily $BOD_f$ load is not expressed completely during the day that it is applied, shrimp and bacteria respire continuously, and waste from previous days is also being decomposed. It seems reasonable to assume that the daily expression of $BOD_f$ is roughly equivalent to the daily $BOD_f$ load. Moreover, feed is applied to shrimp ponds several times per day, and the rate of expression of $BOD_f$ likely is relatively constant – 7.5 kg $O_2 \cdot hr^{-1}$ in this example.

**Table 2**. Effect of different, average early morning dissolved oxygen concentrations on shrimp survival, yield, and feed conversion ratio (FCR) in ponds stocked at 33 $postlarvae \cdot m^{-2}$. Each treatment was replicated three times. Source: McGraw et al. (2001).

| Early morning dissolved oxygen ($mg \cdot L^{-1}$) | Survival (%) | Shrimp yield ($kg \cdot ha^{-1}$) | FCR |
|---|---|---|---|
| 2.32 | 42 | 2,976 | 2.64 |
| 2.96 | 55 | 3,631 | 2.21 |
| 3.89 | 61 | 3,975 | 1.96 |

The standard aerator testing procedure (American Society of Civil Engineers, 1992) measures the kilograms of oxygen per kilowatt·hour that an aerator will transfer to clean freshwater at 20 $^oC$ containing 0 $mg \cdot L^{-1}$ dissolved oxygen. The aeration efficiency under conditions existing in shrimp ponds can be estimated by the equation:

$$AE = SAE \frac{C_{sp} - C_m}{C_{20S}} 1.024^{t-20} \alpha \quad (4)$$

where AE = aeration efficiency (kg $O_2 \cdot kW^{-1} \cdot hr^{-1}$); SAE = standard aeration efficiency (kg $O_2 \cdot kW^{-1} \cdot hr^{-1}$); $C_{sp}$ = concentration of dissolved oxygen at saturation in pond ($mg \cdot L^{-1}$); $C_m$ = measured dissolved oxygen concentration in pond ($mg \cdot L^{-1}$); $C_{20s}$ = concentration of dissolved oxygen at 20 °C and salinity of pond water ($mg \cdot L^{-1}$); $\alpha$ = 0.92. The $\alpha$ value is the rate at which a mechanical aerator will transfer oxygen to pond water divided by the rate that it will aerate clean freshwater. This value averaged 0.92 for water samples from a large number of aquaculture ponds (Shelton and Boyd 1983).

The SAE value for the more efficient Asian style paddlewheel aerators is 1.5 kg $O_2 \cdot kW^{-1} \cdot hr^{-1}$. Enough aerators should be placed in ponds to prevent dissolved oxygen concentration from falling below 3 $mg \cdot L^{-1}$. In a pond with a water temperature of 28 °C and 30 ppt salinity, the AE calculated with Equation 4 for an aerator with an SAE of 1.5 kg $O_2 \cdot kW^{-1} \cdot hr^{-1}$ is 0.792 kg $O_2 \cdot kW^{-1} \cdot hr^{-1}$. Dividing the amount of oxygen necessary to satisfy the BOD imposed by feed (7.5 kg $O_2 \cdot ha^{-1} \cdot hr^{-1}$ in this example) by AE gives an aeration requirement of 9.4 $kW \cdot ha^{-1}$ to maintain a dissolved oxygen concentration of 3 $mg \cdot L^{-1}$ or more. Aerators are usually sized in horsepower (1 kW = 0.746 hp), and about 12.6 $hp \cdot ha^{-1}$ of aeration would be necessary.

The calculations used above for computing aeration requirement may be combined into the following equation:

$$AR = \frac{(BOD_f)(FR)}{(AE)(24\ hr)(0.746)} \quad (5)$$

where AR = aeration requirement (hp/ha); FR = maximum daily feeding rate ($kg \cdot ha^{-1} \cdot day^{-1}$); AE = aeration efficiency (kg $O_2 \cdot kW^{-1} \cdot hr^{-1}$) calculated with Equation 4 for a dissolved oxygen concentration of 3 $mg \cdot L^{-1}$.

When applied to typical conditions for *P. vannamei* culture, the method for estimating aeration requirement outlined above suggests that 1 hp of aeration with typical aerators is adequate for 475 $kg \cdot ha^{-1}$ of shrimp. This agrees well with the common belief that 1 hp of aeration will support 400 to 500 kg of shrimp. However, the use of $BOD_f$ and aerator SAE for estimating aeration requirements would allow for more refined estimates of the amount of mechanical aeration needed in shrimp ponds. It is essential to have sufficient aeration to prevent low dissolved oxygen concentration from stressing the culture species, but electricity for powering aerators is expensive, and excessive aeration is wasteful.

***Use of aeration***

It is suspected that an even greater reduction in aeration cost could be achieved by turning off some aerators during the daytime when dissolved oxygen concentration is high. In catfish farming in the United States, aeration is normally applied only at night. However, shrimp live on the bottom and water circulation caused by aerators is needed during the day to avoid adverse conditions at the sediment water interface. Nevertheless, if daytime aeration could be reduced by only 25%, an electricity savings of 12.5% would be realized. This would reduce the cost of aeration by US$0.052·kg$^{-1}$ in ponds with Asian-style aerators, and by US$0.030·kg$^{-1}$ in ponds with US-style aerators.

An alternative to daytime aeration might be the use of mechanical water circulators (Howerton et al. 1993). These devices could possibly create water circulation at a lower power input than is possible with aerators. They might also conserve dissolved oxygen because surface aerators cause loss of dissolved oxygen from ponds when dissolved oxygen concentration is above saturation during late morning and early afternoon. Research findings are conflicting on the benefits of water circulators in reducing the amount of time that aerators must be operated (Tucker and Steeby 1995); thus, farm trials are needed to ascertain whether or not this technology has merit.

Aerators cause considerable erosion of the insides of embankments and bottoms of ponds. Producers spend considerable effort and expense to remove sediment that accumulates in the central areas of ponds during each crop and disposal of this sediment can cause salination (Boyd et al. 1994). This problem can be lessened if embankments are sloped 2:1 or 3:1 on the wet side and aerators positioned at least 2 or 3 m beyond the toes of the embankments. In addition, aerators should be positioned so that strong currents should not impinge upon embankments. However, the most reliable way of avoiding serious erosion is to cover vulnerable parts of pond bottoms and embankments with plastic liners.

A few farms have resorted to completely covering the insides of ponds with plastic liners to allow greater amounts of aeration. Liners are expensive, and production must be high to make this technology economically feasible. In one production technique used in lined ponds, yields of 12,000 to 15,000 kg·ha$^{-1}$ are achieved in green-water ponds. Dead algae accumulate on pond bottoms, and some producers use suction pumps to remove this material. This reduces the demand for oxygen in the pond, but it does so at the environmental expense of externalizing part of the waste load. The most

environmentally-responsible approach would be to discharge the sludge into a wastewater treatment pond before releasing it into the environment.

The heterotrophic "floc" system (McIntosh 1999, 2008) requires lined ponds, and some shrimp farms in Asia have installed "floc" systems. Production may exceed 20,000 kg·ha$^{-1}$ in such systems, and 30 to 40 hp·ha$^{-1}$ of aeration may be used. Pond water becomes so turbid from suspended organic particles that phytoplankton growth is light limited. The floc is largely suspended in the water by aerator mixing, and it harbors a high abundance of bacteria. Shrimp eat the bacterial floc, thereby lessening the amount of feed that must be applied.

## Acidity from Nitrification

The portion of feed nitrogen not included in shrimp at harvest can potentially enter pond water as ammonia nitrogen. The potential load of ammonia nitrogen in a pond producing 6,000 kg·ha$^{-1}$ shrimp will be estimated assuming that feed is 5.6% nitrogen (35% crude protein), FCR is 1.75 and shrimp contain 2.86% nitrogen. The calculations follow:

Feed N = 6,000 kg shrimp·ha$^{-1}$ × 1.75 × 0.056 kg N·feed$^{-1}$ = 588 kg N·ha$^{-1}$;

Shrimp N = 6,000 kg shrimp·ha$^{-1}$ × 0.0286 kg N·kg$^{-1}$ shrimp = 171.6 kg N·ha$^{-1}$;

Ammonia nitrogen load = Feed N – shrimp N = 416.4 kg N·ha$^{-1}$.

Much of the ammonia nitrogen in pond water is oxidized to nitrate (Equation 2). This oxidation releases hydrogen ions that can reduce the total alkalinity of pond water. Alkalinity is expressed as equivalent calcium carbonate and the loss of alkalinity can be illustrated as follows:

$$CaCO_3 + 2H^+ = Ca^{2+} + CO_2 + H_2O \quad (6)$$

Based on stoichiometry (Equations 2 and 6), oxidation of one unit of ammonia nitrogen can cause a reduction in total alkalinity of 7.14 units. The potential acidity resulting from feed in the example above would be equivalent to 2,973 kg $CaCO_3$ (total alkalinity)·ha$^{-1}$ – 0.28 kg $CaCO_3$·kg$^{-1}$ feed. The actual amount of liming material would vary with feed protein concentration and FCR, but for practical purposes, 0.30 to 0.35 kg $CaCO_3$ equivalent·kg$^{-1}$ feed should suffice. Liming materials do not react completely, so some producers may want to apply about 1.5 times more liming material than the minimum amount needed to neutralize acidity from feed. It is important to

note that liming materials vary in their ability to neutralize acidity, for example, calcium oxide (CaO) and calcium hydroxide [$Ca(OH)_2$] are 135% and 179% more effective, respectively than calcium carbonate ($CaCO_3$). An equation is available for more exact estimation of feed lime requirement (Boyd 2007), but the information needed to use the equation will seldom be available to small-scale, shrimp producers. In highly intensive shrimp culture, an amount of liming material sufficient to neutralize the potential acidity of the feed input of the previous week should probably be calculated and applied at the end of each week of culture.

Seawater and brackishwater are usually high in alkalinity, and in ponds with high water exchange rates, total alkalinity may not decline. Also, in ponds with earthen bottoms that are limed during pond preparation, calcium carbonate in soil may dissolve to replenish alkalinity. However, in intensive ponds without water exchange, and especially in ponds with liners, total alkalinity concentration will decline during the production cycle.

## Water Quality Amendments

Shrimp farms in Asia seldom use high rates of water exchange, as was once common practice. The three most effective measures for maintaining water quality in ponds are conservative feeding practices to avoid overfeeding, mechanical aeration, and liming. Many producers, nevertheless, apply many chemical and biological amendments for the purpose of improving water quality. A study by Gräslund et al. (2003) identified 290 different amendments used by shrimp farmers in Thailand. However, only a few of these amendments are used frequently. Many farmers apply zeolite for ammonia removal, and microbial products such as live bacterial inocula and enzyme preparation to enhance natural microbial processes. There is no sound scientific evidence that either of these practices is effective in pond aquaculture (Boyd and Tucker 1998; Mischke 2003; Boyd and Silapajarn 2006). Fortunately, neither zeolite nor microbial amendments have negative effects on health and growth of aquaculture animals, the environment or food safety. Benzalkonium chloride (BKC) is also commonly used at about 1.5 $mg \cdot L^{-1}$ to control phytoplankton (Lee et al. 1994). The effectiveness of this practice is questionable and the possible negative environmental impacts have not been studied. Sodium nitrate may be applied to ponds to oxidize the sediment-water interface. This practice improved the redox potential at the sediment surface in freshwater ponds (Chainark and Boyd 2010), but its effects on shrimp production are not known. Some producers apply iron or aluminum compounds to remove phosphorus from pond waters in an effort to lessen phytoplankton abundance.

There is some evidence that these products can be effective (Boyd et al. 2008) and further research is needed.

## Low-salinity Shrimp Farming

There is considerable production of shrimp in water of 1 to 5 ppt salinity in several Asian countries. In Thailand, brine solution from coastal seawater evaporation ponds is often added to freshwater in ponds to provide a suitable salinity for shrimp culture (Limsuwan et al. 2002). Ionic imbalances do not occur in pond water because the brine solution contains the full complement of ions of seawater in concentrated form (Boyd et al. 2002). At locations where saline groundwater is used to fill ponds, waters may be deficient in potassium and magnesium. Muriate of potash (fertilizer-grade potassium chloride) and sulfate of potash magnesia (a potassium magnesium sulfate fertilizer sold under the trade name Kmag®) applied to low-salinity shrimp ponds can be highly effective in increasing potassium and magnesium concentrations and correcting ionic imbalances (McNevin et al. 2004).

Low-salinity shrimp culture can cause salination through seepage into groundwater and overflow, or intentional discharge into surface water (Braaten and Flaherty 2001; Boyd et al. 2006). Inland shrimp farming has been banned in freshwater areas in Thailand (Fegan 2001a), but the definition of freshwater areas has been subject to interpretation and the practice persists. Best practices for avoiding salination are to site, design, construct and operate ponds in a way that minimizes seepage, overflow and harvest effluent. Low-salinity shrimp farming is currently being conducted in some freshwater areas without causing serious salination (Boyd et al. 2006).

## Environmental Issues

Complaints by environmentalists have led to the development of codes of conduct and best management practices (BMPs) for shrimp farming in most major shrimp-producing countries (Boyd 2003). Moreover, governments have enacted regulation for reducing environmental impacts (Tookwinas 1996). For example, the Pollution Control Board and National Environmental Board in Thailand have limited biochemical oxygen demand and suspended solids in effluent discharge to a maximum of 20 $mg \cdot L^{-1}$ and 70 $mg \cdot L^{-1}$, respectively (Fegan 2001b). The standards are initially limited to farms larger than 1.5 ha. Governments of several countries prohibited construction of shrimp farms in mangrove forests, established rules about importing broodstock and postlarvae, restricted the use of some antibiotics and implemented effluent regulations. There is increased awareness of environmental protection among

shrimp producers and production practices today are less harmful to the environment than those used in the past.

A study in Bangladesh (Wahab et al. 2003) revealed that effluent water from extensive shrimp farms was of higher quality than the water entering the farms. Feed is not widely used in extensive farms and there is little use of chemicals other than liming materials and fertilizers. Thus, environmentalists tend to favor extensive shrimp production over more intensive culture. Nevertheless, there is a major trade-off. Extensive shrimp farms are more likely than semi-intensive and intensive farms to be constructed in mangrove habitats or other sensitive wetlands, and the area of land devoted to producing 1 tonne of shrimp is 5 to 25 times greater than the area necessary to produce 1 tonne of shrimp by more intensive production (Boyd et al. 2007).

The public is becoming more environmentally aware and there is a growing market for products produced by using environmentally- and socially-responsible methods. There is also great concern by consumers regarding food safety because of many widely publicized incidences of food tainted by microbial and chemical contaminants. Therefore, firms purchasing large amounts of shrimp such as wholesalers, supermarket chains and restaurants are seeking shrimp that are of high quality, free of contaminants and produced by environmentally- and socially-responsible techniques. They want to be able to trace shrimp back to the farm and pond of origin, and in addition, to have access to records of all treatments made to the shrimp should questions arise about food safety.

The governments of importing countries make inspections of shrimp at their port of entry, but they only sample a small percentage of arriving shipments. Some countries also require shrimp and other fisheries products to be labeled with their country of origin and to their means of production (whether they were wild-caught or produced by aquaculture). However, a growing segment of the population wants additional assurance that their food is safe and was produced responsibly.

The Global Aquaculture Alliance (GAA), an aquaculture industry oriented non-governmental organization headquartered in St. Louis, Missouri, USA, developed standards for responsible shrimp production, and the Aquaculture Certification Council (ACC) certifies hatcheries, feed mills, farms and processing plants willing to adopt GAA Best Aquaculture Practice (BAP) standards. Wal-Mart Stores, Inc., the world's largest public cooperation by revenue and several other large shrimp buyers are purchasing ACC-certified shrimp exclusively. GLOBAL G.A.P., a private sector body with main offices in Cologne, Germany, sets voluntary standards for certification of agriculture products worldwide, and recently designed standards for shrimp. In

addition, several large food retailers, e.g. Wegmans Food Markets, Whole Foods and Costco (USA), Marks and Spencer and Tesco (UK) and Carrefour (France) as well as Bon Appetit Management Company, a catering and small restaurant management firm have already developed or are in the process of developing purchasing standards. These buyers seek producers willing to supply shrimp produced according to their purchasing standards. Some shrimp farms in Asia have been certified by ACC or are participating in one of the purchasing standards programs; more are expected to join in the future. The World Wildlife Fund also is developing standards for responsible shrimp culture through dialogues involving a wide range of stakeholders. When completed, a certifying body will be found or established.

It appears that several certification programs may result from the current effort to foster responsible shrimp farming. The Food and Agriculture Organization (FAO) of the United Nations is developing guidelines for aquaculture certification programs, and their guidelines should lead to greater uniformity among the various standards. Nevertheless, most of the standards appear to be consistent with respect to environmental issues. The major issues are as follows: selection of good sites for new farms or farm expansions; protection of mangrove and other wetlands; water pollution control; reduction in use of fish meal and fish oil in feeds; conservative feeding practices; use of farm-reared rather than wild-caught broodstock and postlarvae; more reliance on shrimp health management and less use of antibiotics for disease control; restrictions on imports of broodstock and postlarvae; retention of records on management activities. There are, however, large variations in social standards among the different certification programs, and major differences in how programs are organized, operated and administered.

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# Performance of a closed recirculating system with foam separation, nitrification and denitrification units for intensive culture of kuruma shrimp, *Marsupenaeus japonicus*: a bench scale study

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## Abstract

The development of a closed recirculating aquaculture system that does not discharge effluents would reduce a large amount of pollutant load on aquatic bodies. In this study,kuruma shrimp *Marsupenaeus japonicus* were reared in a closed recirculating system (total water volume, 1.3 $m^3$), which consisted of a culture tank (area, 1.2 $m^2$), a foam separation unit, a nitrification unit and a denitrification unit. The foam separation unit has an inhalation-type aerator and supplies air bubbles to the culture water. The shrimp used in the test (0.8g·individual$^{-1}$) were judged in advance to be free of the white spot disease virus (WSDV) by the method of loop-mediated isothermal amplification (LAMP). The growth of shrimp, which were fed a commercial diet, was satisfactory, with the average weight increases of up to 11 times in 4.5 months. The individual density at the end of the culture period was 51 individuals·$m^{-2}$. The foam separation unit maintained oxygen saturation in the water used for rearing at 101%. Furthermore, contaminants such as suspended solids, chromaticity substances and bacteria absorbed on the stable foam were removed from the culture water by foam separation. The turbidity in culture water was kept at less than two units. Total ammonia-nitrogen (TAN) and nitrite ($NO_2$-N) oxidation were accomplished rapidly and simultaneously in the nitrification unit. TAN and $NO_2$-N concentrations were kept at less than 0.4 mg-N·$L^{-1}$ and 0.02 mg-N·$L^{-1}$, respectively. When the denitrification process was operated, nitrate ($NO_3$-N) that accumulated in the culture water (20 mg-N·$L^{-1}$) was reduced to 4 mg-N·$L^{-1}$. Based on these results, the intensive aquaculture of kuruma shrimp can be achieved using a closed recirculating system under virus-free conditions without emission.

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# Introduction

The technology of a recirculating aquaculture system with a high fish density has been developed (Bovendeur et al. 1987; Heinsbroek and Kamsta 1990; van Rijn and Rivera 1990; Ng et al. 1992; Knosche 1994; Honda et al. 1994; Arbiv and van Rijn 1995; van Rijn 1996; Geiner and Timmous 1998; Yoshino et al. 1999) and the remarkably high productivity and energy efficiency of such a system have become possible (Blancheton 2000). In addition, shrimp, which has the highest demand in the global seafood market, is now produced in closed recirculating systems (Otoshi et al. 2003). However, the frequency of changing rearing water per day is from 5 to 100% in general, thereby having minimal effect on the reduction in pollutant load. In our study that examined the amount of pollution drained from inland aquaculture farms in Japan, it was found that the type of fish being cultivated made minimal difference. On average, the load per unit of cultured fish was 0.8 kg-N·t-fish$^{-1}$day$^{-1}$ and 0.1 kg-P·t-fish$^{-1}$·day$^{-1}$ (Maruyama and Suzuki 1998). This pollutant load corresponded to 73 persons·t-fish$^{-1}$ assuming that the human nitrogen load is equivalent to 11 g-N·person$^{-1}$·day$^{-1}$. It assumed that the load of shrimp culture is equal to that of fish culture. While the aquaculture industry supplies food and livelihood to many, it is likewise a bane to aquatic environments. Particularly, the expansion of shrimp farm affects the pollution load increase, the degradation of the coastal environment and valuable area such as mangrove forests (Mumby et al. 2004).

Recently, the aquaculture industry is likewise urged to convert to a new system that introduces the concept of zero emission and this requires the development of a closed recirculating system using innovative technology for fish production. Zero-emission aquaculture system in an actual shrimp farm has not yet been developed. However, if intensive shrimp culture in a perfectly closed recirculating system becomes technically possible, the development of zero-emission systems can be realized.

With the combined aim of increased fish production and reduced nutrient load in aquatic environments, we have advanced the development of a zero-emission system composed of foam separation, nitrification and denitrification units. Culture trials of Japanese flounder in seawater (Maruyama et al. 1998; Suzuki et al. 2000) and eel in fresh water (Suzuki et al. 2003) were carried out using this almost perfectly closed system. The survival rate was very high, that is, more than 3 months despite the high fish density. The advantage of this system is that it is equipped with an effective foam separation unit as part of its main purification process. Oxygen supply, removal of suspended substances and deaeration can be achieved simultaneously by the foam separation process (Maruyama et al. 1991; Maruyama et al. 1996). By applying the principles of the fish culture system, we tried the development of a closed recirculating

system for culturing kuruma shrimp. An ideal aquaculture system is one which purifies the rearing water while obtaining high biomass productivity. In this study, shrimp growth, function of each water treatment process and load reduction were examined.

## Materials and Methods

### *System description*

A closed recirculating system with foam separation, nitrification and denitrification units is shown in Fig.1. This system consisted of a shrimp rearing tank (water volume 0.7 $m^3$; water surface area 1.2 $m^2$), a foam separation tank (0.2 $m^3$) equipped with an inhalation-type aerator (200 V, 0.2 kw), a nitrification tank (0.16 $m^3$) and a denitrification tank (0.22 $m^3$). The total amount of water in this system was 1.3 $m^3$. A heater (100 V, 1 kw) and a pH control pump (Iwaki Co., EH.W-PH, 5% sodium hydrogen carbonate solution) for adjusting the conditions of the rearing water (25 $^oC$ and pH 7.5) were set in the foam separation tank and a water conditioner was set on the recirculating pipe. First, sand filtered seawater was introduced to the system and one cycle was carried out for 15 min at 56 $L \cdot min^{-1}$. The rearing water was transported to the foam separation tank by a circulating pump and oxygen supply and foam separation processing were simultaneously carried out using this unit. The rearing water was then introduced into the nitrification tank with an up-flow style and the treated rearing water was returned to the rearing tank. The system was located indoors and the rearing tank was shielded from light.

The difference between fish and kuruma shrimp rearing is that kuruma shrimp lives in the sand during daytime. Therefore, the sand must be laid at the bottom of the rearing tank. The sand used in the rearing tank accumulated evacuated matters and residual feed and large amount of labor is required to clean the sand. To reduce the accumulation of polluted substances in the sand, the rearing tank was made with a double bottom and the rearing water was made to flow upward under the sand layer. Coral sand (grain size, 2 mm) was used for this system.

The main core of this system for rearing water treatment was the foam separation unit (Suzuki et al. 2003), which was equipped with an inhalation-type aerator (Plesca Co., Japan). Surface-active materials in the rearing water were adsorbed on bubbles and the bubbles were carried to the water surface. Then, foam was generated on the water surface. The foam generated continuously was spontaneously removed from the foam duct placed at the upper part of the tank equipped with air exhaust. Furthermore, air bubbles were vigorously mixed into the water and oxygen was

efficiently dissolved into the rearing water until it passed through the foam separation unit.

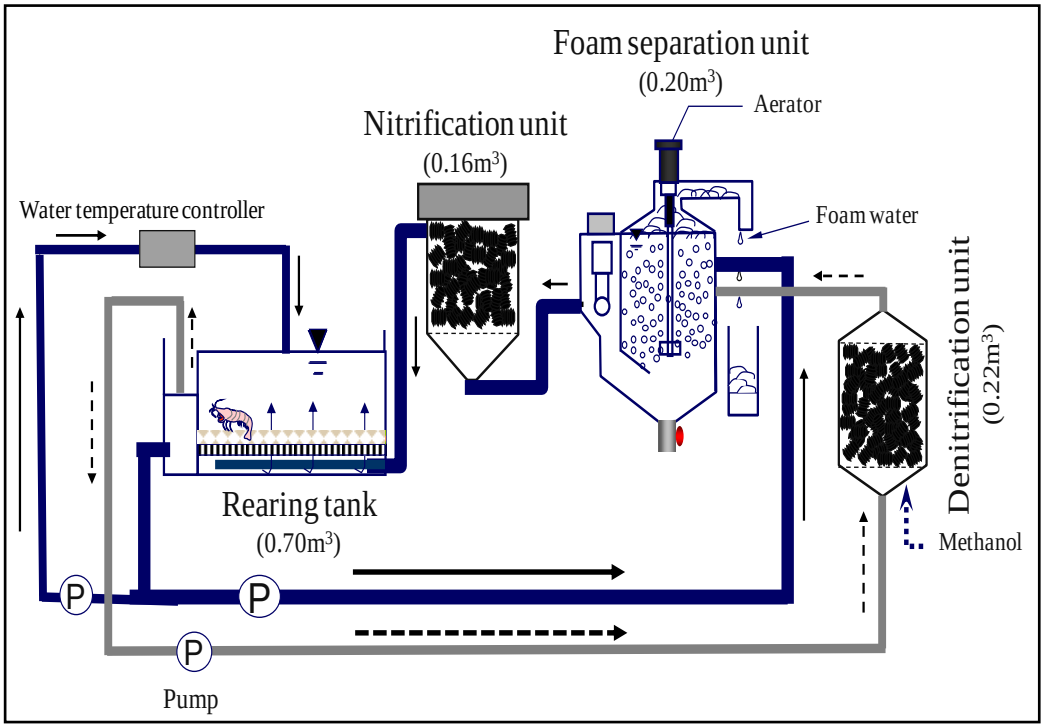


**Fig. 1**.Schematic diagram (not to scale) of the closed recirculating system with foam separation, nitrification and denitrification units.

A cylindrical medium made of polyethylene (Furukawa Electrician Industry Co., 14 mm diameter, 11 mm inside diameter, 14 mm length, 0.93 specific gravity) was used to fill the nitrification tank up to the 0.16 $m^3$ (surface area 0.93 $m^2$) mark. Nitrifying bacteria were immobilized onto the medium prior to the shrimp-rearing experiment.

In the denitrification process, a portion of the rearing water was made to flow into the denitrification tank using another line via a circulating pump. The same medium as that used in the nitrification process was used as the denitrification medium. The methanol dose tube was established at the midpoint of the inflow line to the denitrification tank and methanol was continuously injected by a metering pump (Iwaki Co., EH-B15) with an appropriate amount of methanol corresponding to five times the concentration of $NO_3$-N in the rearing water. Then, the mixture of rearing water and methanol was introduced into the denitrification tank. Methanol injection was adjusted taking into account the $NO_3$-N concentration in the rearing water every week. The

treated water that passed through the denitrification tank was returned to the foam separation tank.

The above system was considered an almost perfectly closed system because water was added only to replace that which was lost to evaporation and foam generation.

***Shrimp rearing***

Juvenile kuruma shrimp *M. japonicus* (total gross weight 100 g, 125 individuals, about 0.8 g·individual$^{-1}$) were placed in the rearing tank. The shrimp used in the test were judged in advance to be free of the white spot disease virus (WSDV) by the method of loop-mediated isothermal amplification (LAMP) (Kono et al. 2004). Throughout the rearing experiment, the shrimp were fed a commercial diet (crude protein, above 51.0%; oil, above 5.0%; fiber, less than 5.0%; ash, less than 22.0%; calcium, above 0.5%, phosphorus, above 0.080%; Kyowahakko Co., Japan) daily. In the initial stage, 5 g of the feed was given once daily, every evening. Shrimp were cultured for 135 days.

***Analytical methods***

To determine the quality of rearing water, a sample was collected every 2 or 3 days from the rearing tank before feeding. Dissolved oxygen (DO), turbidity as kaolin standard (Mitsubishi Kagaku Co., SEP-PT-706D), total organic carbon (TOC, Shimadzu Co.,TOC-5000), color as cobalt platinum standard, absorbance at 260 nm (E260, Shimadzu Co., UV-2200), TAN (HACH Co., DR-2000), $NO_3$-N (HACH Co., DR-2000), $NO_2$-N (HACH, DR-2000), total nitrogen (T-N), phosphate ($PO_4$-P) and total phosphorus (T-P) were analyzed. The standard platinum-cobalt method of measuring color was used, in which the unit of color is produced by 1 mg-Pt·L$^{-1}$ in the form of chloroplatinate ion. The collapsed-foam water samples were also obtained and TOC, color, E260, suspended solids (SS), T-N and T-P were analyzed. The analytical methods followed that of the Japanese Industrial Standard (JIS K 0102) or HACH Co. analytical manual.

Nitrogen in the solid samples, such as feed, shrimp tissue, ecdysis shell and dried sludge, were analyzed using an elemental analyzer (CHNS.O Analyzer 2400, Perkin Elmer Co.). Phosphorus in solid matter was decomposed in a mixture of perchloric acid and nitric acid and analyzed in the same way as T-P in rearing water.

## Results and Discussion

### *Growth of shrimp*

The shrimp growth during the experimental period (135 days) is shown in Fig. 2. Throughout the rearing period, the shrimp fed actively and their total weight increased over time.

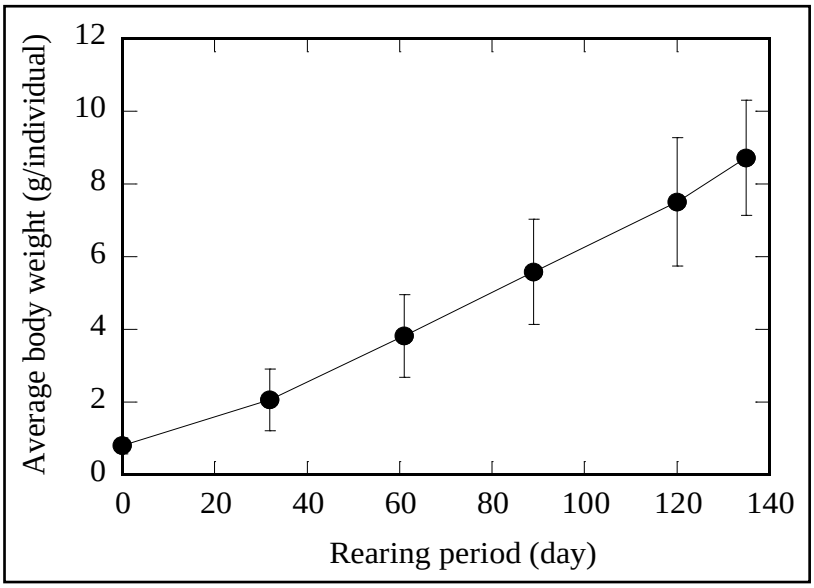


**Fig. 2.** Shrimp growth during the rearing period.

The individual weight increased from an average of 0.80 g to 8.72 g during the experimental period. The growth rate was 1.98 g·month$^{-1}$ and the rearing density was 51 individuals·m$^{-2}$ by the end of the study. Although there was no dead individual throughout the rearing period, total population decreased from 125 to 61 individuals. The survival rate through the trial was 49% because of cannibalism.

### *Quality of rearing water*

The changes in DO of the rearing water are shown in Fig. 3. The oxygen saturation percentage was kept above approximately 101% throughout the experimental period.

The maximum and minimum DO concentrations were 7.0 mg·L$^{-1}$ and 6.0 mg·L$^{-1}$, respectively, with a mean of 6.55 mg·L$^{-1}$. While this system does not provide an

extraneous source of oxygen except for the aerator in the foam separation tank, a high DO concentration was properly maintained in the rearing water.

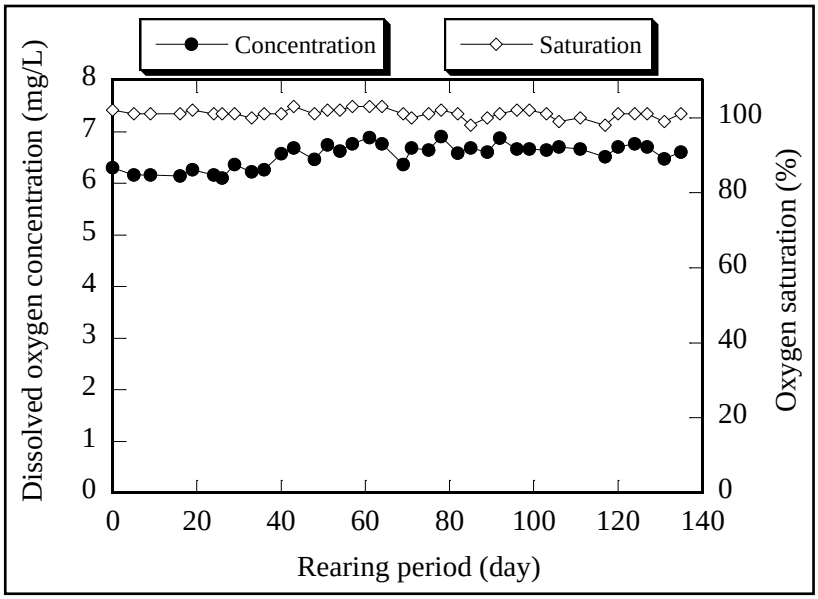


**Fig. 3**. Dissolved oxygen level in the rearing water.

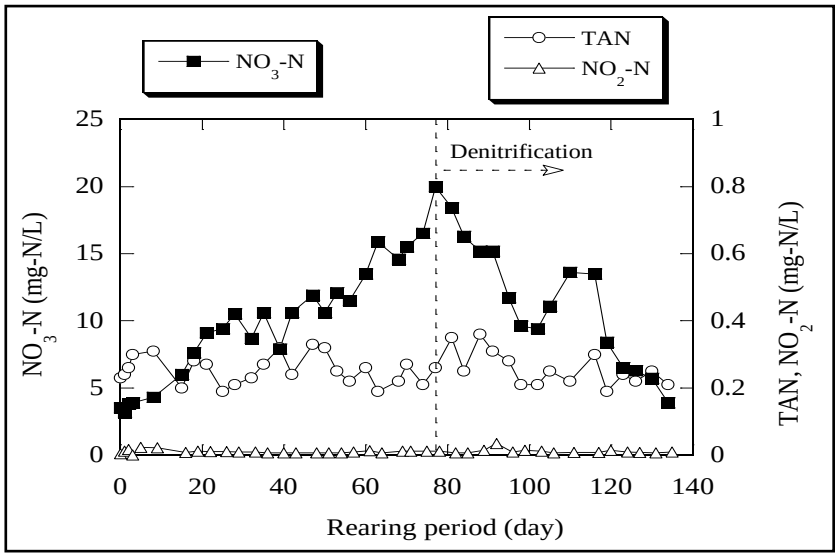


**Fig. 4.** Concentration of nitrogen compounds in the rearing water during the rearing period.

The changes in the concentrations of TAN, $NO_2$-N and $NO_3$-N in the rearing water are shown in Fig. 4. The TAN concentration was kept low throughout the study period at less than 0.4 mg-N·$L^{-1}$. The $NO_2$-N concentration was maintained at a very

low level less than 0.02 mg-N·$L^{-1}$ for 135 days. In contrast, $NO_3$-N was formed via TAN oxidation in the absence of denitrification and $NO_3$-N steadily accumulated in the rearing water. However, when the denitrification process was initiated on the 78th day, $NO_3$-N concentration began to decrease after about 1 week and was reduced to 4 mg-N·$L^{-1}$ by the end of the study. In the period without denitrification, the cumulative amount of feed intake (x) and the amount of $NO_3$-N (y) in the system showed a good correlation (y=0.031x, r=0.934). For example, when 100 g of feed were given to the shrimp, 3.1 g of $NO_3$-N accumulated continuously in the rearing water without denitrification process.

The fundamental parameters such as pH, water temperature and salinity were kept at 8.0, 28 ºC and 2.8%, respectively throughout the trial.

***Characteristics of foam separation process***

The foam which concentrated the polluted substances was generated from the duct of the foam separation unit during the rearing period. The average quantity of water discharged per day was 293 mL (n=36, 0.02% per day, 0.293 L. 1300 L=0.0002). Total volume of foam water was less than 40 L during the rearing period. It has been reported that foam generation of fish mucus is dependent on the concentrations of mucus and coexisting solvent ions (Suzuki et al. 2003). It was proven that the mucus substance acts on the foam separation process for not only fish rearing but also shrimp rearing.

The suspended solids were significantly concentrated in the separated foam water and the turbidity of the separated foam water was two orders of magnitude higher than that of the rearing water (Fig. 5). The turbidity of the rearing water was maintained in the range of 1-2 units, whereby almost no suspended substances could be observed. The turbidity in the foam water changed irregularly and varied from 50 to 600 mg·$L^{-1}$, making it necessary to remove suspended substances from the system by a foam separation process. Suspended solids was not analyzed in the rearing water since only small amounts were observed and that the turbidity of the rearing water was retained at less than 1.0 unit. Moreover, a brown material was significantly concentrated in the foam water. The color unit of the foam water (average, 369 units; n=36) was 100 to 1000 times higher than that of the rearing water (Fig. 5). The foam separation process was able to remove the color components, which are difficult to remove by biological treatment or physical filtration. Furthermore, an analysis of the effect of bacterial removal was undertaken (Fig. 6). The bacteria was concentrated markedly and suspended in the foam water.

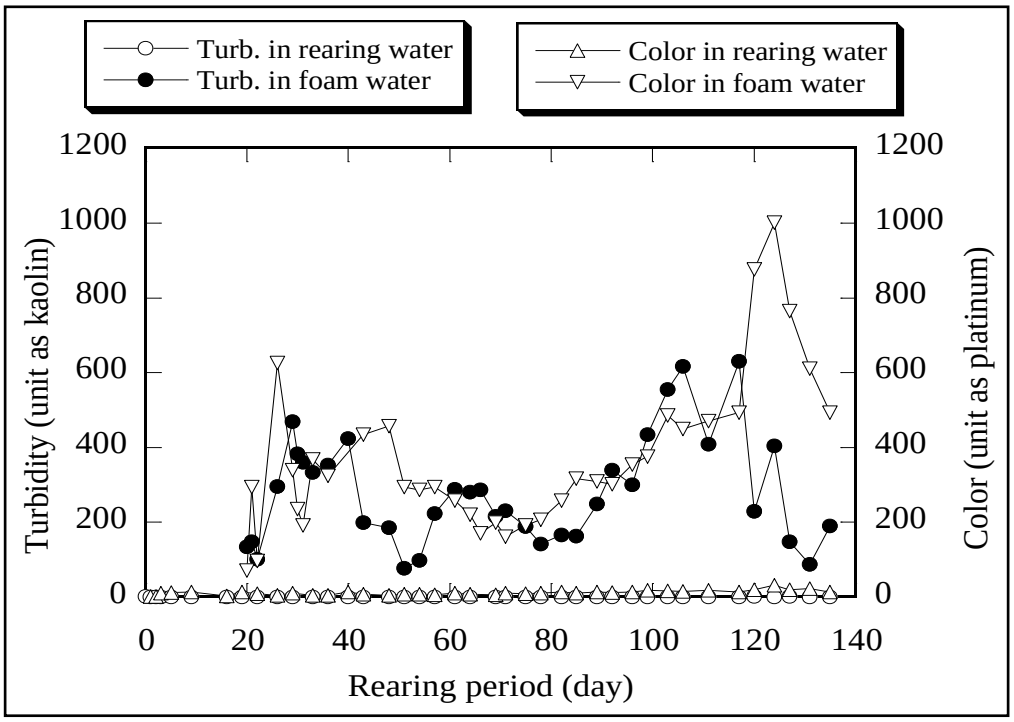


**Fig. 5.**Changes in turbidity and color in the rearing water and the foam water during the rearing period.

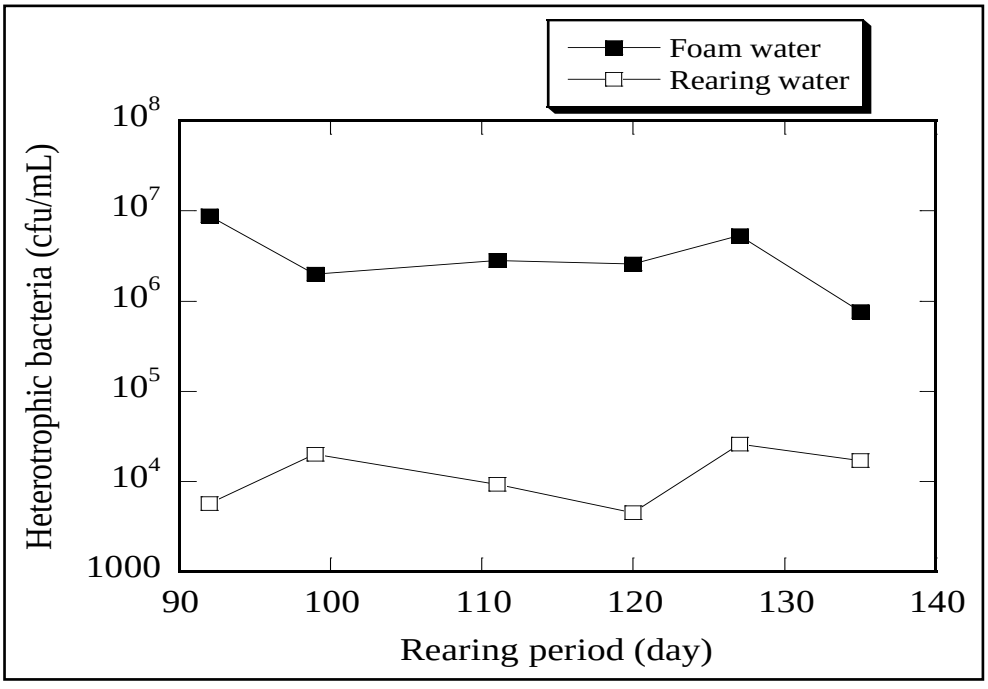


**Fig. 6.** Changes in bacteria counts in the rearing water and the foam water during the rearing period.

***Mass balances***

About 25% of the total weight of feed remained in the system as suspended solids. The total of the residual amount of SS was considered as 100%. The total N and P contents in the feed were considered as 100%. The mass balances of this system for SS, N and P are shown in Fig. 7. The N and P contents in the dried feed were 9.4% and 1.5%, respectively. The N and P contents in the shrimp body were 11.7 % and 1.0%, respectively.

In the case of the SS, about 60% was accumulated in the nitrification tank and 5.9% was removed by foam separation (Fig. 7a). The nitrification tank functioned as both nitrification and sedimentation units. The point to be noticed is that the accumulation of SS in the sand was small, less than 3%. In general, a huge amount of labor is needed for cleaning and maintenance of the sand in the shrimp culture tank. With the upflow type of rearing tank, it is possible to drastically ease the maintenance of rearing tank or pond for shrimp culture.

In the case of total nitrogen, 51% was utilized for shrimp growth, 3.1% was accumulated in the rearing water as $NO_3$-N and organic nitrogen, 2.2% was removed by foam separation and 9.5% was accumulated in the nitrification and denitrification tanks as sediment (Fig. 7b). Regarding mass balances in the culture, the assimilation of nitrogen in the fish body varied from 25 to 35% of the total nitrogen input irrespective to the difference in fish species (Folke and Kautsky 1989; Hall et al. 1992; Maruyama and Suzuki 1998; Skjølstrup et al. 1998; Suzuki et al. 1999). The nitrogen assimilation in shrimp was higher than that in fish. Almost all the nitrogen that must be treated in this system was present as a dissolved fraction. In this study, the remaining 34.2% of nitrogen in the system was removed as nitrogen gas by denitrification. Denitrification could have removed the residual $NO_3$-N in the rearing water if the operation was continued for a few days after the shrimp was harvested.

In the case of phosphorus, 27% was utilized for shrimp growth, 5% was accumulated in the rearing water, 2.1% was removed by foam separation and 60% was accumulated in the nitrification and denitrification tanks as sediment (Fig. 7c). Because of analytical error, the total percentage exceeded 100%. In case of a recirculating aquaculture system for sea fish, it has been reported that the phosphorus accumulates in high concentration in the sludge, because the phosphorus reacted with calcium and magnesium in the sea water and formed insoluble compounds (Suzuki et al. 2000).

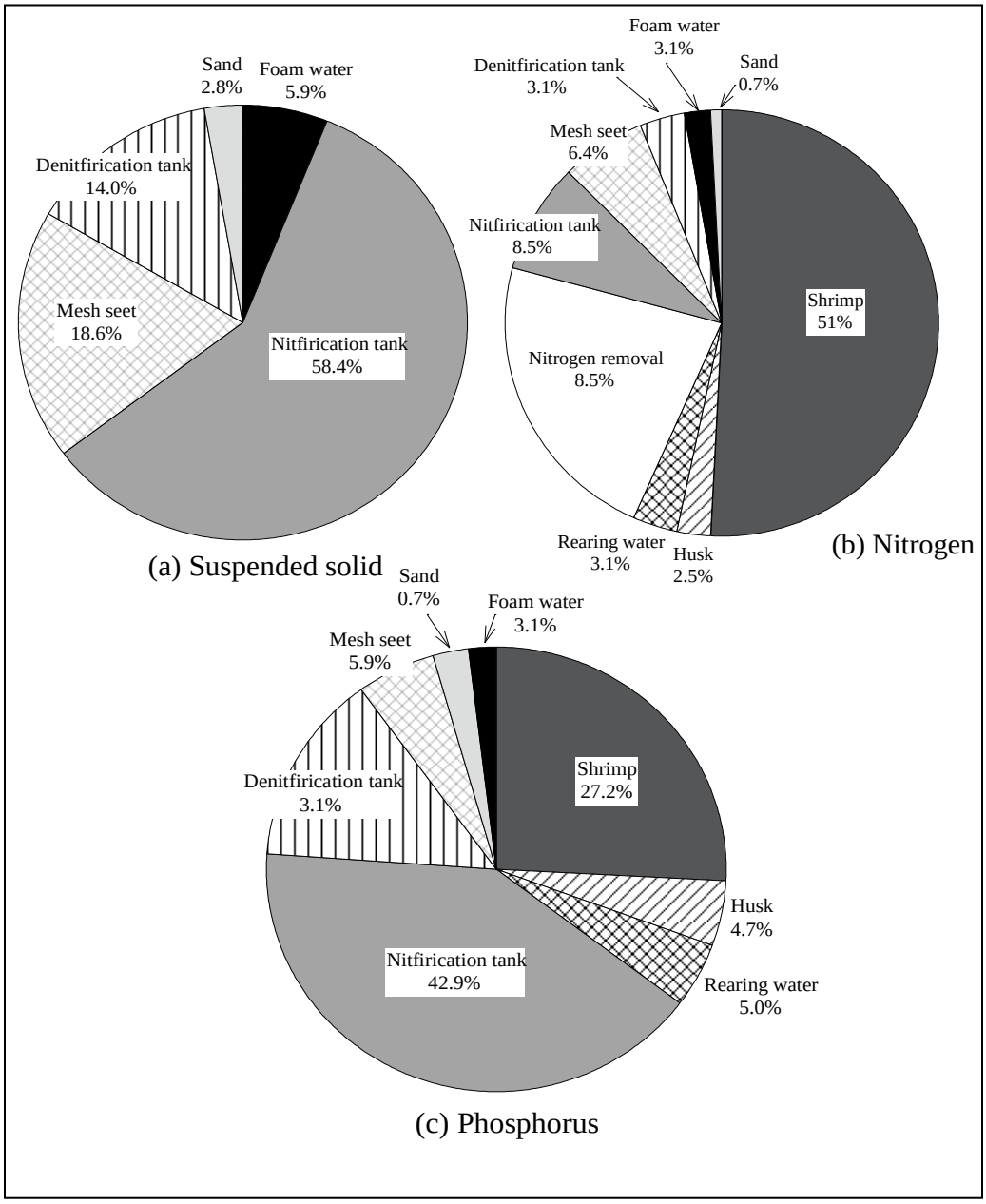


**Fig. 7.** Mass balances of suspended solid (a) nitrogen (b) and phosphorus (c) in the closed recirculating system.

## Conclusions

Our proposed system achieved kuruma shrimp culture in a perfectly closed cycle for more than 4 months. Shrimp growth was satisfactory and the average weight of individual increased more than ten-fold during the study period. Oxygen was efficiently supplied to the rearing water by a foam separation unit and oxygen saturation was maintained at 100% throughout the experiment. Simultaneously, the foam separation process removed the brown colloidal substances generated by shrimp mucus. The nitrification tank removed suspended solids and likewise rapidly nitrified TAN. While $NO_3$-N accumulated in the rearing water in the absence of denitrification, after it was initiated on the 78th day, $NO_3$-N was effectively removed and reduced to less than 4 mg-N·$L^{-1}$ at the end of the study.

In this study, the rearing trial serves as a starting point. Further development to improve the low survival, to increase stocking density and shrimp growth rate and to minimize the capital and operating costs of this system will be necessary prior to potential commercial viability. This system has an application potential for production of kuruma shrimp under a perfectly closed rearing condition free of WSDV. Furthermore, the closed recirculating system can be utilized for maintaining pathogen free broodstocks since this system makes it easy to control the condition of a specific pathogen free environment.

## Acknowledgement

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# Integration of quantitative and molecular genetics in shrimp breeding

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## Abstract

The increasing quantity of high quality DNA sequence, and proteomic, data is providing more efficient means of selecting strains and understanding physiology. Whole genome selection and the demonstration that high density single nucleotide polymorphism (SNP) analysis provides more accurate pedigree information in dairy cattle than the paper pedigree trail has revealed the prospective strengths of these approaches. In principle applicable to shrimp, fundamental differences between shrimp and vertebrate biology means the level of information available for shrimp is far less than that for cattle, pigs, chickens or finfish. Pedigreed data is far less in spatial and temporal extent and covers a relatively limited number of traits. Molecular markers allow parentage tracking and assessment of diversity levels in breeding programs. Work is in progress on the molecular and genetic mechanisms controlling key aspects of performance, including growth, reproduction and disease response. The success of the few attempts to integrate available molecular tools is limited by the lack of depth of information on shrimp and a lack of investment in the process. More effort will be required to obtain the critical research mass and quality of information needed to achieve true integration of molecular and quantitative genetics in shrimp breeding.

## Introduction

The increasing quantity of high quality DNA sequence data, and proteomic data, is leading to ever greater amounts of molecular information on a variety of organisms. Most information is available for humans, in which considerable investment has been made, but agricultural species, both plants and animals have increasing amounts of molecular information (e.g. Collins et al. 2003; Varshney et al. 2009). However, investment levels in agriculture are an order of magnitude less than for humans, and this investment has been spread over several species. Nevertheless, molecular approaches are playing a leading role in understanding physiological

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processes, and providing molecular markers to assist selective breeding (Guimarães et al. 2007). The latter includes selection on traits that could not be selected previously because the phenotypes could not be distinguished by observation, and traits for which the molecular information increases the efficiency of selection. The ability to use whole genome selection and the demonstration that high density SNP analysis provides more accurate pedigree information in cattle than the paper pedigree trail, has revealed the potential power of the new approaches to genetic improvement in agriculture.

In principle, these approaches are equally applicable to aquaculture (Davis and Hetzel 2000). However, the level of investment in molecular genetics work in aquaculture species is an order of magnitude less than that for agriculture species. These issues are particularly acute for shrimp. Molecular markers are available that allow parentage tracking and assessment of diversity levels in breeding programs. There is work in progress on the molecular and genetic mechanisms controlling key aspects of performance, such as growth, reproduction and disease response. It is clear that integrating these approaches will benefit the shrimp industry, but the extent to which developments in quantitative and molecular genetics are integrated in shrimp aquaculture is not obvious.

This paper provides a brief review of the history of shrimp farming, the available information on shrimp quantitative and molecular genetics and assesses the extent to which these approaches have been integrated in shrimp genetic improvement. The scientific names used in this paper follow the recommendations of Alderman et al. (2007), using *Penaeus* as the preferred generic name for the common farmed species, but noting their sub-generic name (elevated by some authors to generic level) at first usage. All species discussed here belong to the family Penaeidae.

## Brief History of Shrimp Farming and Shrimp Breeding Programs

The domestication and genetic improvement of penaeid shrimp has been reviewed recently (Benzie 2009). More details can be obtained from that source, but the main points are summarized here. Although some form of rearing shrimp through wild capture of larvae has been performed for many hundreds of years, the earliest development of large scale farming was in Japan in the 1950's and 1960's (Fast and Lester 1992; Liao and Chien 1994; Rosenberry 2002). This innovation took some time after the key technology development in the 1930's and 1940's, when rearing techniques for the kuruma prawn, *Penaeus (Marsupenaeus) japonicus* were developed in Japan by Hudinaga (1935, 1942). The development of large scale hatchery processes and better understanding of the rearing of shrimp in farms was required first (Liao and

Chien 1994). Since then, hatchery methods for more than 20 penaeid species have been developed and farm trials have been undertaken for many of these (Fast and Lester, 1992; Rosenberry, 2002; Briggs et al. 2004) (Table 1). Although a number of these species continue to be farmed locally, few have played a significant role in aquaculture production. Today, seven penaeid species, *Penaeus (Fenneropenaeus) chinensis*, *Penaeus (Fenneropenaeus) indicus*, *Penaeus (Fenneropenaeus) merguiensis*, *Penaeus (Litopenaeus) stylirostris*, *Penaeus (Litopenaeus) vannamei*, *Penaeus (Marsupenaeus) japonicus* and *Penaeus monodon*, provide 99% of the world's farmed marine shrimp and two of these, *P. vannamei* and *P. monodon*, contribute 87% (Table 1).

**Table 1.** Species of penaeid shrimp for which hatchery technologies were developed and/or farm trials were carried out in the last 40-50 years, and the percent of world production for 2006, the latest available statistics (FAO 2009), for the main farmed species today.

| Species for which hatchery technologies and/or farm trials were carried out | Principal species farmed today | % of market |
|---|---|---|
| *Metapenaeus affinis* | *Penaeus (Fenneropenaeus) chinensis* | 2 |
| *Metapenaeus ensis* | *Penaeus (Fenneropenaeus) indicus* | 1 |
| *Metapenaeus monoceros* | *Penaeus (Marsupenaeus) japonicus* | 2 |
| | *Penaeus (Fenneropenaeus) merguiensis* | 3 |
| *Penaeus (Farfantepenaeus) aztecus* | *Penaeus monodon* | 25 |
| *Penaeus (Farfantepenaeus) brasiliensis* | *Penaeus (Litopenaeus) stylirostris* | <1 |
| *Penaeus (Farfantepenaeus) californiensis* | *Penaeus (Litopenaeus) vannamei* | 62 |
| *Penaeus (Farfantepenaeus) duorarum* | | |
| *Penaeus esculentus* | (all other species) | <5 |
| *Penaeus (Farfantepenaeus) notialis* | | |
| *Penaeus (Litopenaeus) occidentalis* | | |
| *Penaeus (Farfantepenaeus) paulensis* | | |
| *Penaeus penicillatus* | | |
| *Penaeus plebejus* | | |
| *Penaeus (Litopenaeus) schmitti* | | |
| *Penaeus semisulcatus* | | |
| *Penaeus (Litopenaeus) setiferus* | | |
| *Penaeus (Farfantepenaeus) subtilis* | | |

The relative production levels of all of these species have fluctuated considerably through time (Fig. 1). *Penaeus japonicus*, the first species farmed, dominated for some 15-20 years, but now only supplies 2% of the world market; it required an expensive high protein diet and was displaced by shrimp that could be produced more cheaply. Other species became dominant depending on the status of disease, a major influence on shrimp farming (Walker and Mohan, 2009). A domesticated stock of *P. stylirostris*, resistant to infectious hypodermal and hematopoietic necrosis virus (IHHNV) was introduced to Central and South America

and to some Asian countries in the late 1990's under the name of "supershrimp" (SEAFDEC, 2005).

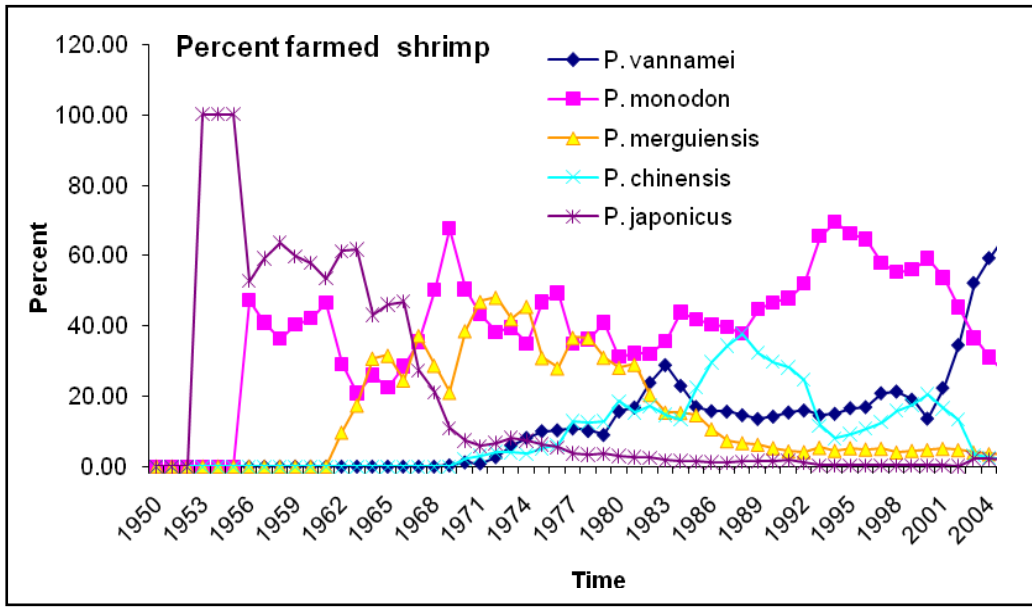


**Fig. 1**. Graph demonstrating the changing proportion of different species in the world production of shrimp, based on published FAO data (FAO 2009). A graph of total shrimp production is provided below for comparison. The category for unidentified farmed shrimp was excluded in calculating the proportion of production attributable to particular species (this effectively assumes that the proportion of particular species of shrimp in the unknown category reflects their proportions otherwise in the world market). The value of unidentified shrimp is less than 10% (usually less than 5%) for most of the time period so the figures would be affected little, if at all, should that assumption be violated. The figure for unidentified shrimp is much higher in earlier years 1950's and 1960's, but in this period *P. japonicus* and *P. monodon* were the only farmed species of note. *Penaeus japonicus* was dominant in the 1950's and 1960's, declining to less than 2% by the late 1970's; *P. merguiensis* provided around 30% of world production in the mid-1970's declining to less than 5% by the late 1980's; *P. chinensis* rose in the late 1980's to about 35% declining to less than 3% by 2004. *Penaeus monodon* first appeared in significant volume in the late 1950's, maintaining a dominant market share until the late 1990's when the introduction of domesticated SPF strains of *P. vannamei* to Asia, led to a huge increase in production of this species. Comparison with the graph for total production (Fig. 2) shows that the volumes of *P. japonicus* and *P. merguiensis* production, although dominant in the early years was never high compared with the large volumes that developed since the mid 1980's.

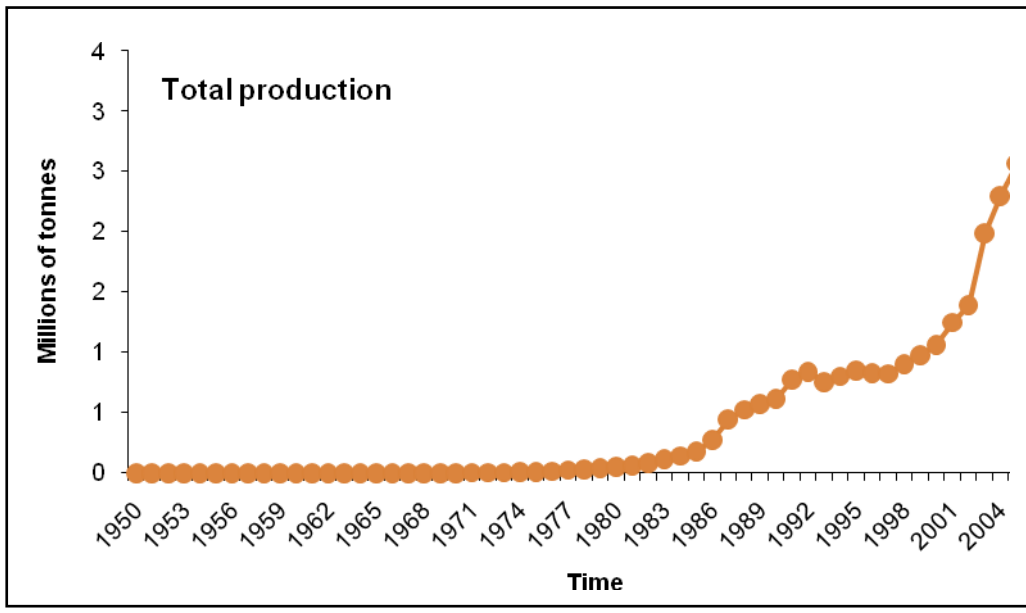


**Fig. 2.** Graph showing the total world production of shrimps from 1950-2004.

The stock proved susceptible to other diseases such as Taura syndrome virus (TSV) and white spot syndrome virus (WSSV), preventing this species from developing a dominant role in world production, despite being one of the first species for which domesticated strains were developed (Bedier et al. 1998). In contrast, a domesticated strain of *P. vannamei* resistant to TSV meant production of this species increased. The difficulty of developing domesticated stocks of *P. monodon*, a species with excellent farming traits now hampered by poor performance as a result of disease and lack of specific pathogen free (SPF) stocks, has seen this species' dominance of the market replaced by *P. vannamei*. History has therefore seen a succession of dominance in global supply by *P. japonicus*, *P. monodon* and *P. vannamei* respectively (Fig. 1). The relative production of other species through time depended on the geographic regions being brought into aquaculture production as a response to disease elsewhere and other market variable.

The history of breeding programs for shrimp is relatively short, with the first attempts to maintain domesticated populations over a number of generations being documented in the late 1980's (Benzie 1998, 2000). Given the widely known benefits of genetic improvement in agriculture, one might have thought that carefully planned approaches to breeding in aquaculture would have been used to achieve a major and rapid improvement in production. However, the actual history of development was one in which the key factors driving domestication were reliability of supply (which could not be achieved by accessing seasonal wild stocks) and the maintenance of production

in the face of disease through the development of disease tolerant or disease free stocks (Benzie, 2009). The development of domesticated strains of shrimp was therefore a response to critical issues affecting production, rather than a proactively built strategy for genetic improvement.

The animals used to make up the founding populations were usually those obtained from stocks available to the farmer/industry, including those derived from other farms and those which may have been under culture for a various number of generations (Benzie 2009). The founding populations were not established with particular forethought as to the genetic diversity present in the population, or with identified strategies for genetic improvement in mind. It is not a surprise that early genetic work highlighted concerns over the deleterious effects of unintended inbreeding through poor stock management (Sbordoni et al. 1986; Sbordoni et al. 1987). Domesticated stocks available to the industry were developed for their general production characteristics and were not designed to capture a particular subset of natural variation. As a result, a number of major domestication programs, which later morphed into genetic improvement programs, introduced new stocks to increase their genetic diversity, such as the U.S. Shrimp Consortium Program, which focused on *P. vannamei* (Alcivar-Warren et al. 2009) and the New Caledonia program for *P. stylirostris* (Goyard et al. 2003, 2008). More recent programs for *P. monodon* and *P. vannamei* have included a wide range of genetic diversity given the experience of the earlier programs (Gitterle et al. 2005a, 2005b; Argue et al. 2008) and larger programs have implemented improved stock handling techniques to prevent breeding of close relatives. The general history of the major domesticated strains has been documented in a range of published information summarized in Benzie (2009). It is clear that the genetic diversity in domesticated stocks depends on the original variation in the founder stocks and the nature of the regime used to manage the population. Most of the few existing major programs appear to have robust genetic and biosecurity management regimes (Lotz et al. 1995; Lotz 1997; Moss and Argue 2001; Le Moullac et al. 2003).

Domesticated stocks exist today for all of the main farmed species, often for private use of the company or industry owning the stocks, but not all are subject to genetic improvement programs (see Benzie 2009). Nevertheless, SPF genetically improved stocks are now available and openly traded for the two most commonly farmed species *P. vannamei* and *P. monodon* and almost all of the small *P. stylirostris* industry relies upon a domesticated stock. Domesticated stocks are playing an increasing role as a source of seed to farms with more than 99% of *P. vannamei* stocks being supplied from improved strains (Benzie 2009). Therefore, in total, about 70% of farmed shrimp are derived from domesticated stocks now, compared with the 1-2% estimated by Gjedrem (2000) a decade ago. Strains of SPF *P. vannamei*, improved for

growth and disease resistance in the U.S. Shrimp Consortium Program, have been spread worldwide and comprise all or parts of various breeding populations around the world. These populations and two strains developed separately by a U.S. Company and by an industry-wide program in Colombia have improved growth performance and comprise the main source of *P. vannamei* seed. An industry-wide program in New Caledonia has developed improved *P. stylirostris* and supplies a small, but important regional industry in the Pacific (Goyard et al. 2003, 2008). An SPF population of *P. monodon* improved for growth has recently been developed by the private sector in the U.S.A. (Argue et al. 2008), and breeding populations of *P. monodon* with improved reproductive performance, growth rates and farm yields have been established in Australia (Preston et al. 2009). Selectively improved growth of *P. chinensis* in China has been reported for a government-industry supported breeding program (Zhang et al. 2005; Huang et al. 2008), but the extent to which this strain supplies the industrial market is unknown.

The major point emerging from this brief review of shrimp farming is that the industry has few defined genetic improvement programs, although domestication is widespread. The number of species farmed has reduced greatly, with nearly all production now derived from seven species and most from only two. Production is increasingly dependent upon domesticated stocks and future production will demand more sophisticated approaches to genetic improvement of these populations.

## Quantitative Genetic Information

Data on quantitative genetic parameters has been published for only a subset of the seven species most commonly farmed today. There are estimates for heritability of a variety of characters for *P. chinenesis* (Huang et al. 2008; Zhang et al. 2008), *P. japonicus* (Hetzel et al. 2000), *P. monodon* (Benzie et al. 1997; Benzie 1998; Jarayabhand et al. 1998; Kenway et al. 2006; Macbeth et al. 2007), *P. stylirostris* (Bedier et al. 1998; Goyard et al. 2002) and *P. vannamei* (Carr et al. 1997; Fjalestad et al. 1997; Pérez-Rostro et al. 1999; Argue et al. 2002; Pérez-Rostro and Ibarra 2003a, 2003b; Arcos et al. 2004, 2005; Ibarra et al. 2005, 2007a; 2009; Gitterle et al. 2005a, 2005b, 2006a, 2006b, 2006c; Castillo-Juarez et al. 2007; Ibarra and Famula 2008). Fewer report realized heritabilities or responses to selection (Hetzel et al. 2000; Argue et al. 2002; Goyard et al. 2002; Preston et al. 2004; De Donato et al. 2005; Li et al. 2005), genetic correlations (Pérez -Rostro et al. 1999; Pérez -Rostro and Ibarra 2003a, 2003b; Arcos et al. 2004, 2005; Kenway et al. 2006; Macbeth et al. 2007; Ibarra et al. 2009) or genotype by environment interactions (Argue et al. 2002; Jerry et al. 2006b; Ibarra and Famula 2008). All these genetic parameters provide valuable information upon which to design genetic improvement programs. In a review of genetics in shrimp

breeding written more than a decade ago, Benzie (1998) reported fewer than ten papers giving quantitative genetic data for shrimp. Since then, there has been an increase in the number and accuracy of estimates of heritability and for a greater range of characters. However, progress over the decade has been less than might have been expected, and there are still fewer than forty original papers publishing detailed quantitative genetic information for penaeid shrimp (Benzie 2009). The majority of published data refers to *P. monodon* and *P. vannamei* (Table 2).

Heritability provides an assessment of the extent to which characters are under additive genetic control (i.e. due to allelic effects passed on from parents to progeny in gametes) and can be selected in the test populations, with the upper limit of 1.0 indicating that all of the variability in a population observed for the trait is due to allelic effects (i.e. complete additive genetic control) and the lower limit of 0 indicating no additive genetic control (all observed variation is then due to non-additive genetic effects e.g. dominance, epistatic or environmental effects). There would be no prospect of genetic improvement through selection for characters with heritabilities of 0. In general, characters with heritabilities less than 0.1 are unlikely candidates for economically viable selection, but those with values of 0.2 or above would be likely to show a good response to selection. Estimates for heritability have focused principally on various measures of growth (weight, length and size increments) and data exists for four species, with most estimates clustering around 0.3-0.5 (Table 2), indicating considerable scope for selection to be effective. Realized heritabilities and responses to selection confirm these levels of heritability in growth related characters with positive responses to selection for growth in *P. chinensis* (Li et al. 2005 ), *P. japonicus* (Hetzel et al. 2000; Preston et al. 2004), *P. stylirostris* (Goyard et al. 2002) and *P. vannamei* (Argue et al. 2002; De Donato et al. 2005).

Similarly, heritabilities of 0.14-0.62 have been measured for TSV resistance (Table 2) and positive responses to selection have been observed, with reductions in viral titre suggesting disease resistance rather than disease tolerance (Srisuvan et al. 2006). In contrast, heritabilities for resistance or tolerance to WSSV have been very low (<0.1 to 0), suggesting genetic selection is not possible, or will be very expensive, for that trait (Gitterle et al. 2005b, 2006a, 2006b, 2006c).

In *P. monodon* and *P. vannamei*, a number of reproductive traits such as number of spawns, days to spawn, egg number, nauplii number, hatch rate and biochemical characteristics of hemolymph (vitellogenin), of the eggs (protein, acylglyceride and vitellin levels) and stress related traits such as survival under hypoxia, have been measured and have high heritabilities (Table 2). Interestingly, egg size itself did not have a high heritability but oocyte size does (Arcos et al. 2005; Ibarra

et al. 2009). Genetic correlations between these reproduction related characters and between them, and weight or growth measurements were variable (see Ibarra et al. 2007b for a review of reproductive work), but those between oocyte diameters and hemolymph vitellogenin correlation was high (Ibarra et al. 2009). These data indicate the potential to improve a number of reproduction related characters through selection.

Genetic correlations between various weight and length measurements are generally high and positive (range -.20-+1.00, majority >0.80), indicating that selection of any one of a number of measurements of growth would be effective. Positive or non-significant correlations of growth with TSV resistance are also reported (Carr et al. 1997). In contrast, the genetic correlations measured between growth and WSSV resistance (-.94-+0.33, mean -0.24) are largely negative (Gitterle 2005b, 2006c). These results, together with data for low heritability of WSSV resistance, indicate selection for WSSV resistance would select for slow growth and vice versa. This suggests the development of WSSV tolerant strains are likely to be uneconomic and other management processes must be developed for many shrimp diseases (Cock et al. 2009).

Selected stocks have been demonstrated to perform significantly better in production than unselected populations for growth and disease resistance (e.g. Argue et al. 2002; Preston et al. 2004; Srisuvan et al. 2006). In general, genetic gains achieved through selected stocks appear to average about 5% per generation (Benzie 2009). The performance of given stocks in different environments appears to be similar where specific tests have been carried out, but these have usually involved environments that show few differences (different shapes of tanks or ponds). However, there is evidence for weak but significant genotype by environment (GxE) effects for *P. japonicus* in commercial ponds (Jerry et al. 2006b) and in tanks (Coman et al. 2004) and evidence of GxE interactions in *P. vannamei* at different stocking densities (Ibarra and Famula 2008). Strains improved in one program have performed well over wide geographical regions, but solid data on genotype by environment interactions in shrimp are still needed to determine whether regional strains will need to be developed.

These results have shown the power of genetic improvement in shrimp and provided information to determine the most efficient approach to future gains. Given the geographical and commercial scale of shrimp farming, and the fact that genetic work has been undertaken now for more than two decades in some of these species, there is still remarkably limited information published in this field.

**Table 2.** Heritabilities mean, (range) for selected characters in farmed penaeid shrimp. The data are means from the published literature. Sources additional to those listed in 1. Benzie (1998) are: 2. Kenway et al. (2006), 3. Jarayabhand et al. (1998), 4. Fjalestad et al. (1997), 5. Carr et al. (1997), 6. Pérez-Rostro and Ibarra (2003a), 7. Argue et al. (2002), 8. Gitterle et al. (2005a), 9. Gitterle et al. (2005b), 10. Gitterle et al. (2006a), 11. Gitterle et al. (2006b), 12. Hetzel et al. (2000), 13. Goyard et al. (2002), 14. Pérez-Rostro and Ibarra (2003b), 15. Ibarra et al. (2005), 16. Pérez-Rostro et al. (1999), 17. Castillo-Juarez et al. (2007), 18. Gitterle et al. (2006c), 19. Arcos et al. (2005), 20. Arcos et al. (2004), 21. Macbeth et al. (2007), 22. Ibarra et al. (2007a), 23. Huang et al. (2008), 24. Zhang et al. (2008), 25. Ibarra et al. (2009).

| Character | Species | | | | |
|---|---|---|---|---|---|
| | *chinensis* | *monodon* | *japonicus* | *stylirostris* | *vannamei* |
| Growth | | | | | |
| Weight measures | 0.14 | 0.37 (0.05-0.56) | 0.28 | 0.11 | 0.51 (0.00-1.42) |
| Length measures | (0.44-0.53) | 0.29 (0.07-0.59) | - | 1.03 (0.64-1.31) | 0.71 (0.00-1.42) |
| Growth rate | | 0.51 (0.48-0.55) | - | - | 0.69 (0.00-1.27) |
| Survival | | 0.53 (0.36-0.72) | - | - | 0.05 (0.00-0.21) |
| Diseases | | | | | |
| TSV tolerance | - | - | - | - | 0.31 (0.14-0.62) |
| WSSV tolerance | - | - | - | - | 0.03 (0.00-0.21) |
| Reproduction | | | | | |
| Days to spawn | - | 0.47 | - | - | 0.48 (0.41-0.54) |
| Egg number | - | 0.41 | - | - | 0.13 (0.09-0.17) |
| Nauplii number | - | 0.27 | - | - | - |
| % Hatch | - | 0.18 | - | - | - |
| Number of spawns | - | - | - | - | 0.20 (0.06-0.43) |
| Vitellogenin hemolymph (adult) | - | - | - | - | 0.29 |
| Egg diameter 1st spawn | - | - | - | - | 0.04 (0.00-0.07) |
| Egg vitellin 1st spawn | - | - | - | - | 0.38 (0.28-0.47) |
| Egg protein 1st spawn | - | - | - | - | 0.16 (0.13-0.18) |
| Egg acylglycerides 1st spawn | - | - | - | - | 0.18 (0.20-0.35) |
| Oocyte diameter (juvenile) | - | - | - | - | 0.57 |
| Oocyte diameter (adult) | - | - | - | - | 0.23 |
| Number of oocytes (juvenile) | - | - | - | - | 0.00 |
| Number of oocytes (adult) | - | - | - | - | 0.11 |
| Ovary maturity (juvenile) | - | - | - | - | 0.71 |
| Stress | | | | | |
| Resistance to hypoxia | - | - | - | - | 1.44 (1.08-1.73) |
| Sources | 23, 24. | 1, 2, 3, 21. | 12. | 1, 13. | 1, 4-11, 14-20, 22, 25. |

# Molecular and Genomic Resources for Shrimp

### *Molecular markers*

Molecular variants have been used to assess the diversity and structure of wild stocks and domesticated populations for some time, and more recently to determine parentage in domesticated stocks and to develop genetic maps. Molecular genetics work on shrimp first focused on using molecular markers to assess levels of diversity in wild and cultured stocks and to assess population structure of wild stocks (Benzie 2000). The emphasis on wild stock structure reflected the strong fisheries influence on shrimp research at that time and the concern over levels of genetic variety in cultured stocks following production failures in early domesticated populations (Sbordoni et al. 1987; Sunden and Davis, 1991). The early studies used allozyme electrophoresis to assess variation at the protein level, but subsequent studies assessed variation in mitochondrial DNA and nuclear DNA using the latest tools as they became available, from random amplified polymorphic DNA (RAPDs), restriction fragment length polymorphisms (RFLPs) and microsatellites, to full sequence of DNA fragments and single nucleotide polymorphisms (SNPs). Today, a suite of nuclear or mitochondrial sequences from particular genes, intergenic sequences such as mtDNA control region are available for analysis depending on the specific question being addressed by the study.

The later DNA tools have generally proved more sensitive than allozymes and have shown evidence of a variety of genetic structures in wild shrimp populations. Some species show large geographical regions with high levels of gene flow, separated by sharp genetic disjunctions from other such areas. Other species show marked shifts in gene frequency over smaller geographical scales. The genetic disjunctions can either be related to large-scale biogeographical boundaries of considerable age, or to reflect present-day barriers to gene flow related to circulation patterns or differences in the temperature or salinity of water masses. More details on the structure of wild populations can be found in the reviews by Benzie (2000, 2009). However, key points with respect to discussion of domesticated shrimp are that molecular markers have demonstrated 1) the existence of species not distinguishable (or not easily distinguishable) using morphological characters (cryptic species) in *P. japonicus*, the earliest species farmed in the centre of its north-west Pacific range (Tsoi et al. 2005; 2007) and in *P. merguiensis* in the centre of its range (Hualkasin et al. 2003); 2) major genetic differentiation between *P. monodon* populations from the south-west Indian and Pacific Oceans (Benzie et al. 2002; You et al. 2008); and 3) more subtle but significant genetic differences between *P. vannamei* populations from different parts of its natural range in the east Pacific (Valles-Jimenez et al. 2005, 2006). These results suggest the possibility of local adaptation in shrimp populations, the possibility of genotype by

environment interactions, the need for care in choosing the source(s) for establishing domesticated populations and possible issues for interactions between domesticated and local stocks.

An immediate application for molecular markers was their use to assess levels of genetic diversity in domesticated stocks. This showed that marked declines in the reproductive performance of *P. japonicus* were associated with reductions of molecular genetic diversity indicative of considerable inbreeding (Sbordoni et al. 1986, 1987). Since then, a number of studies have shown marked reductions in genetic diversity in cultured populations (reviewed in Benzie 2000 and Benzie 2009). However, no decline in genetic diversity was reported for stocks of *P. chinensis* (Zhang et al. 2004) and *P. vannamei* (Cruz et al. 2004; Soto-Hernandez and Grijalva-Chon 2004; Luvesto et al. 2007; Lima et al. 2008; Perez-Enriquez et al. 2009). Therefore, not all reductions in levels of molecular variation are associated with declines in production performance, and there is only one report of a positive association of DNA heterozygosity with individual performance in inbred *P. stylirostris* populations (Bierne et al. 2000). The genetic diversity in domesticated stocks obviously depends on the original variation in the founder stocks and the nature of the regime used to manage the population. It is also clear that the overall level of molecular diversity, measured using a relatively small number of loci (as is usual) provides only a general guide as to the diversity in the cultured population and is not necessarily directly linked to variation at the often polygenic morphological characters of importance in genetic improvement.

## Parent Assignment

The avoidance of the deleterious consequences of unintended inbreeding demanded the ability to avoid inbreeding of close relatives. This ability is not provided by population level assessments of molecular variation, but by managing mating between parents. It was then a short step to using molecular markers to assist parent assignment in cultured populations, particularly once highly variable markers such as microsatellites, became available. Panels of microsatellite markers have been developed for *P. chinensis* (Dong et al. 2006), *P. japonicus* (Sugaya et al. 2002; Jerry et al. 2004, 2006b), *P. monodon* (Jerry et al. 2006a; Li et al. 2007) and *P. vannamei* (Alcivar-Warren et al. 2003). In general, these have shown that sufficient variation is provided by approximately 10 loci to allow 99% accuracy of assigning parentage to shrimp sampled from a mixed population. The levels of relatedness of the sampled shrimp can also be assessed and allow breeders to avoid mating individuals that are too closely related. The markers also allow for traceability of stocks through production chains, thereby increasing confidence in food security (Maldini et al. 2006). These methods have been used as the primary assessment of consanguinity in populations of *P.*

*japonicus* and *P. monodon* in Australia subject to mass selection in order to avoid inbreeding (Preston et al. 2009). More commonly, these methods appear to be used as backups where physical tagging is used preferentially to track pedigrees.

## Functional genomics and candidate genes

Relatively few shrimp genes had been characterized more than ten years ago and DNA sequences were limited (Benzie 1998), but increasing numbers of sequences and gene characterizations have been published in the last decade (Benzie 2009). The hundreds to thousands of sequences available at the end of the 20th century can be compared with the hundreds of thousands available today. There is also an increasing number of genomic tools including BAC libraries for *P. vannamei* (Zhang et al. 2010), *P. monodon* (Wuthisuthimethavee et al. 2009) and *P. japonicus* (Koyama et al. 2010), and FOSMID libraries for *P. monodon* and *P. vannamei* (Saski et al. 2009). Development of markers a few years ago was characterized by processes resulting in less than ten to hundreds of markers (e.g. microsatellites: Vonau et al. 1999; Moore et al. 1999; Cruz et al. 2002; Meehan et al. 2003; Glenn et al. 2005) but now, use of advanced techniques of DNA marker development and the plethora of sequence data coming from next-generation DNA sequencing platforms (i.e. Roche 454 FLX, Illumina GIIx) can result in hundreds to thousands of potential DNA markers being rapidly isolated (e.g. O'Leary et al. 2006; Gorbach et al. 2008; Ciobanu et al. 2009).

A detailed review of the genes being characterized for shrimp is beyond the scope of this paper, but studies have tended to focus on genes that might be related to response to bacterial (e.g. Destoumieux et al. 2000; De Lorgeril et al. 2005; Amparyup et al. 2010) and viral (e.g. He et al. 2005; Pan et al. 2005; Wang et al. 2006, 2008; Prapavorarat et al. 2010) diseases or both (Gross et al. 2001; Robalino et al. 2007; Wang et al. 2008; Ma et al. 2008), reproduction (see reviews by Lo et al. 2007; Ibarra et al. 2007b), growth (e.g. Lyons et al. 2007) and a number of other genes that have attracted the interest of particular researchers (e.g. Zhang et al. 2007b). Around the year 2000, conference presentations were focused on providing a list of possible gene identities from searches of DNA data bases using shrimp sequences (e.g. BLAST searches) (Benzie 2005). Since then, more than a hundred papers have been published where genes have been sequenced, their amino-acid sequence inferred and corroborated and their expression in one or more tissues and/or in different environments assayed. In the case of antimicrobial activity, a substantial database has been set up (Gueguen et al. 2006). However, there are no publications yet, where any of these possible candidate genes are finely mapped or where they are used in selection programs. Targeted approaches to understanding biochemical or metabolic pathways have not yet emerged, although work in disease response and reproduction is moving in that direction.

Functional genomics is being undertaken using species of shrimp that are commonly farmed, but results are still fragmented.

## Genetic Maps

First order genetic maps exist for only four of the main domesticated species: *P. chinensis*, *P. japonicus*, *P. monodon* and *P. vannamei* (Table 3). These maps are largely based on AFLP markers and the spacing of markers simply reflects the number of markers mapped, with more recent studies generally mapping more markers than earlier studies for a given species.

The number of linkage groups observed is now approaching that expected from the chromosome counts (Chow et al. 1990) for most species. The advantage of AFLP markers is that they can provide a large number of markers quickly and cheaply, but the markers vary from species to species, even family to family and cannot be used for comparative genomics. More recently, maps using microsatellites (You et al. 2010) or SNPs (Du et al. 2009) as the main markers have been published for *P. monodon* and *P. vannamei* respectively. These provide a crude basis for comparative mapping but to date few markers have been used in common. Marker density is still sparse in even the densest of shrimp maps (around 500 markers relative to thousands or millions in vertebrate maps). This lack of depth means there is no knowledge currently on genome structure such as rates of recombination across the genome, haplotype blocks and so on, that are needed for the current maps to be practically useful in breeding programs. There is a need to increase the density and coverage of the shrimp genome using type 1 genetic markers (microsats, SNP) to allow detailed comparative mapping and provide the capability to undertake effective genome-wide selection.

The maps developed to date have largely mapped molecular markers alone and few have included phenotypic characters, although sex has been scored in three species with associations to single linkage groups in each case: *P. vannamei*, (Zhang et al. 2007a; Alcivar-Warren et al. 2007; Du et al. 2009), *P. japonicus* (Li et al. 2006a) and *P. monodon* (Staelens et al. 2008). Putative QTLs have been identified for growth in *P. chinensis*, *P. japonicus*, *P. monodon* and *P. vannamei*, but the lack of marker density means these are not well defined. The level of linkage indicates the distance between the marker(s) and the locus of interest spans several million base pairs and that much more work needs to be done to identify the candidate genes. A quantitative trait locus associated with growth has been reported for *P. japonicus* and a possible candidate gene with a role in fatty acid metabolism, *ELOVL-MJ* identified (Lyons et al. 2007). However, much more work needs to be done to establish whether *ELOVL-MJ* is responsible for the observed QTL effects. Only one effective marker for a phenotypic

trait has been developed for any shrimp species as yet and that is for sex in *P. monodon* (Staelens et al. 2008). This marker was used by You et al. (2010) to identify their linkage group 26 as the W chromosome in their *P. monodon* study, but the site was not variable and could not be used to distinguish sex in a *P. vannamei* mapping study (Du et al. 2009).

**Table 3**. Summary data on the genetic maps for penaeid shrimp. Number of maps refers to the total number of combined sex (c), female (f) or male (m) maps reported. More detailed information is given for the most detailed map available for the species with the data given for combined sex, female and male maps in that order. The source for the more detailed information is given in italics in the last column of the Table, with other sources given in regular font. The markers used for the map for which details are listed are given in italics in column three. Where there is more than one map that has advanced features not shared by the others, separate data is given in a row below. Sources are 1. Li et al. (2006b), 2. Moore et al. (1999), 3. Li et al. (2003), 4. Wilson et al. (2002), 5. Maneeruttanarungroj et al. (2006), 6. Staelens et al. (2008), 7. Pérez et al. (2004), 8. Li et al. (2006a), 9. Wuthisuthimethavee et al. (2005), 10. You et al. (2010), 11. Yue et al. (2004), 12. Sun et al. (2008), 13. Tian et al. (2008), 14. Zhang et al. (2007a), 15. Alcivar-Warren et al. (2007), 16. Du et al. (2009).

| Species (Haploid chromosome number) | No of maps | Type of markers (and total number mapped by all studies) | No. of markers mapped c, f, m | Average space between markers (cM) c, f, m | % of genome mapped c, f, m | No of linkage groups c, f, m | Sources (time span of publication) |
|---|---|---|---|---|---|---|---|
| *P. chinensis* (44) | 11 c,f,m | AFLPs (638+)<br>Msats (36)<br>RAPDs (237) | -, 197, 194 | -, 13.5, 11.0 | -, 74, 73 | -, 35, 36 | *1*, 11, 12, 13 (2006-2009) |
| *P. japonicus* (43) | 5 c,f,m | AFLPs (865) | -, 139, 245 | -, 7.8, 8.3 | -, 44, 88 | -, 33, 43 | 2, 3, *8* (1999-2006) |
| *P. monodon* (44) | 17 c,f,m | AFLPs (1817)<br>Msats (12)<br>EST-SSRs (36)<br>EPICs (1)<br>SSCPs (6)<br>SNPs (1)<br>SCAR (1) | -, 405, 547 | -, 4.6, 4.1 | -, 92, 113 | -, 45, 42 | 4, 5, *6*, 9, 10 (2002-2010) |
| | | AFLP (85)<br>Msat (347) | -/-, 36/171, 49/176 | -, 13.8, 11.2 | -, 63, 68 | -, 36, 37 | *10* |
| *P. vannamei* (43) | 6 c,f,m | AFLPs ( )<br>Msats (43)<br>EST-SSRs (5)<br>SNPs (418) | -/-, 319/18, 252/14 | -, 15.1, 14.5 | -, 88, 90 | -, 45, 45 | 7, *14*, 15, 16 (2004-2009) |
| | | SNPs (418) | 418, -, - | 5.4. -, - | 43, -, - | 45, -, - | *16* |

The principal finding of this review of recent progress is that an increasing amount of molecular genetics work is being undertaken in shrimp, including marker development, gene characterization and functional genomics. These tools are being used to increase understanding of physiology, growth and response to disease in shrimp. However, as far as utilizing marker assisted selection, whole genome selection or identification and use of candidate genes for marker assisted selection to meet the potential of molecular tools to provide more efficient means of selecting improved strains, work has just begun.

## Discussion

This paper has provided brief reviews of key components of genetic improvement in shrimp aquaculture as they relate to assessing the extent to which quantitative and molecular genetics are integrated in shrimp aquaculture production. The results have shown that some quantitative genetic information is available for several shrimp species, but that these data are still limited in extent and sophistication. Despite the examples of the value and practice of genetic improvement programs available in agriculture, shrimp production has been driven by short-term economic factors and domesticated stocks have been developed from necessity, rather than through strategic planning. The application of quantitative genetic methods has been primarily through public sector research in the first instance, although work was conducted often with individual industry members. These have led to more structured approaches to genetic improvement through the use of pedigreed populations and the use of quantitative genetic methods by the major genetic improvement companies now operating in the shrimp industry.

Similarly, the use of molecular tools has been pioneered by the research community and public sector; first, to assess the genetic structure of wild stocks and second, to assess the genetic diversity in cultured stocks. Investigations of wild stocks demonstrated considerable structure including the existence of cryptic species. They have not resulted in any particular change in approach by the commercial industry, although domesticated stocks that have been established more recently have deliberately accessed more diverse wild stocks. However, this decision was more likely to have been affected by the finding of reduced molecular variation in stocks established from small founding populations, and the concerns for deleterious levels of inbreeding. Although molecular markers have proved to be effective in tracking parentage in cultured populations, there are still questions concerning their cost and their application in the industry is limited to programs supplying less than 1% of improved stock worldwide. Pedigreed populations are usually tracked by rearing families in separate containers and by tagging them at later stages of development.

The isolation and characterisation of an increasing number of individual shrimp genes is being reported, but more concentrated research on biochemical or physiological pathways, despite the consolidation of work in reproduction and disease response has not yet been achieved. Work to better understand the molecular basis for shrimp cellular and physiological function is in progress but there are still few candidate genes identified. Anonymous molecular markers have played a great role in developing genetic maps for shrimp, but almost all of the characters mapped are molecular markers. Only sex determining regions have been mapped in a couple of shrimp species and a few regions indicating relatively large chromosomal regions as ill-defined QTLs for growth identified. In that sense, integration between quantitative and molecular genetics is being undertaken, but is still limited. The majority of this work is also undertaken largely by public sector research, although private sector involvment is growing. The best shrimp maps developed have relatively few molecular markers mapped (<500) when compared with terrestrial livestock and plants (with several thousand to millions of markers mapped), and few associations with morphological, physiological or metabolic traits have been established.

The relatively short time over which industrial shrimp aquaculture has developed means that other information which is available for agricultural organisms is lacking for shrimp. First, the extent and depth of information on the biology, physiology, metabolism and biochemistry of shrimp is far less than that for cattle, pigs, chickens or finfish, for example. Second, the amount of information from pedigreed data sets is far less in spatial and temporal extent and, because of the lack of primary biological information, covers a relatively limited number of traits. However, domestication programs have existed for some 20 years and the relative paucity of quantitative and molecular data also reflect a lack of strategic approach by the industry and the limited investment in genetics in the sector.

The advantages of integrating molecular and quantitative genetics are clear, and the directions of the research community reflect these. The ability to translate these technological achievements into competitive commercial outcomes is harder, made more so by the limited investment and limited information available in the sector. Moves to integrate quantitative and molecular genetics in shrimp genetic improvement are being undertaken, but the limited scale of activity slows this process and their integration into industrial activity.

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# Identification and expression analysis of nitric oxide synthase gene, Mj NOS, in kuruma shrimp *Marsupenaeus japonicus*

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## Abstract

Nitric oxide (NO) signaling is involved in many physiological processes in vertebrates and invertebrates. In mammals, NO plays as an endothelium-derived relaxing factor in the form of a simple unstable gas, as a neurotransmitter in the central and peripheral nervous systems and as an immune effector mediated by macrophages. In crustaceans, nitric oxide synthase (NOS) plays a significant role in innate immunity and in the regulation of the nervous system. We describe the full-length cDNA sequence (4,616 bp) of the kuruma shrimp *M. japonicus* NOS (Mj NOS). The open reading frame of Mj NOS encoded a protein of 1,187 amino acids with an estimated mass of 134 kDa, and had an 82.3% sequence homology with the NOS gene of the land crab *Gecarcinus lateralis*. In the brain, gill, intestine, thoracic ganglion and testis of the kuruma shrimp Mj NOS, mRNA was constitutively expressed. When *Vibrio penaeicida* was injected into the kuruma shrimp, Mj NOS was expressed in the brain, gill, heart, lymphoid organ, intestine and thoracic ganglion. In the gill, Mj NOS expression reached its peak at 12 h and decreased to its normal level 24 h after *V. penaeicida* injection.

## Introduction

In vertebrates, nitric oxide (NO) serves as a primary immune activator molecule and a signal messenger with multifaceted roles (Aktan 2004; Bogdan et al. 2000). In invertebrates, NO is predominantly associated with antibacterial, antiviral and antiparasitic interactions as a cytotoxic molecule functioning directly or after

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interaction with other free-radical intermediates (Torreilles and Guérin 1999; Nappi 2000 et al.; Beck et al. 2001; Jiang et al. 2006). NO works as an endothelium-derived relaxing factor in the form of a simple unstable gas (Palmer et al. 1987; Furchgott 1990) as a neurotransmitter in the central and peripheral nervous systems (O'Dell et al. 1991) and as an immune effector mediated by macrophages in mammals (Nathan and Hibbs 1991; Fang 1997).

NOS has a C-terminal reductase domain and an N- terminal oxygenase domain. The C-terminal reductase domain has a binding motif for flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH). The N-terminal oxygenase domain has a binding motif for P450-like cysteine thiolate-ligate heme and tetrahydrobiopterin ($BH_4$). Both the oxygenase and reductase domains are linked by a calmodulin (CaM) binding motif (Nathan 1992; Schmidt et al. 1992; Andrew and Mayer 1999; Nishida et al.1992).

NOS has three isoforms in mammals. They are neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). By the concentration of intracellular $Ca^{2+}$ and binding with CaM, nNOS and eNOS are constitutively expressed and regulated. In contrast, iNOS lacks an autoinhibitory loop and binds with CaM at a high affinity and low $Ca^{2+}$ levels; this action is predominantly regulated at the transcriptional level (Andrew and Mayer 1999). Once binded with the NOS enzyme, CaM stimulates the rate of electron transfer from the reductase domain to the oxygenase domain (Abu-Soud and Stuehr 1993; Abu-Soud et al. 1994).

Insect and vertebrate NOS genes have a high sequence homology with the invertebrate NOS gene and have proved to be involved in many physiological reactions (Luckhart et al. 1998). In the fruit fly *Drosophila melanogaster* and the tobacco hornworm *Manduca sexta*, NO regulates the morphogenesis of the nervous system (Gibbs and Truman 1998; Truman et al. 1996). As a neurotransmitter, NO also functions in the central nervous system and in the antennal lobe of the honeybee *Apis mellifera* and fruit fly (Müller 1996; Nighorn et al. 1998).

In crustaceans, signal transduction using NO and cyclic guanosine monophosphate (cGMP) plays a role in neuronal development and in neuron and cardiac muscle regulation. In crabs, the NO/cGMP signaling pathway is essential for the systematic assembly of the neuronal circuit that drives rhythmic movements (Scholz et al. 2001; Scholz et al. 2002). NOS is found in the land crab *Gecarcinus lateralis*, a crustacean species with a high NOS gene sequence homology with the insects. In addition, *Gecarcinus lateralis* NOS shows significantly high NOS mRNA expression levels in the ovary, testis and eyestalk ganglia (Kim et al. 2004).

In *Fenneropenaeus chinensis* and the kuruma shrimp *M. japonicus*, NOS activity during viral infection has been studied (Jiang et al. 2006). However, the full-length cDNA of NOS from shrimp has yet to be reported. Additionally, only the *Litopenaeus vannamei* EST database encoding the partial NOS gene had been registered at the time of this study. Consequentially, the cloning of the full-length cDNA of Mj NOS comprises the first cloning report on the shrimp NOS gene. In this report, we describe the full-length cDNA encoding of the NOS gene from the kuruma shrimp denoted as Mj NOS. Under the auspices of our research, we investigated the gene expression after *V. penaeicida* injection.

The shrimp culture industry has experienced worldwide growth since the 1970's (Momoyama and Muroga 2005). With its expansion, various viral and bacterial diseases that have caused serious damage to the shrimp culture industry have been reported (Momoyama and Muroga 2005). *V. penaeicida* infection was spread from the late 1980's to the early 1990s' and caused an average 20-30% loss of shrimp production annually (Momoyama and Muroga 2005). This indicates that *V. penaeicida* infection is a serious problem in the shrimp culture industry.

## Materials and Methods

### *Animals*

Adult kuruma shrimp, *M. japonicus* (average weight: 15 g) were obtained from a shrimp farm in Miyazaki, Japan. They were acclimatized in aerated seawater at 22 °C and fed a commercial diet equaling 1% of their body weight once daily.

### *Designing of degenerate primers*

A partial gene of NOS cDNA was initially obtained by RT-PCR with degenerate primers. It was designed from the conserved regions of the land crab (*G. lateralis*) NOS gene and *L. vannamei* in the EST database (GenBank accession numbers: AY552549 and FE061797) using ClustalW alignment with the ClustalW program (Hall 1999). The degenerate primers NOS-F1 and NOS-R1 (Table 1) were designed to anneal DNA sequences encoding the LALSREP and QMRDEN region, respectively. Additionally, another set of degenerate primers, NOS-F2 and NOS-F3 (Table 1), was designed on the basis of the conserved regions. It aligned with the lobster, parasitic bee and honeybee NOS genes in the EST databases (GenBank accession numbers: EW703101, GE409919, and NM_00101296, respectively) to anneal the DNA sequences encoding TELVYGAK and WSKLQVFD, respectively.

***RNA extraction and cDNA preparation***

Total RNA was extracted from a pool of gill tissues of three individual kuruma shrimp using Trizol Reagent (Invitrogen, USA) in accordance with the manufacturer's instructions. It was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Purity was determined by measuring $OD_{260\ nm}/OD_{280\ nm}$. The RNA samples were treated with RNase free DNase I (Invitrogen, USA) and cDNA was synthesized using a ReverTra Ace qPCR RT Kit (Toyobo, Japan) in accordance with the manufacturer's instructions. It was used as the template for PCR analysis.

***Cloning and sequencing***

Initially, on the conserved regions of the crustacean NOS domain, one set of degenerate primers, NOS-F1 and NOS-R1 (Table 1) was designed. PCR was performed with cDNA prepared using these primers to amplify the initial predicted sequence. Two forward degenerate primers, NOS-F2 and NOS-F3 (Table 1) were designed from three invertebrate NOS genes to perform RACE-PCR using a SMART RACE cDNA Amplification Kit (Takara Bio, Inc., Japan) in accordance with the manufacturer's instructions (Takara Bio, Inc., Japan). This was used to further identify the gene sequences. Following the determination of a partial sequence of the NOS gene, the entire length was obtained using 5′- and 3′-RACE-PCRs with the gene-specific primers (Table 1). The PCR products were cloned into the pGEM-T Easy vector (Promega, USA). They were then transformed into DH5α (Promega, USA). Recombinants were identified using the red-white color of colonies on MacConkey agar (Sigma-Aldrich, USA). Plasmid DNA from at least three independent clones was recovered using the QIAprep Spin Miniprep Kit (QIAGEN, Japan). Sequencing was performed using a CEQ 8000 Automated Sequencer (Beckman Coulter, Inc., USA).

The structural domains and signal peptide of Mj NOS amino acid sequences were predicted using a simple modular architecture research tool (SMART; Version 6.0) (http://smart.embl-heidelberg.de/help/smart_about.shtml). The sequence generated was analyzed for similarity with other known sequences. FASTA and the basic alignment search tool suite of the MatGat 2.02 program were used. Direct comparisons between cDNA sequences were performed using the gap program of BioEdit where multiple sequence alignments were generated using Clustal W (http://www.ebi.ac.uk/clustalw/). A phylogenetic chart was constructed. Full-length amino acid sequences of previously published NOS molecules using the neighbor-joining (NJ) method with MEGA 4 (http://www.megasoftware.net) were used for phylogenetic analysis.

**Table 1.** PCR primers used for Mj NOS analysis.

| Primers | Sequence (5′–3′) |
|---|---|
| **Degenerate PCR** | |
| NOS-F1 | TGGCCCTGTCTCGTGAAC |
| NOS-R1 | GTTTTCATCCCKCATCTG |
| NOS-F2 | AGAAYTGGTTTWCGGMGCTA |
| NOS-F3 | GGTCHAAGTTACAGGTVTTCGA |
| 5′-RACE | |
| 5′-R1 | CTGACATTGCTGAGAGGACCAAAGT |
| 5′-R2 | ACTGGATACGGCCGATACACCTT |
| 3′-RACE | |
| 3′-F1 | CCAGGATCTTCTTGTTGGTGTTGG |
| 3′-F2 | GTGTACCGTCAAGTTGTAGAGCAGAAGG |
| 3′-F3 | AAAGTGCAAGAGTGAAGATGTCC |
| **Cloning for partial sequence primer** | |
| c-R1 | CGACCATGCTCCTGGACAATAGACT |
| c-R2 | CACACATTCAGCCATGGTACAGTCAC |
| c-R3 | CACCATAATGTAGTGGTCCCTTTCCA |
| c-R4 | GTCAGCTTCACTAGCCTCTC |
| c-F1 | CTTCAACGGCTGGTACATGGTGT |
| c-F2 | GTTGGTGGTCACGTCTACTT |
| **RT-PCR analysis** | |
| Mj NOS-F | GCCCTGTCTCGTGAACCTAC |
| Mj NOS-R | TTTTCATCCCTCATCTGTAGCA |
| Mj EF1α-F | GTCTTCCCCTTCAGGACGTA |
| Mj EF1α-R | GAACTTGCAGGCAATGTGAG |

### *Mj NOS expression by RT-PCR*

Various tissues from body parts including the brain, gill, heart, hemocytes, hepatopancreas, intestine, lymphoid organ, muscle, thoracic ganglion, stomach, hematopoietic organ, ovary and testis were obtained from healthy shrimp (n=3). *Vibrio penaeicida* was obtained from the National Institute of Technology and Evaluation (NITE) Biological Resource Center (NBRC No. 15640). It was cultured in marine broth (Becton, Dickinson, USA) liquid medium. The concentration of the bacterial solution was adjusted at $2\times10^5$ CFU·mL$^{-1}$ on the basis of the McFarland No. 1. For the expression analysis of the Mj NOS gene, 100 μL of the bacterial solution was injected into second abdominal segment of each shrimp (n=6) and PBS was injected for control. Total RNA was extracted from each organ using Trizol Reagent in accordance with the manufacturer's instructions. The cDNA was synthesized using a ReverTra Ace qPCR

RT Kit after DNase I treatment. The gene-specific primers Mj NOS-F and Mj NOS-R (Table 1) were designed and used to amplify the conserved regions in the reductase domain of the Mj NOS gene. The kuruma shrimp elongation factor 1α (EF1α) gene (Table 1: Mj EF1α -F, -R) served as the control to confirm the quality and quantity of the cDNA used. A time course (0 - 24 h) expression analysis of Mj NOS was performed using pooled gill tissues from three individual shrimp that were injected with *V. penaeicida*. By semiquantitative analysis, the relative expression ratio of the Mj NOS was calculated.

## Results

### *Cloning and sequencing*

The entire cloned sequence of the Mj NOS cDNA is 4,616 bp with a 3,561 bp open reading frame coding for 1,187 (Fig. 1) amino acids. Additionally, it has an estimated mass of 134 kDa.

### *Sequence homology and domain structure*

To determine the percent homology with other NOS genes, sequence alignment was performed. The sequence homologies of Mj NOS were as follows: 82.3% with land crab NOS (Gl NOS); 60.1% with cricket *Gryllus bimaculatus* NOS (Gb NOS); and 40.3% with rat nNOS (Table 2A). The Mj NOS amino acid sequence was aligned with the published NOS sequences of the land crab, mollusks (cuttlefish), chordates (i.e., amphioxus and sea squirt), insects (i.e., cricket, silkworm, fruit fly and honey bee) and rat (Fig. 2). Mj NOS consists of an oxygenase domain, CaM binding domain and a reductase domain with 372, 24 and 668 amino acids, respectively (Fig. 2).

```
MKEVNKPQRLQNISSGNEVYDSLHTRTQTEGVCTRHLCNGALMVPRKRGTEPRSRDEVLKLAKDFIDEYY 70
QSIKRFNSEQHRQRWEQITREIDDRGTYDLTQTELVYGAKLGWRNSPRCIGRIQWSKLQVFDARYVTTAS 140
GMFEALCNHIKYGTNKGDLRSAITIFPPRTDGKHDFRVWNSQLISYAGYKQEDGTIIGDPINVEFTEVCV 210
RLGWKPKGGRWDVLPLVLSANGHDPEWFDIPQDLILTIPISHPEYKWFSDLDLQWYALPAVASLMFDCGG 280
LECPAAPFNGWYMVSEIGTRDLCDPHLFNILKTVGRSMGLDTRSPTNLWKDKALVEVNIAVLHSFQSLNV 350
TIVDHHSAAESFMKHFENEQRLRGGCPADWVWIVPPLSGSITPVFHQEMSLYYLKPSYEYQDPAWKTHVW 420
KKTKDVNRNSVRKTKRKFRFKEIARAVKFTSKLFGKALSKRIKATILYATETGKSEMYAKKLGEIFGHTF 490
NAQVCCMADYDLINIEHEALVLVVTSTFGNGDPPENGEEFAKNLYAMKVSGTAAGIDDVTSSMHRSLSFM 560
RMNSLTEGAGAPSTPLENGLASCNLRGSITSDIMSEDNFGPLSNVRFAVFALGSSAYPNFCAFGKYVDNL 630
LSELGGERLVKLTCGDELAGQEQAFKQWAGDVFTVGCETFCLDDDVAMKEATAALKIEATAALKIEATAS 700
ANKIKLAPCTKTDGIDIGLSRMHGKRVRSCQVLASRNLHGENASRWTQQVILTTGGVNELNYSPGDHVAI 770
LPANRKELIDAVLARLDNCPNPDEPIQVQVQKEVHSLNGVIQTWEPHERLPSTTVRELLTRYLDITTPPT 840
PNFLHLLAEYAYDNDQRTRLDQLATDPHEYEEWKHLRYPHLKEVLEEFPSVVLDAGLLLTHLPLMGPRFY 910
SISSSPDAHPGQIHITVAVVIYNTENGKGPLHYGVCSNYLKEVKAGNHIELFVRSASSFHMPRDPNVPII 980
LVGPGTGVAPFRGFWHHRHYMLKHKKENAGKMTLFFGCRTRALDLYADEKEAMQRTGVLSQTYLALSREP 1050
TIKKTYVQDLLVGVGSEVYRQVVEQKGHFYVCGDCTMAECVYQKLKSIVQEHGRLSDQEVENFMLQMRDE 1120
NRYHEDIFGITLRTEEIHRQKRESARVKMSSISQAGPPTPPVTQAPTNFAQEAAAVTDGGSTAAPVE 1187
```

**Fig. 1**. The amino acid sequence of kuruma shrimp nitric oxide synthase (Mj NOS) cDNA (4,616 bp). It contained a complete open reading frame (ORF) encoding a protein of 1,187 amino acids (residue number indicated on the right). GenBank accession # AB485762.

**Table 2.** By the entire NOS or each domain, amino acid identity and similarity of the Mj NOS gene compared to other known NOS sequences. Upper triangle is identity. Lower triangle is similarity. A : entire NOS, B : CaM binding domain, C : oxygenase domain, D : reductase domain. The full name, abbreviation and accession number: *M. japonicus* NOS, Mj NOS and AB485762; *Gecarcinus lateralis*, Gl NOS and AY552549; *Gryllus bimaculatus* NOS, Gb NOS and AB477987; *Bombyx mori* NOS, Bm NOS and NM_001043498; *Drosophila melanogaster* NOS, Dm NOS and U25117; *Apis mellifera* NOS, Am NOS and NM_001012962; *Sepia officinalis* NOS, So NOS and AY582749; *Branchiostoma floridae* NOS, Bf NOS and AF396968; *Ciona intestinalis* NOS, Ci NOS and XM_002120231; *Rattus norvegicus* nNOS, Rn nNOS and X59949.1; *Rattus norvegicus* eNOS, Rn eNOS and NM_021838.2; *Rattus norvegicus* iNOS, Rn iNOS and NM_012611.3.

A

| **Entire NOS** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1**. Mj NOS | | 82.3 | 60.1 | 49.7 | 47.3 | 58.2 | 48.5 | 40.6 | 38.6 | 40.3 | 45.7 | 41.9 |
| **2**. Gl NOS | **90.8** | | 59.4 | 49.2 | 46.8 | 57.0 | 48.7 | 40.7 | 38.2 | 40.2 | 45.8 | 42.3 |
| **3**. Gb NOS | **76.0** | **75.1** | | 55.4 | 56.5 | 71.3 | 48.0 | 42.4 | 40.3 | 42.2 | 46.8 | 43.8 |
| **4**. Bm NOS | **68.8** | **68.2** | **71.5** | | 47.1 | 53.7 | 43.8 | 39.9 | 39.1 | 39.8 | 44.7 | 42.2 |
| **5**. Dm NOS | **63.6** | **63.6** | **69.6** | **63.3** | | 52.2 | 40.2 | 41.2 | 41.1 | 42.9 | 41.3 | 39.8 |
| **6**. Am NOS | **74.8** | **74.4** | **84.4** | **70.1** | **66.7** | | 48.0 | 40.6 | 39.7 | 40.1 | 46.8 | 44.0 |
| **7**. So NOS | **67.3** | **66.6** | **67.6** | **62.9** | **58.8** | **68.2** | | 44.5 | 38.6 | 42.0 | 45.4 | 42.5 |
| **8**. Bf NOS | **58.4** | **58.3** | **58.9** | **58.9** | **60.7** | **57.7** | **60.4** | | 44.6 | 46.8 | 42.9 | 41.8 |
| **9**. Ci NOS | **57.2** | **56.7** | **57.9** | **57.7** | **61.2** | **57.2** | **56.3** | **63.5** | | 48.4 | 42.7 | 41.1 |
| **10**. Rn nNOS | **57.5** | **56.3** | **58.9** | **57.5** | **61.0** | **56.2** | **56.8** | **64.0** | **67.0** | | 48.8 | 43.5 |
| **11**. Rn eNOS | **65.1** | **64.6** | **63.9** | **63.7** | **57.4** | **64.2** | **63.1** | **58.8** | **59.1** | **62.6** | | 48.5 |
| **12**. Rn iNOS | **61.4** | **60.2** | **62.8** | **61.1** | **55.8** | **62.1** | **63.6** | **58.0** | **57.7** | **57.9** | **65.1** | |

*Table 2. (contd.)*

B

| CaM binding domain | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1**. Mj NOS | | 95.8 | 79.2 | 75.0 | 79.2 | 79.2 | 62.5 | 33.3 | 45.8 | 50.0 | 45.8 | 37.5 |
| **2**. Gl NOS | **100** | | 75.0 | 75.0 | 75.0 | 75.0 | 66.7 | 33.3 | 45.8 | 45.8 | 41.7 | 41.7 |
| **3**. Gb NOS | **91.7** | **91.7** | | 87.5 | 95.8 | 100 | 62.5 | 33.3 | 41.7 | 54.2 | 41.7 | 33.3 |
| **4**. Bm NOS | **83.3** | **83.3** | **87.5** | | 83.3 | 87.5 | 58.3 | 29.2 | 37.5 | 50.0 | 37.5 | 29.2 |
| **5**. Dm NOS | **91.7** | **91.7** | **100** | **87.5** | | 95.8 | 62.5 | 33.3 | 37.5 | 54.2 | 41.7 | 33.3 |
| **6**. Am NOS | **91.7** | **91.7** | **100** | **87.5** | **100** | | 62.5 | 33.3 | 41.7 | 54.2 | 41.7 | 33.3 |
| **7**. So NOS | **83.3** | **83.3** | **83.3** | **70.8** | **83.3** | **83.3** | | 37.5 | 29.2 | 58.3 | 41.7 | 33.3 |
| **8**. Bf NOS | **50.0** | **50.0** | **58.3** | **54.2** | **62.5** | **58.3** | **66.7** | | 20.8 | 37.5 | 41.7 | 34.6 |
| **9**. Ci NOS | **66.7** | **66.7** | **70.8** | **66.7** | **70.8** | **70.8** | **66.7** | **45.8** | | 30.4 | 31.8 | 27.3 |
| **10**. Rn nNOS | **70.8** | **70.8** | **70.8** | **66.7** | **70.8** | **70.8** | **75.0** | **54.2** | **65.2** | | 52.2 | 43.5 |
| **11**. Rn eNOS | **62.5** | **62.5** | **58.3** | **54.2** | **58.3** | **58.3** | **58.3** | **58.3** | **63.6** | **60.9** | | 22.7 |
| **12**. Rn iNOS | **54.2** | **54.2** | **50.0** | **45.8** | **50.0** | **50.0** | **54.2** | **54.2** | **59.1** | **60.9** | **45.5** | |

C

| Oxygenase doamin | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1**. Mj NOS | | 89.0 | 73.7 | 64.0 | 70.8 | 71.5 | 66.6 | 61.8 | 57.7 | 65.2 | 58.8 | 61.2 |
| **2**. Gl NOS | **96.0** | | 73.7 | 66.1 | 70.2 | 71.8 | 68.4 | 63.1 | 58.2 | 66.0 | 62.2 | 62.8 |
| **3**. Gb NOS | **86.3** | **85.8** | | 67.3 | 75.6 | 81.5 | 65.2 | 61.8 | 56.5 | 64.6 | 61.7 | 60.6 |
| **4**. Bm NOS | **81.2** | **80.4** | **83.1** | | 63.5 | 68.3 | 63.4 | 61.6 | 56.3 | 64.9 | 58.2 | 60.4 |
| **5**. Dm NOS | **85.8** | **85.8** | **87.7** | **81.8** | | 73.7 | 62.6 | 59.6 | 55.8 | 64.9 | 59.3 | 59.0 |
| **6**. Am NOS | **87.1** | **87.4** | **92.2** | **82.5** | **87.1** | | 66.0 | 61.0 | 59.3 | 64.6 | 60.4 | 62.5 |
| **7**. So NOS | **84.0** | **84.2** | **81.6** | **78.6** | **81.3** | **81.6** | | 65.5 | 57.8 | 63.8 | 59.6 | 64.1 |
| **8**. Bf NOS | **81.2** | **81.0** | **78.3** | **79.4** | **78.6** | **78.0** | **81.3** | | 54.4 | 60.1 | 54.8 | 59.3 |
| **9**. Ci NOS | **77.2** | **76.7** | **76.1** | **74.8** | **75.1** | **76.7** | **77.7** | **73.5** | | 60.4 | 59.0 | 59.8 |
| **10**. Rn nNOS | **79.5** | **80.3** | **80.1** | **79.8** | **79.5** | **80.9** | **80.1** | **76.6** | **78.5** | | 69.1 | 66.8 |
| **11**. Rn eNOS | **76.1** | **76.1** | **74.7** | **73.1** | **74.7** | **75.3** | **75.3** | **70.7** | **74.8** | **82.4** | | 64.4 |
| **12**. Rn iNOS | **78.2** | **78.5** | **77.1** | **76.1** | **75.3** | **76.6** | **77.9** | **76.3** | **75.6** | **82.2** | **77.7** | |

*Table 2. (contd.)*

D

| Reductase domain | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1**. Mj NOS | | 83.0 | 59.6 | 49.3 | 50.9 | 55.9 | 43.7 | 42.4 | 43.0 | 45.7 | 45.1 | 40.0 |
| **2**. Gl NOS | **91.5** | | 59.3 | 48.0 | 51.0 | 54.7 | 43.6 | 42.6 | 42.7 | 45.5 | 44.3 | 40.1 |
| **3**. Gb NOS | **74.9** | **74.7** | | 53.1 | 61.6 | 68.2 | 43.6 | 43.5 | 44.0 | 46.9 | 45.6 | 41.3 |
| **4**. Bm NOS | **68.1** | **67.6** | **70.0** | | 48.6 | 49.8 | 40.1 | 37.7 | 40.7 | 40.7 | 42.9 | 36.9 |
| **5**. Dm NOS | **68.9** | **69.3** | **77.8** | **67.1** | | 55.0 | 41.5 | 42.7 | 42.6 | 43.8 | 42.5 | 40.3 |
| **6**. Am NOS | **72.3** | **72.7** | **84.4** | **69.1** | **74.3** | | 41.4 | 41.7 | 42.5 | 44.4 | 46.4 | 40.2 |
| **7**. So NOS | **63.9** | **63.4** | **64.9** | **61.8** | **64.5** | **63.7** | | 48.8 | 43.8 | 50.9 | 45.4 | 39.4 |
| **8**. Bf NOS | **60.3** | **61.0** | **63.4** | **58.8** | **62.9** | **62.9** | **67.7** | | 48.8 | 51.1 | 46.9 | 42.3 |
| **9**. Ci NOS | **62.7** | **62.4** | **65.5** | **62.0** | **63.9** | **64.9** | **64.8** | **67.0** | | 52.1 | 47.4 | 42.5 |
| **10**. Rn nNOS | **66.0** | **64.5** | **68.8** | **62.0** | **64.4** | **65.3** | **68.8** | **69.4** | **72.5** | | 56.3 | 46.7 |
| **11**. Rn eNOS | **62.7** | **63.0** | **64.0** | **61.1** | **61.2** | **64.9** | **65.1** | **66.2** | **66.8** | **74.2** | | 46.9 |
| **12**. Rn iNOS | **56.3** | **55.6** | **57.6** | **56.3** | **57.1** | **56.6** | **60.0** | **60.8** | **62.8** | **65.1** | **64.8** | |

The heme binding and tetrahydrobiopterin ($BH_4$) domains were well-conserved (77.3-95.5% and 72.7%-100%) in the oxygenase domain (Table 3). In terms of the CaM binding domain, Mj NOS demonstrated the highest homology with Gl NOS (95.8%) followed by insect NOSs (75.0-79.2%) (Table 2B) The CaM binding domain of Mj NOS showed a lower homology than those of amphioxus NOS and rat iNOS (Table 2B). In both the oxygenase and reductase domains, Mj NOS is found to have the highest homology with Gl NOS at levels of 89.0% and 83.0 %, respectively; however, in the oxygenase and reductase domains, when the Mj NOS gene was compared with other NOS genes, it showed 57.7-73.7% and 40.0-59.6% homologies, respectively (Tables 2C and 2D).

To determine the similarity among them, the complete domain structure of Mj NOS was compared with those of other NOSs (Fig. 3). Regarding the domain structure, the reductase domain of Mj NOS contained conserved binding motifs for FMN, FAD and NADPH which are typical structures of NOS. When compared among species, the arrangements of those domains were found to be common (Fig. 3). However, Mj NOS does not has the glutamine-rich sequence that has been observed in the N-terminal region of Dm NOS, the PDZ domain in chordate NOSs (i.e., Bf and Ci NOSs) and rat nNOS.

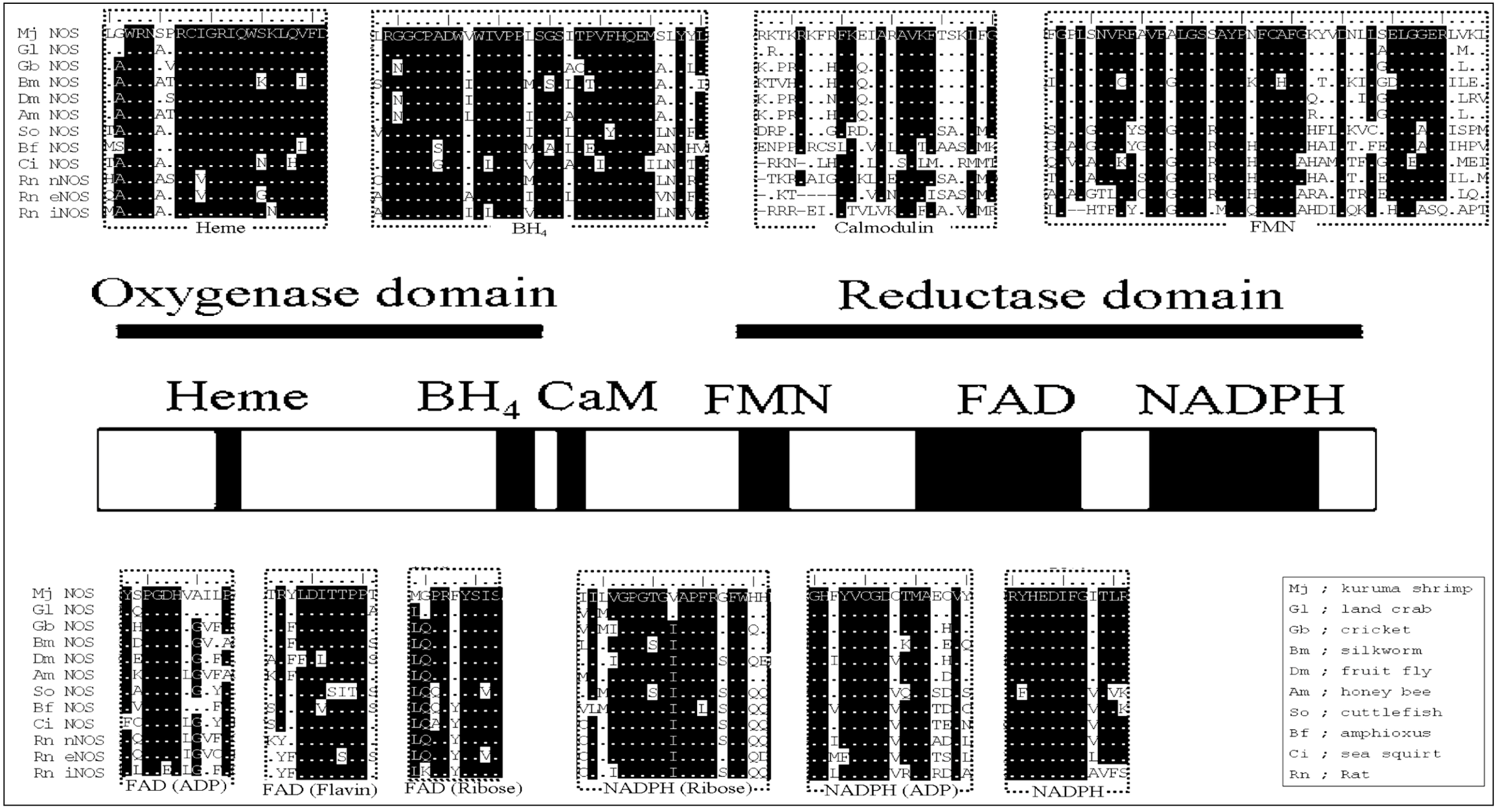


**Fig. 2.** Comparison of the deduced amino acid sequence of NOS from kuruma shrimp, (*Marsupenaeus japonicus*), land crab (*Gecarcinus lateralis*), insects, mollusks, chordates and rats. Kuruma shrimp NOS was aligned with NOS sequences from the land crab, insects (*Gryllus bimaculatus, B. mori, Drosophila melanogaster* and *Apis mellifera*), mollusks (*Sepia officinalis*), chordates (*Branchiostoma floridae* and *Ciona intestinalis*) and rats (*Rattus norvegicus* iNOS, nNOS, eNOS) by the ClustalW program (see Materials and methods). In all 12 sequences, these identities are highlighted in black. Boxes with broken borders identify highly conserved binding sequences for heme, tetrahydrobiopterin ($BH_4$), calmodulin, FMN, FAD and NADPH. The full name, abbreviation and accession number: *M. japonicus* NOS, Mj NOS and AB485762; *Gecarcinus lateralis*, Gl NOS and AY552549; *Gryllus bimaculatus* NOS, Gb NOS and AB477987; *B. mori* NOS, Bm NOS and NM_001043498; *Drosophila melanogaster* NOS, Dm NOS and U25117; *Apis mellifera* NOS, Am NOS and NM_001012962; *Sepia officinalis* NOS, So NOS and AY582749; *Branchiostoma floridae* NOS, Bf NOS and AF396968; *Ciona intestinalis* NOS, Ci NOS and XM_002120231; *Rattus norvegicus* nNOS, Rn nNOS and X59949.1; *Rattus norvegicus* eNOS, Rn eNOS and NM_021838.2; *Rattus norvegicus* iNOS, Rn iNOS and NM_012611.3.

**Table 3**. In the heme, $BH_4$ in the oxygenase domain and FMN, FAD and NADPH in the reductase domain, amino acid identity in the MjNOS gene compared to other known NOS sequences.

| | **Mj** | | | | |
|---|---|---|---|---|---|
| | **Oxygenase domain** | | **Reductase domain** | | |
| | **hem** | **$BH_4$** | **FM** | **FA** | **NADP** |
| **Gl** | **95.5** | **100.0** | **95.5** | **90.3** | **95.7** |
| **Gb** | **90.9** | **84.8** | **95.5** | **77.4** | **87.2** |
| **Bm** | **77.3** | **75.8** | **70.5** | **74.2** | **87.2** |
| **Dm** | **90.9** | **90.9** | **86.4** | **67.7** | **83.0** |
| **Am** | **86.4** | **84.8** | **93.2** | **67.7** | **93.6** |
| **So** | **86.4** | **78.8** | **61.4** | **64.5** | **68.1** |
| **Bf** | **86.4** | **72.7** | **59.1** | **71.0** | **68.1** |
| **Ci** | **77.3** | **72.7** | **59.1** | **67.7** | **78.7** |
| **Rn** | **77.3** | **84.8** | **70.5** | **67.7** | **76.6** |
| **Rn** | **77.3** | **78.8** | **61.4** | **58.1** | **76.6** |
| **Rn** | **81.8** | **78.8** | **47.7** | **67.7** | **66.0** |

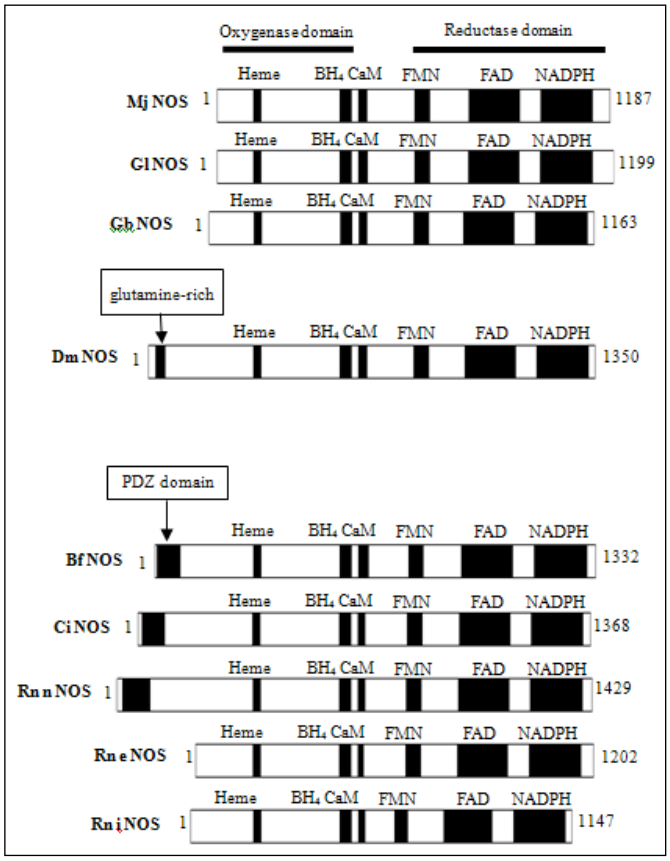


**Fig. 3.** Domain organization of NOS from the animals shown in Fig. 2. The ORFs from *M. japonicus* NOS (Mj NOS) and other NOS are compared. The oxygenase domain contains heme-binding and tetrahydrobiopterin ($BH_4$) domains. The reductase domain contains binding domains for FMN, FAD and NADPH. A calmodulin (CaM) binding domain is located between the oxygenase and reductase domains and is involved in dimerization and regulation of catalytic activity (Regulski & Tully 1995 and Stasiv *et al.* 2001). Near the amino terminus, *D. melanogaster* NOS (Dm NOS) has a Gln-rich sequence. *B. floridae* NOS (Bf NOS), *C. intestinalis* NOS (Ci NOS) and rat Rn nNOS have a PDZ domain. The full name, abbreviation and accession number: *M. japonicus* NOS, Mj NOS and AB485762; *Gecarcinus lateralis*, Gl NOS and AY552549; *Gryllus bimaculatus* NOS, Gb NOS and AB477987; *Drosophila melanogaster* NOS, Dm NOS and U25117; *Branchiostoma floridae* NOS, Bf NOS and AF396968; *Ciona intestinalis* NOS, Ci NOS and XM_002120231; *Rattus norvegicus* nNOS, Rn nNOS and X59949.1; *Rattus norvegicus* eNOS, Rn eNOS and NM_021838.2; *Rattus norvegicus* iNOS, Rn iNOS and NM_012611.3.

### *Phylogenetic analysis*

Known NOS sequences were compared using sequence alignments to perform a phylogenetic analysis. The data showed that Mj NOS was closely related to Gl NOS, and that the NOSs of insects of major taxonomic groups including Orthoptera (*G.*

*bimaculatus*), Hymenoptera (*A. mellifera*), Diptera (*D. melanogaster*) and Lepidoptera (*B. mori*) formed a cluster. Mollusks (*S. officinalis*), chordates (*B. floridae* and *C. intestinalis*) and vertebrate NOSs formed another distinct cluster. Within the vertebrates, the inducible NOS (iNOS) and noninducible NOSs (i.e., nNOS and eNOS) were in separate clusters (Fig. 4).

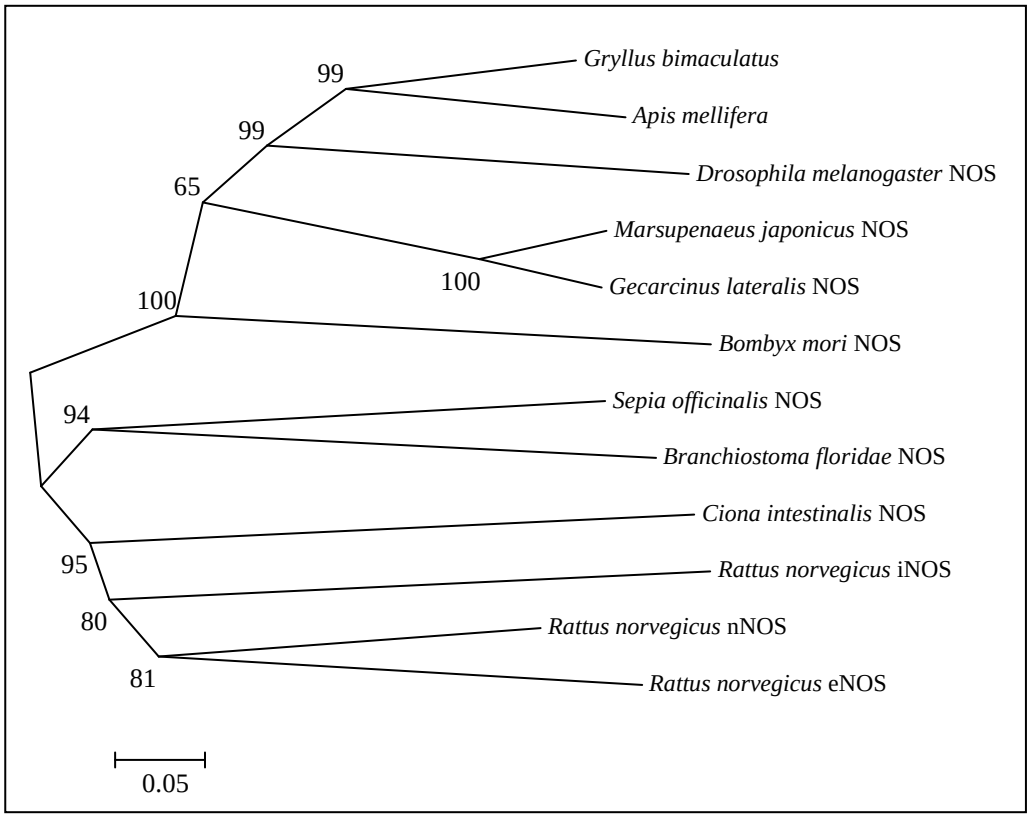


**Fig. 4.** Phylogenetic relationships of NO synthases from crustacean, insects, mollusks, chordates and mammals. By the ClustalW and Tree view programs, the deduced amino acid sequences were analyzed. Crustacean NOS sequences form a group divergent from molluscan and mammalian NOS sequences. The land crab NOS diverged from the insects within the arthropods. Accession numbers are the same as those given in the legend for Fig. 2.

### *Expression analysis*

A marked increase was observed in the Mj NOS expression level in the gill, heart, lymphoid organ and thoracic ganglion tissues following *V. penaeicida* injection, whereas a decrease was observed in the testis (Fig. 5). The relative expression level of Mj NOS in the gill of the *V. penaeicida*-injected group began increasing 1 h post *V. penaeicida* injection and peaked 12 h after; it then decreased 24 h after the injection in a time course experiment on Mj NOS expression (Fig. 6).

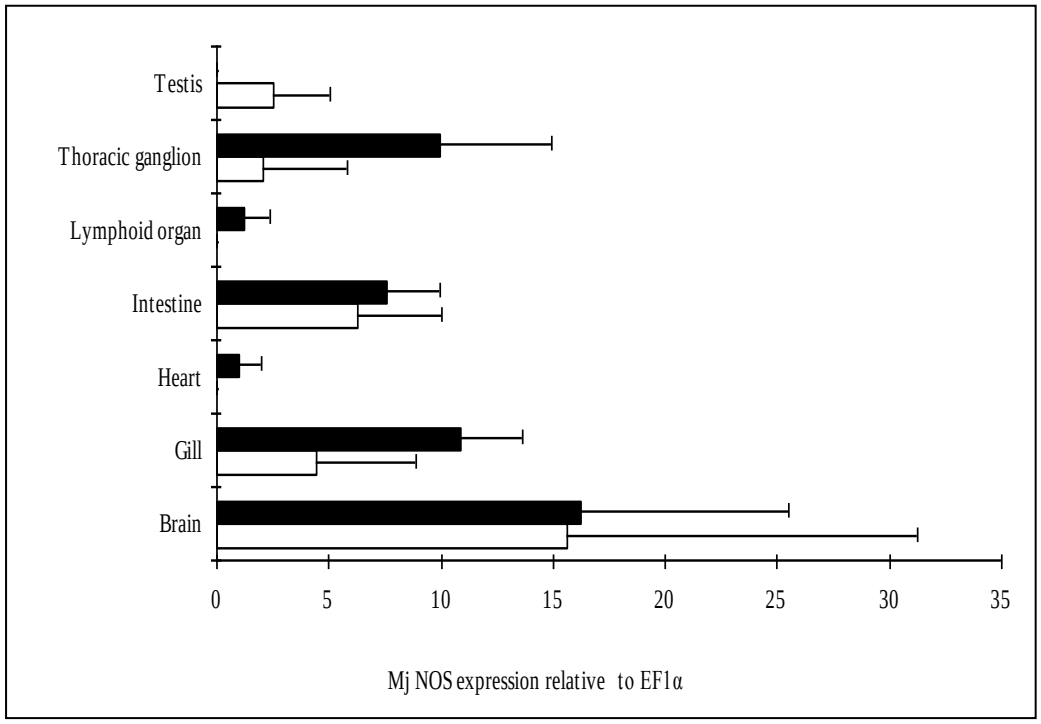


**Fig. 5.** The expression of Mj NOS from shrimp organs after injection of *V. penaeicida*. The expression ratio of Mj NOS was based on the quantity of expression of Mj NOS and EF1α. It was used a semi-quantitative analysis in the organs of kuruma shrimp. As a control, PBS injection was used.

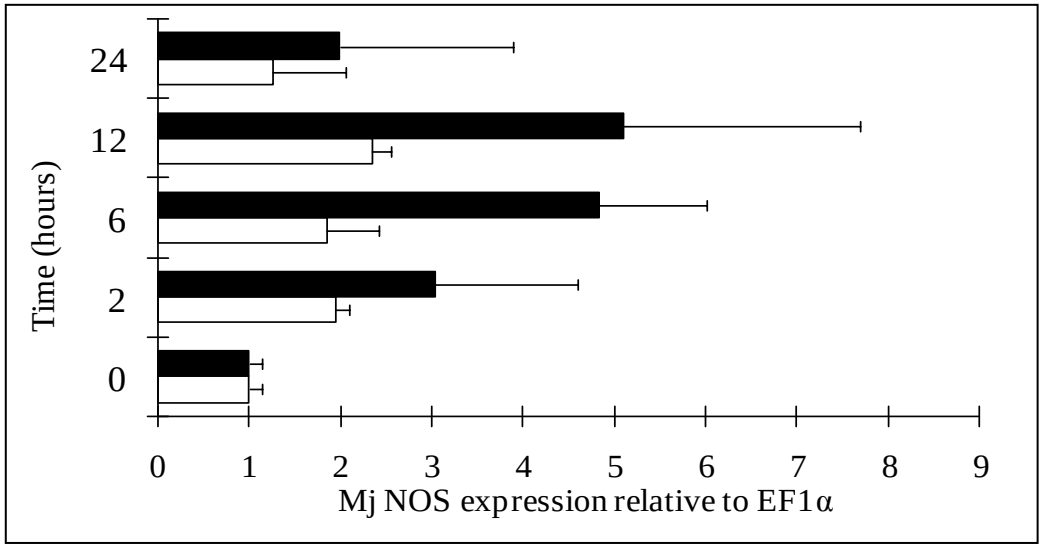


**Fig. 6.** Expression analyses of Mj NOS in kuruma shrimp gill after *V. penaeicida* injection. By a semi-quantitative analysis, the expression ratio of Mj NOS was analyzed over time (0 – 24 h) based on the quantity of expression of Mj NOS and EF1α.

## Discussion

The cDNA of the NOS gene of the kuruma shrimp (Mj NOS) from gill tissue mRNA was cloned. The ORF encoded a 1,187 amino acid protein with an estimated mass of 134 kDa. The molecular mass of Mj NOS was similar to that of land crab and crayfish NOSs (Lee et al. 2000) at ~136 and ~138 kDa, respectively and showed the highest identity with the land crab NOS (82.3%) and insect NOS (47.3-60.1%). The highest identity of Mj NOS with land crab (82.3%) shows that our original prediction of the high conservation of NOS in crustaceans is correct. The Mj NOS oxygenase domain showed an 89.0% sequence homology with Gl NOS and a 64.0-73.7% sequence homology with insect NOS. The Mj NOS reductase domain showed the highest (83.0%) sequence homology with Gl NOS followed by the insect NOS (49.3-59.6%). The Mj and Gl NOSs CaM binding domains showed 75.0-79.2% homologies with those of insect NOSs, particularly those with similar amino acid sequences: (KF(R/H/N)FK(E/Q)IARAVKFTSKLFG). The amino acid sequence variation shown above is found to involve arginine (R) and glutamic acid (E) in crustacean NOSs and histidine (H) or asparagine (N) and glutamine (Q) in insect NOSs. Conversely, the CaM binding domains of amphioxus NOS and rat iNOS show lower similarities (33.3 and 37.5%) than those of crustacean NOSs. Among animal species, this suggests the CaM domain has higher variations than the oxygenase and reductase domains.

*Drosophila* NOS has 24 glutamine residues at its N-terminal end and this segment is a glutamine-rich domain that is involved in protein-protein interactions for the regulation of transcription activation (Regulski and Tully 1995; Yeh et al. 2006). However, no glutamine-rich domain was observed in Mj NOS, Gl NOS or other insect NOSs, except Dm NOS.

In the N-terminal end of amphioxus Bf, sea squirt Cl and rat nNOSs, analysis of secondary structures showed a PDZ domain. In the rat, the interaction of PDZ-containing domains mediated the synaptic association of nNOS. It plays a central role in the formation of macromolecular signaling complexes (Brenman et al. 1996) which may function differently from amphioxus NOS and rat nNOS since Mj NOS and other invertebrate NOSs have no PDZ domain.

Phylogenetic analysis showed that the NOSs studied are divided into two distinct clusters. One cluster includes crustacean and insect NOSs and the other cluster includes mollusk, chordate and vertebrate NOSs.

After *V. penaeicida* injection in the gills, heart, lymphoid organ and thoracic ganglion of the kuruma shrimp, we observed the up-regulation of Mj NOS expression.

However, in heart or lymphoid organs of the control (PBS-injected) shrimp, there was no Mj NOS expression. No Mj NOS expression was observed in the muscle or hepatopancreas of the injected or control group. In the crab tissues, no Gl NOS expression was observed (Kim et al. 2004). The NOS gene was detected in the ovaries of the crab, but not in either of the injected groups of the shrimp. In contrast, in the thoracic ganglion, the shrimp NOS gene was found, but the crab NOS gene was not. Interestingly, in the testis, the crab and control shrimp NOS genes were detected, but the NOS gene was not detected in the *V. penaeicida*-injected shrimp, suggesting the down-regulation of NOS expression. In the heart, no NOS genes were detected, except in the *V. penaeicida*-injected shrimp, suggesting the up-regulation of NOS expression after *V. penaeicida* injection. In the expression of the NOS gene, these differences between the shrimp and the crab may be due to differences in the species, molting cycle and the capability of up- or down-regulation by stimulation, as observed in insect NOS genes (Imamura et al. 2002). In shrimp hemocytes, none Mj NOS was found. However, the NOS activity has been reported in kuruma shrimp hemocytes following white spot syndrome virus infection (Jiang et al. 2006). In hemocytes, the non-detection of Mj NOS is considered off-target sampling timing of shrimp. Shrimp hemocytes were sampled 6 h after *V. penaeicida* injection. Mj NOS expression could be found 1 and 2 h after *V. penaeicida* injection. Additionally, NO production was also confirmed 4 h after the injection of LPS which was extracted from *V. penaeicida* in unpublished data.

The Mj NOS expression level began increasing 1 h after injection and then gradually peaked after 12 h after, determining the changes in Mj NOS expression level in the gill after *V. penaeicida* injection. It then decreased down to the normal level 24 h after injection. The *V. penaeicida*-injected group showed a higher expression level than the control group during the entire period. These results indicate that Mj NOS is clearly inducible using a *V. penaeicida* injection. NOS is also inducible after LPS injection in insects (Imamura et al. 2002). This inducible NOS has been demonstrated to be common in arthropods (Yeh et al. 2006 and Imamura et al. 2002). Further study is necessary to clarify the function and structure of inducible NOSs that may differ between various organs after transcription.

In the lungs of rats, LPS stimulation induced a large effect on iNOS gene expression with dose dependence. However, it induced a small effect on eNOS and nNOS gene expressions (McCluskie *et al.* 2004). In mammals, nNOS and eNOS are $Ca^{2+}$/CaM-dependent; however, iNOS is $Ca^{2+}$-independent. NOS only has the $Ca^{2+}$/CaM-dependent form (Davies 2000; Korneev et al. 1998; Luckhart and Rosenberg 1999) similar to nNOS or eNOS in the system of enzyme activation by CaM in invertebrates. However, in crayfish hemocytes, $Ca^{2+}$-independent LPS-inducible NOS

activity has been reported. This inducible NO activity promotes bacterial adhesion and enhances bactericidal activity (Yeh et al. 2006).

Mj NOS had a higher similarity to mammalian nNOS than to iNOS or eNOS as determined on the basis of sequence and phylogenetic analyses in this study. However, Mj NOS was induced by *V. penaeicida* injection, suggesting that it is an inducible NOS after stimulation and has a higher similarity to mammalian iNOS in function. These contradictory observations suggest that nNOS may be the ancestor of eNOS and iNOS. More studies are required to better understand the molecular function and structure of Mj NOS in shrimp. The presence of NOS in crustacean non-neuronal tissues suggests that NO signaling is involved in physiological processes in addition to neuromodulation. In gonadal tissue, NOS may regulate gametogenesis and/or steroidogenesis (Kim et al. 2004).

## Acknowledgements

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# Genetic improvement and farming technological innovation on fleshy shrimp *Fenneropenaeus chinensis* in China

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## Abstract

The farming of the fleshy shrimp *Fenneropenaeus chinensis*, one of the indigenous and most important mariculture species in the coastal areas of Northern China, has experienced a dramatic fluctuation in the last two decades. The outbreak of white spot syndrome virus (WSSV) in 1993 resulted in a production collapse of this species; many efforts have been made to restore the industry thereafter. In order to improve the growth performance and disease resistance of shrimp, systematic selective breeding and technological innovation on farming practices were introduced. Two new varieties of *F. chinensis*, named "Huanghai No. 1" and "Huanghai No. 2" were developed after 7 and 10 years of selective breeding, respectively. The characteristic trait of "Huanghai No. 1" is faster growth. Compared with the unselected population, its average body length and body weight increased by 8.40% and 26.86% respectively. The new variety "Huanghai No. 2" was developed using a combined individual/family selection methods and the multi-trait BLUP (Best Linear Unbiased Prediction) strategy. This new variety was WSSV tolerant, as evidenced by higher survival rate and longer survival time after WSSV infection when compared to commercial seedling. Meanwhile, efforts were made to innovate and/or improve farming technologies to cope with the local shrimp farming conditions. Currently, the farming industry of *F. chinensis* in Northern China is recovering, and the farmers' confidence reveals a prosperous future for farming this shrimp species in Northern China, even though challenges still exist and much more efforts are needed for further development.

## Introduction

The fleshy shrimp, *F. chinensis*, with its relatively big size, high quality meat, good taste and large market share, has played an important role in shrimp farming and fisheries in Northern China, especially in provinces along the coasts of Bohai Sea and Yellow Sea before 1995 (Deng 1997; Deng and Ye 2001). The natural distribution of this shrimp is confined to the Bohai Sea, Yellow Sea, East China Sea and a small area

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of the northern part of the South China Sea (Deng et al. 1990). Researches on the reproductive biology, larval rearing and farming technology of *F. chinensis* has been conducted since the 1950's in China. In the 1970's, a series of technical breakthroughs dramatically accelerated the development of shrimp farming industry. From the middle of the 1980s to the early 1990s, China was the world leader in terms of larval rearing and the scale of shrimp farming with a production area around 150,000 ha and an annual production of 200,000 tons (Wang et al. 1995). Shrimp farming had become one of the most important industries in the coastal areas for local economic development. Unfortunately, *F. chinensis* farming has declined since 1993 due to serious viral disease outbreaks caused by white spot syndrome virus (WSSV), which has resulted in a dramatic decrease of *F. chinensis* production (Huang et al. 1995c; Wang et al. 2006).

Systematic research was carried out to determine the causes of the WSSV outbreak and production decline. The factors that contributed to the decline of *F. chinensis* farming included: a) *Lack of genetically improved shrimp stocks*: From experience with crop and animal husbandry, developing genetically improved stocks with characteristics of faster growth rate and/or disease resistance (or tolerance) is a basic component of maintaining a sustainable and healthy shrimp farming industry; b) *Available disease diagnosis techniques could not meet the needs of shrimp farming practices*: There was still a lack of available early disease (WSSV) warning mechanisms with which farmers could take necessary measures as early as possible and reduce economic loss; c) *Traditional larva rearing and pond farming technology could not control pathogen introduction and transmission*: Research reports suggested that the natural seawater, with live food organisms such as rotifer and several other invertebrates that are involved in shrimp larval rearing and pond farming were all possible WSSV carriers (Lo et al. 1996; Zhang et al. 2006; Yan et al. 2007); d) *Related techniques involved in farming practice needed urgent improvement.*

The US Marine Shrimp Farming Consortium had developed specific pathogen-free (SPF) populations of *Litopenaeus vannamei* to provide healthy seed to commercial farmers (Carr et al. 1994; Pruder et al. 1995; Wyban et al. 1995) as a means to support aquaculture of the species in the United States since 1990's. In order to revitalize *F. chinensis* farming industry in China, systematic research has been carried out since the mid 1990's to mitigate the situation and meet the urgent needs of the shrimp farming industry including selective breeding, pathogen detection and disease control, new modes of shrimp farming and complementary technique development. Based on more than 10 years of efforts, two new varieties of *F. chinensis* named "Huanghai No.1" with a registration No. GS-01-001-2003 and "Huanghai No.2" with a registration No. GS-01-002-2008, respectively and new modes of shrimp farming tailored to the local conditions were developed (Li et al. 2005; 2006a). With the full support from

governmental agencies and shrimp farmers, the new varieties have been commercially cultured and new farming modes are practiced in the coastal areas of Northern China. A prosperous future for the fleshy shrimp *F. chinensis* farming is anticipated, even though challenges still exist and much more efforts are needed for further development. This paper provides an updated overview of *F. chinensis* breeding program and farming practices in China.

## Selective Breeding for Faster Growth-- "Huanghai No. 1".

### *Materials and Technical Approachesh*

Growth performance improvement is undoubtedly the first target in any shrimp selective breeding program, as it is a trait with significant economic impacts. Mass selection was carried out on *F. chinensis* to improve its growth rate from 1997 to 2004. The initial population was selected from the wild catch in the Yellow Sea, where *F. chinensis* overwinter and migrate to the coastal waters to spawn. For each of the selected generations, usually over 5000 individuals were taken from ponds of different farming areas and transferred to overwintering tanks during late October to early November, after the shrimp completed their natural mating in farming ponds. By March of the next year, about 500 individuals were re-selected from the overwintered population and used as spawners to produce the next generation. During the re-selection process, individual shrimp was screened for WSSV infection using techniques such as T-E staining (Trypan blue-Eosin Y straining) (Huang et al. 1995a), ELISA with monoclonal antibody and/or dot blot test kit with gene probe (Huang et al. 1995b; Lei et al. 2001) and PCR technology. Only individuals with negative results were kept for spawning. Considering the large quantity of farmed shrimp from which the selection was made, an estimated selection intensity of 1~3% was applied through the whole process of selective breeding. In addition to the traditional selective breeding techniques, modern molecular biotechnologies were applied to assist the selection which included random amplified polymorphic DNA(RAPD), simple sequence repeats (SSR) and amplified fragment length polymorphism (AFLP) technologies for genetic diversity assessment of selected population (He et al. 2004; Zhang et al. 2005; Li et al. 2006b), RAPD, SSR and AFLP for genetic linkage mapping construction (Li et al. 2006a； Liu et al. 2010), identifying two molecular markers associated with desired traits detection (He et al. 2007).

### *Growth Performance in Selected Generations*

Selective breeding was conducted from 1997~2004. At the time of harvest, which was usually carried out in October in Shandong Province, random samplings

were made from experimental ponds that belonged to the same farm with the same pond managements to evaluate the results in each generation (Table 1). The sampled data showed that in the second through to the fifth generation, the average body length of the selected populations increased steadily, and shrimp body length ranges also showed a trend of progressive increase.

The selected variety passed the appraisement of the National Certification Committee for Aquatic Varieties in the end of 2003 after seven generations' selection, and was named "Huanghai No. 1" with a registration No. GS-01-001-2003. It was the first new variety of *F. chinensis* ever genetically improved and also the first successfully artificially bred marine aquatic animal variety in China (Li et al. 2005) (Fig. 1). Since 2006, "Huanghai No 1" has been recommended by the Ministry of Agriculture of China to be cultured with priority in the coastal areas of China.

**Table 1.** Sampling data of *F. chinensis* at harvest of the second through fifth generation of selection.

| Selected generation | Sampling date | Sampling No. | Average body length (cm) | Range of body length (cm) |
|---|---|---|---|---|
| The 2nd generation | October 2~3, 1998 | > 300 | 12.13 | 9.3-14.2 |
| The 3rd generation | October 2~4, 1999 | > 300 | 13.29 | 9.7-15.5 |
| The 4th generation | October 3~5, 2000 | > 300 | 13.59 | 11.0-16.4 |
| The 5th generation | October 2~4, 2001 | > 300 | 15.10 | 12.9-18.3 |

**Fig.1.** New variety of *F. chinensis* "Huanghai No.1". (top: ♀, bottom: ♂)

## Main Characteristics of "Huanghai No. 1"

### *Growth Rate and Morphology*

The first important characteristic of the new variety "Huanghai No. 1" was faster growth rate. Compared to the unselected population, the average body length of the new variety increased by 8.40% and the average body weight increased by 26.68% at the time of harvest (Li et al. 2005) (Fig. 2). This was based on the data collected from a dozen of experimental farming ponds during an appraisement by a group of renowned scientists working in this field.

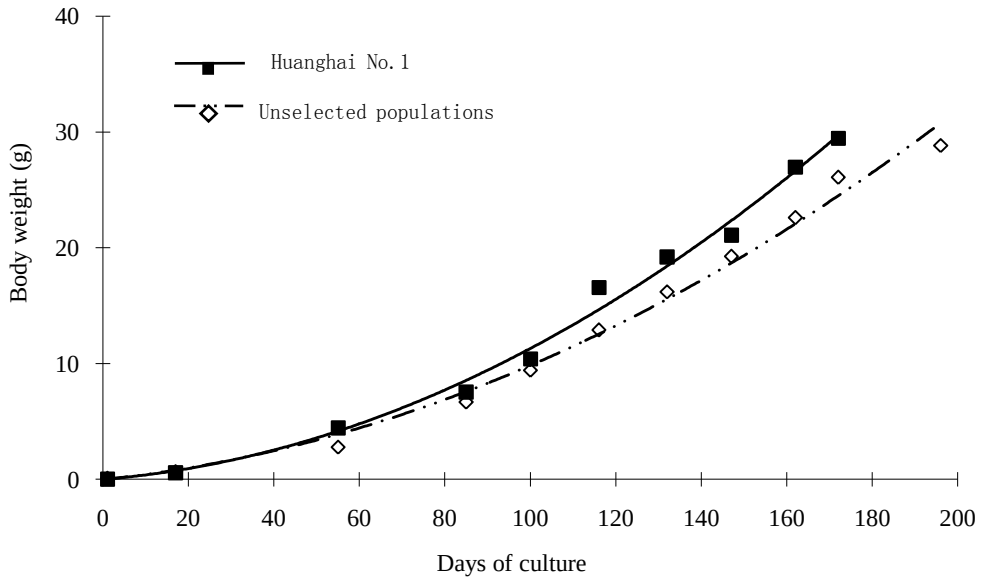


**Fig. 2.** Comparison of body weights between the 6th generation of "Huanghai No.1" and unselected populations during their growth.

After seven generations of orientated selection, the new variety showed a somewhat different morphology from the wild population (Li et al. 2006a). The length of the total abdominal section increased significantly and the total somites were longer while the widths of the carapace and the first somite were narrower. Compared to the wild population, the length of the 3rd, 5th and 6th somites increased by 2.41%, 2.27% and 3.41%, respectively, and the widths of carapace and the first somite were narrowed by 1.97% and 5.76%, respectively. Based on statistical analysis, the morphological characteristics of "Huanghai No. 1" and the wild populations were significantly different. Two formulas were constructed to differentiate the morphology between "Huanghai No. 1" and the wild population, and the correct rate could reach 70.67%.

"Huanghai No. 1" : $Y = -371.7165 + 2334x_1 + 3083x_2 + 184.3125x_3$

Wild populations : $Y = -387.2686 + 2248x_1 + 3234x_2 + 218.4082x_3$

Where, $Y$ : the discriminant accuracy; $x_1$ : the length of third somite /body length; $x_2$ : the width of carapace / body length; $x_3$ : the width of first somite / body length.

***Genetic Characteristics***

In order to evaluate the genetic characteristics of "Huanghai No. 1", related research on genetic variation, genetic structure and growth-related molecular markers was conducted successively (He et al. 2007; Li et al. 2006b). A total of 240 RAPD primers were used to scan 150 shrimp individuals belonging to three groups: unselected populations (body length was between 11 and 14 cm); big size group (body length was longer than 15 cm) and small size group (body length was less than 15 cm) of the sixth generation of "Huanghai No. 1". Two candidate molecular markers, one positively associated with growth and the other negatively associated with growth, were detected and validated, then designed as sequence characterized amplified regions (SCAR) markers (He et al. 2007). The results could be useful in molecular marker assisted selection (MAS) of *F. chinensis* in the near future.

Genetic variation and genetic structure of "Huanghai No. 1" were also analyzed. Results revealed that there was lower genetic difference within the selected variety ($Gst<0.05$), which indicated that the genetic structure after seven successive generations of selection tends to stabilize (Li et al. 2006b).

**Commercial Farming of "Huanghai No.1"**

"Huanghai No. 1" had been recommended as the "Major Aquaculture Variety" by the Ministry of Agriculture of China since 2006. From 2006 to 2008, "Huanghai No.1" was cultured in more than 10,000 ha in Northern China, including Hebei, Shandong, Jiangsu and Liaoning provinces, and was widely accepted by shrimp farmers. Utilization of this improved variety in commercial farming practices showed at least a 20% increase in economic return.

## Selective Breeding on Disease Resistance –"Huanghai No. 2"

Since 1998, arduous efforts were made to select a WSSV-resistant variety of *F. chinensis*. After 10 years of hard work, progress was achieved in selective breeding of disease resistant traits, and the new variety was named as "Huanghai No. 2". This new

variety showed better survival rate after WSSV infection in the laboratory and at the farm. Furthermore, farming practice also proved its tolerance to unfavorable culture conditions.

***Breeding Scheme***

The breeding scheme of the disease-resistant variety "Huanghai No. 2" is presented in Fig. 3.

The selection began in 1998. Initial spawners were collected from ponds that were seriously infected with WSSV and the virus caused heavy shrimp mortality. The survivors were collected and used as candidates for the WSSV-resistant selection. In each of the following generations, a WSSV-challenge-test in the laboratory was applied to select the WSSV-resistant individuals (Kong et al. unpubl. data). Pilot scale farming practice showed that the survival rate increased by more than 30% via this individual selection compared with 0-30% survival rate of unselected ones. Since 2001, family selection was introduced and 3~5 families that showed higher survival rate after artificial WSSV infection of around 30 families were added into the base population. After 5 years' selection, a WSSV-resistant line of *F. chinensis* was produced and named as "Jikang 98" in 2003. Since 2005, "Huanghai No. 1" and other four distinct wild populations were introduced into the breeding program, and breeding value estimates of multi-traits using BLUP (Best Linear Unbiased Prediction) were employed. Based on an unbalanced nested design, about 100 families were produced each year in the Selective Breeding Centre, Yellow Sea Fisheries Research Institute, and the target traits were not only WSSV-resistance, but also faster growth and higher survival rates, with weighing values of 15%, 80% and 5%, respectively. After three more generations of selection via BLUP, multi-traits breeding technology was set up and the new variety of *F. chinensis* named as "Huanghai No. 2" was developed in 2008. During the breeding of "Huanghai No. 2", the inbreeding coefficient was controlled to less than 1% in order to avoid inbreeding.

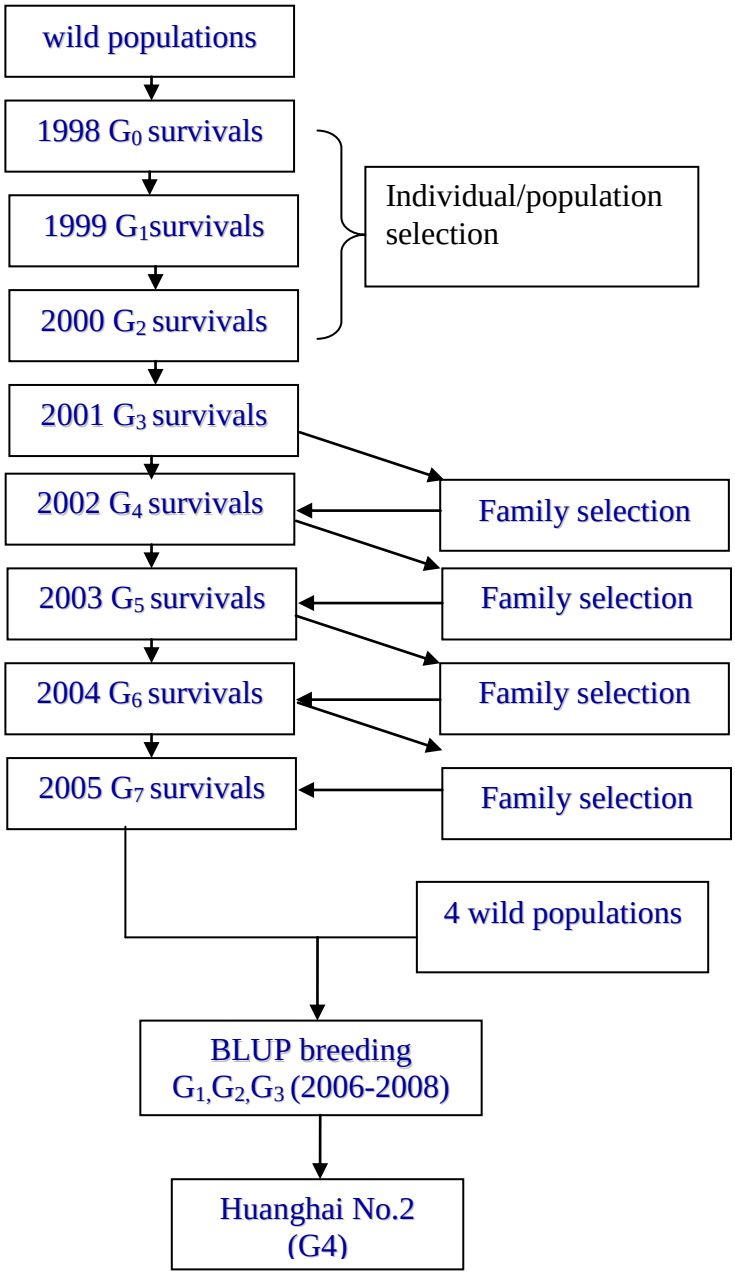


**Fig. 3.** Breeding scheme of "Huanghai No. 2"

***Performance and Genetic Gain of "Huanghai No. 2"***

The new variety Huanghai No. 2 was mainly characterized by a better WSSV-resistance/tolerance, which means longer survival time and higher survival rate after

artificial WSSV infection in the laboratory and in the field when compared with the unselected ones, as well as faster growth rate, which is preferred by farmers for producing bigger shrimp.

In order to evaluate the genetic gain of "Huanghai No.2", experiments were conducted to compare the main traits between the selected families (with higher breeding value among all families) and control families (with median breeding value among all families) under the same culture conditions during 2006-2008 (Zhang et al. 2008). Results showed that all the target traits have been improved to varied extents. The genetic gain of grow-out body weight, survival time post WSSV infection and grow-out survival rate was 13.56%, 6.67% and 5.05%, respectively, after 3 years' selection (Table 2). Comparison on the performance of "Huanghai No. 2" and commercial shrimp were also made in 2008. The results showed that the superiority of "Huanghai No.2" in harvest body weight, survival time and survival rates was 35.85%, 15.85% and 5.35%, respectively. These data demonstrated that the breeding procedure of "Huanghai No. 2" was effective and feasible.

**Table 2**. The genetic gain of main traits of "Huanghai No. 2"

| Genetic gain (%) | Body weight | Survival time[1] | Survival rate[2] |
|---|---|---|---|
| 2006 | 12.23 | 4.15 | 3.49 |
| 2007 | 15.56 | 6.38 | 5.40 |
| 2008 | 12.91 | 9.74 | 6.26 |
| Mean | 13.56 | 6.76 | 5.05 |
| Heritability ($h^2$)[3] | 0.22±0.16 | 0.14±0.12 | 0.03±0.021 |

Note: [1] The genetic gain of survival time between selected and control families after artificial infection of WSSV;
[2] The genetic gain of survival rate between selected and control families when shrimp were harvested in experimental pond without WSSV infection;
[3] Heritability ($h^2$) was estimated based on the data of 2008.

## Pathogen Detection and Disease Control

It was difficult to set up totally virus (WSSV) free conditions for *F. chinensis* larvae rearing in hatcheries, especially in the small-scale hatcheries. Several special measures were taken to reduce the risk of WSSV infection, which allowed shrimp larvae suppliers to offer larvae without a specific pathogen. All of these measures developed had been summarized technically and could be found from several domestic patents in China, for example, shrimp embryo disinfection of WSSV technique (Patent No. ZL01107747.6) and SPF shrimp larvae rearing technique (Patent No. ZL02135494.4). At the same time, a series of quick and accurate WSSV detection

technologies had been set up. T-E staining technique could diagnose WSSV in 10 min (Huang et al. 1995a), the transmission route was revealed by ELISA with monoclonal antibody (Huang et al. 1995b), commercialized dot blot kit with gene probe and PCR-gene probe technology (Lei et al. 2001) and LAMP technology (Patent No. ZL200810139949.4) were applied for large-scale investigation (Lei et al. 2001).

### *Immuno-Enhancement Technology*

Like all crustaceans, *F. chinensis* lacks a full-evolutionary specific immune system. However, experiments had shown that by adding some special elements with biological activity into formulated feed, it was possible to increase shrimp's disease-resistance/tolerance to a certain extent. The ongoing related research included the development of peptidoglycan as immuno-stimulant, the selection and utilization of probiotics for disease resistance and a combination of probiotics and bio-floc technology. Preparation of low molecular-weight peptidoglycan is accomplished by two-enzyme method, enhancement of phenoloxidase, alkaline phosphatase, hemocyte phagocytosis activities, etc. In farming practice, it was reported that adding 0.2% biological activity elements of peptidoglycan from $G^+$ bacteria to feed could increase the survival rate of farmed shrimp by 30% (Song et al. 2005).

### *Farming Technological Innovation*

Tremendous efforts were made to explore the optimal approaches to sustainable shrimp farming in China in recent years. The principle of biosecurity was applied to the farming industry and field practices proved that the principle was applicable technically and both economic and ecological benefits could be achieved if the principle was properly implemented. The main techniques involved in shrimp farming practice include: stocking SPF larvae, adopting a limited water exchange system, using aerators properly, applying water quality improving reagents such as probiotics, properly using water quality management measures, adding additives to feeds to enhance anti-stress and immune abilities of shrimp, controlling pathogenic bacteria in farming ponds and properly managing the effluent from shrimp farming ponds. After years of gradual improvement, several farming modes were set up technically and used in the farming of *F. chinensis*.

### *Deep-Pond-Farming Mode*

Farming ponds were built above the high tide zone along the coastal area. Typically, the ponds were 0.33~0.4 ha in size and 2.5~3 m in depth. The pond bottom was built with a 0.2%-slope to outlet, so discharged water can flow out easily. Waterproof materials were used to prevent collapsing and leaking of ponds, and

farming water was pumped from an uncontaminated sea area. The stocking density of 7-day postlarvae ($PL_7$) ranged at 100~150 seed·$m^{-2}$ and SPF postlarvae were strongly recommended to be used to prevent introduction of shrimp virus. The limited water exchange system was applied and daily exchange ratio was kept at 5~10%. High quality feeds (with additives to improve shrimp immunity and anti-stress) were used. WSSV and other pathogenic bacteria (i.e. *Vibrio* spp.), as well as feed and water quality was routinely monitored. Farming practices proved that the above measures when implemented collectively and properly could control the water quality and pathogenic agents within an acceptable level and achieve a good harvest. In Shandong Province, deep pond farming played an important role for Chinese shrimp farming (Li et al. 2003). Even though the costs to build such ponds were relatively high, their merit was evident, a yield of 8~15 ton·$ha^{-1}$ per crop could be harvested.

***Indoor-Industrialized-Farming Mode***

Cement and/or fibreglass-reinforced plastic tanks were built indoor to facilitate farming activities such as stocking the larvae ahead of main culture season and postponing harvest in Northern China where natural conditions permit only one crop. The following characteristics define this mode: a) farming tanks are usually 50~200 $m^2$ in size; b) shrimp were stocked at a higher density of 200~300 individual·$m^{-2}$; c) pure liquid oxygen was usually used; d) the farming yield in each crop was 2.5~4 kg·$m^{-2}$; e) water exchange ratio daily was usually about 30%; f) higher investment in facility, feed cost, energy supply, etc. Li et al. (2006) found that the quantity of phytoplankton, zooplankton and benthos were higher in semi-intensive farming ponds than in indoor industrialized farming tanks, but DO, $NH_4$-N and $PO_4$-P concentrations were the opposite and the concentrations of N and P were seldom affected by the DO and the stocking density in industrialized shrimp culture system (Liu et al. 2008). Shrimp growth rate and survival rate in indoor industrialized farming tanks were lower than those in semi-intensive farming ponds, but indoor industrialized farming gained higher production yield because of the higher stocking density. This farming mode has obtained a higher success rate because it experienced limited influence from the weather and many farming factors were controlled artificially. As such it was practiced in the coastal areas in Northern China. Usually the shrimp harvested were smaller as a result of the higher stocking density, but the yield was higher in terms of per unit farming area.

***The Integrated Farming Mode***

The integrated farming mode (also termed polyculture, ecological culture, etc.) means several aquatic species (in most cases two or three species) were cultured in the same pond based on their feeding behavior and ecological adaptation. Usually different

species of fishes, crabs, shellfishes and seaweeds are considered for farming with the appropriately-varied ratio (Wang et al. 2001; Liu and Li 2007). The purpose of this farming mode was to improve the efficiency of energy and feed, utilize the water body three-dimensionally, form a mutually beneficial ecological system among the culture species and consequentially increase the output per farming unit area. This system practically emphasizes a rational integration of farming species with different trophic levels, accelerating the circulation of matter and energy in a farming pond, making full use of the artificial and natural products such as nutrients, salts, dissoluble organic substances, particle organic matters, planktons, benthos, etc., to achieve optimum outputs. In most cases in Northern China, ponds practicing the integrated farming mode usually range from 2 to 20 ha in size with a water depth of 1.5 to 2 m. This mode requires less investment and begins with a lower stocking density (15~20 shrimp postlarvae·$m^{-2}$ usually), and production yield could be as low as 750 kg·$ha^{-1}$, but this mode could also produce larger size shrimp which means much better market value. At the same time, lower stocking density means less feed, less labor, better water quality and most importantly, less impact to the environment.

## Discussions

Traditional selective breeding based on phenotype has achieved remarkable progress in genetic improvement of *F. chinensis* especially on growth performance. However, there are still problems that the selective strategies have to address. For example, high-intensity selection could increase the probability of inbreeding if the relationship or genetic correlation among individual candidates was ignored in successive generations. Selective breeding strategies including BLUP genetic evaluation could not achieve ideal progress for traits that have low heritability such as WSSV-resistance of *F. chinensis*. Therefore, a long-term strategy should be established to breed the new varieties characterized by faster growth rate, stronger disease resistance /tolerance, better feed efficiency, etc. We believe that we have initiated such exploration with a good start but realize that more challenges still lie ahead. At the same time, a healthy shrimp farming approach is strongly recommended for sustainable development which includes high-health seed rearing, water quality management, probiotics application, high quality feed and optimal feeding regime, organic waste biodegradation, pathogen diagnosis and integrated disease control, effluent management, etc. Some suggestions for further research are presented below:

**a) *Establishing and optimizing the technical approaches of new variety breeding and extension*:** To evaluate the farming performance and economic benefits of a genetically improved variety, an optimized and traceable management system should be established by which the possible gene or genetic contamination could be avoided. Moreover, this

type of system would be helpful to accelerate the genetic gain of shrimp by screening candidate brooders with molecular markers that associate with commercially important traits especially growth performance and disease resistance. Research on SNP (Single Nucleotide Polymorphism) and genomics of *F. chinensis* are expected to contribute more knowledge at the molecular level.

**b)** ***Extending the limited water exchange system and bio-security concepts to shrimp farming*****:** With the advancement of technology, it is acceptable to apply limited water exchange system in large-scale shrimp farming. Enclosed farming systems permit re-circulation of a large proportion of sea water during the growout season and enable the shrimp farms be kept relatively isolated, which can prevent pathogen transmission from external sources and reduce potential cross-contamination within the system.

**c)** ***Enforcing standardization of shrimp farming activities, and establishing and refining related regulations and laws*****:** A responsible shrimp farming industry means not only producing sufficient quantity of shrimp to meet the needs of a continuously expanding market, but also high quality shrimp products to satisfy consumers. By enforcing the related regulations and/or laws, it will help to standardize the mariculture activities and essentially protect the shrimp farming industry from deviating from the way of sustainable development.

**d)** ***Developing an ecosystem approach for shrimp farming*****:** In farming areas where social and/or economic conditions are not suitable for developing industrialized shrimp farming, ecosystem-based or integrated shrimp farming mode could make full use of farming resources and garner an ideal economic return.

## Acknowledgements

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# Development of rapid, simple and sensitive real-time reverse transcriptase loop-mediated isothermal amplification method (RT-LAMP) to detect viral diseases (PRDV, YHV, IHHNV and TSV) of penaeid shrimp

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## Abstract

A one-step, single tube, real-time accelerated loop-mediated isothermal amplification (real-time LAMP) assay was developed separately to detect major shrimp viral diseases such as penaeid rod shaped DNA virus (PRDV), hypodermal and haematopoietic necrosis virus (IHHNV), Taura syndrome virus (TSV) and yellow head virus (YHV). Real-time LAMP method is more sensitive than other conventional PCR, RT-PCR and LAMP methods. The applicability of this assay was validated with plenty of viral samples collected from Japan and Thailand. Highly conserved regions of each viral genome developed separately were used to design the real-time LAMP primers. The real-time LAMP assay reported in this study is simple and rapid, where specific amplification is obtained for PRDV, IHHNV, TSV and YHV in 60 min under isothermal conditions at 63 $^{o}$C employing six distinct sequences of the target gene. The quantification of viral load in the infected samples was determined from the standard curve based on their threshold time required for turbidity to occur in the reaction by precipitation of magnesium pyrophosphate. Sensitivity analysis revealed that all of these viruses can be detected up to 100 copies of template DNA, rendering it ten-fold more sensitive than conventional LAMP assay.

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# Introduction

Shrimp viral diseases are a major impediment to commercial shrimp farming. The diseases affecting shrimp culture are white spot syndrome virus (WSSV) or penaeid rod shaped virus (PRDV), yellow head virus (YHV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) and Taura syndrome virus (TSV). PRDV of shrimp was first discovered in northern Taiwan around 1992 and is currently the most serious viral pathogen in farm reared *Marsupenaeus japonicus* (Nakano et al. 1994; Takahashi et al. 1994) throughout the world (Chou et al. 1995; Lo et al. 1996; Flegel 1997). The International Committee on Taxonomy of Viruses (ICTV) determined PRDV is the type species of the genus *Whispovirus*, family *Nimaviridae* (Mayo 2002). The typical clinical signs of PRDV include lethargy, reduced food intake and the appearance of white spots on the carapace (Lightner 1996). This virus causes 100% mortality within 3 to 10 days in all life stages of both wild and cultured *Penaeus monodon* and *M. japonicus,* and has a wide host range which includes penaeid shrimp, crabs, copepods and other arthropods (Chen et al. 2000; Syed Musthaq et al. 2006).

YHV was first reported as a virulent pathogen in the early 1990's causing a 100% crop loss within 3 to 5 days post-infection in *P. monodon* in Thailand (Limsuwan 1991 ; Chantanachookin et al. 1993). Since then, several researchers have reported the occurrence of YHV in farmed and wild shrimp in Taiwan and many other Asian countries. The causative agent is a rod-shaped, enveloped virus with positive stranded ssRNA containing four open reading frames classified under the genus *Okavirus*, family *Ronivirida*. So far, six genotypes have been identified and only genotype 1 is known to cause typical signs of YHV infection in shrimp. Other genotypes have been found to be widespread in *P. monodon* shrimp populations throughout the Indo-Pacific region, but have not been associated with farm disease outbreaks (Wijegoonawardane et al. 2008).

IHHNV is a cosmopolitan virus infecting penaeid shrimp in the Asian-Pacific area and the Americas, but it is not reported in Japan. The virus causes 90% mortality in *P. stylirostris* juveniles (Lightner et al. 1983) and is detected in other penaeid shrimps (Flegel 1997). The typical symptoms of the viral disease include reduction in growth and a runt deformity (Lightner et al. 1992; Primavera and Quinitio 2000). The virions of IHHNV are non-enveloped icosahedrons (22 nm in diameter) containing a single-strand linear DNA of 4.1 kb (Bonami et al. 1990). Genome homology suggests IHHNV may be related to mosquito Brevidensovirus (Shike et al. 2000). Taura syndrome virus (TSV) is a causative agent of Taura syndrome, a major disease of the white leg shrimp, *P. vannamei*. TSV was originally placed in the family Picornaviridae, but was later transferred to the Dicistroviridae. The complete TSV genome is a linear, positive-sense,

single stranded RNA virus of 10,205 bases. Taura syndrome was first recognized in shrimp farming in Ecuador in mid-1992. The loss was estimated to be close to US$ 100 million. Although the TSV originated in Ecuador, it was subsequently discovered in Taiwan in 2000.

Viral diseases are particularly difficult to control after the onset of infection; therefore, prophylaxes to prevent or reduce the losses through vertical and horizontal transmission are most important (Bell and Lightner 1988; Lotz 1997). Various diagnostic methods have been developed to detect shrimp viral diseases, including bioassays, histopathology, dot blot with *in situ* hybridization, polymerase chain reaction (PCR) (Lightner and Redman, 1998) and quantitative real-time PCR (Tang and Lightner 2001). Although the PCR based methods are sensitive and highly specific, they require expensive equipment, costly reagents and are time consuming. Therefore, a simple, quick and quantitative detection method is urgently needed to prevent the invasion of shrimp viral diseases into Japan from other countries. The LAMP assay is a novel approach to nucleic acid amplification that amplifies DNA with high specificity, selectivity and rapidity under isothermal conditions, thereby obviating the need for a thermal cycler. The LAMP assay is based on the principle of autocycling strand displacement DNA synthesis (Notomi et al. 2000). The reaction is performed by DNA polymerase with strand displacement activity and two sets of specially designed inner and outer primers. This assay is highly specific for the target sequence, because the target sequence is recognized at six independent sequences in the initial stage and at four independent sequences during the later stages of the LAMP reaction. The LAMP method has been used without quantitation for diagnosis of various shrimp viruses, such as WSSV (Kono et al. 2004), YHV (Mekata et al. 2006), *Mr*NV & XSV (Pillai et al. 2006), infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Sun et al. 2006) and TSV (Kiatpathomchai et al. 2007). These qualitative methods, however, cannot determine the copy number of the viral particles present in the sample.

Real time loop mediated isothermal amplification (RT-LAMP) assay produces large amounts of the target DNA as well as an insoluble by-product, magnesium pyrophosphate, during the reaction, making it possible to perform a real-time measurement of turbidity using an inexpensive photometer (Mori et al. 2001). Real-time LAMP assay has been used for many non-shrimp viruses, such as West Nile virus (Parida et al. 2004), severe acute respiratory syndrome (SARS) virus (Poon et al. 2005), dengue virus (Parida et al. 2005) and hepatitis A virus (Yoneyama et al. 2007). In the present study, a comparatively less expensive quantitative real-time RT-LAMP assay was successfully applied for detection of shrimp viral diseases in the field and proven to have high sensitivity and specificity.

# Materials and Methods

### *Shrimp*

Black tiger shrimp (*P. monodon*) and white leg shrimp (*L. vannamei*) with prominent signs of PRDV, YHV infection and IHHNV, TSV infection respectively were collected from shrimp farms in Songkhla, Thailand and Miyazaki, Japan. Shrimp samples were collected in separate sterile tubes and transported to the laboratory on dry ice for the RT-LAMP assay for each viral pathogen.

### *Nucleic acid extraction*

Extraction of DNA was performed from the corresponding viral homogenates prepared from PRDV and IHHNV sources. From each sample, 200 µL of the homogenate was added to 600 µL of DNAzol reagent (Invitrogen, Carlsbad, CA, USA) and further steps were carried out according to the manufacturer's instructions. Total RNA was extracted from a pool of heart tissues from the infected shrimp using an RNA extraction kit (High Pure RNA Tissue Kit; Roche Diagnostics, Germany) according to the manufacturer's instructions. Extracted nucleic acid samples were quantified using Nanodrop UV Spectrophotometer ND-100 (NanoDrop Technologies, USA). Synthesis of cDNA for quantitation analysis was carried out using ReverTra Ace qPCR RT Kit (Toyobo, Japan) with 1 µg of total RNA as per the manufacturer's instructions.

### *Design of primers for real-time LAMP procedures*

RT-LAMP primers specific to PRDV, YHV, IHHNV and TSV were designed according to the published sequences using Primer Explorer Software version 4 (https://primerexplorer.jp/lamp4.0.0/index.html, Fujitsu, Japan). The target regions of each shrimp viral pathogen include: ORF36 of PRDV, replicase polyprotein gene of YHV, non-structural protein of IHHNV and coat protein gene of TSV (GenBank Accession No. AF369029, EU487200, AF218266 and AF277378 respectively). A set of four primers, two outer (F3 and B3) and two inner primers (FIP and BIP), were designed according to the guideline provided. The oligonucleotide primers used for the amplification are shown in Table 1. Each primer has two distinct sequences corresponding to the sense and antisense sequences of the target: one for priming in the first stage and the other for self-priming in later stages. The specific primer regions were selected because of their early expression following the specific viral pathogen invasion.

**Table 1**. Primers used for the RT-LAMP assay for diagnosis of PRDV, YHV, IHHNV and TSV

| Primer name | Sequence 5'-3' |
|---|---|
| WSDV-FIP | TCCGTCTTCAGGGAATACATATGCTCAGGGAAGAAATAGACCATG |
| WSDV-BIP | GGACCCAAATCGAAATATAAGGCCTATGTTGCCCAAGATCCAC |
| WSDV-F3 | AAACACCGGATGGGCTAA |
| WSDV-B3 | CAAGGCAATACAGAATGCG |
| | |
| YHV-FIP | GACGTGTGGATGCATAAATTTCATGTATCAAATTTTCCTTGAACCTGG |
| YHV-BIP | CGATTGCCGTTACAACATGATGTTGGAATGGTCTCATGAAGG |
| YHV-F3 | TTCCGTAATGCCGGTGAT |
| YHV-B3 | AACGCTAGGAGTATAACCG |
| | |
| IHHNV-FIP | GAAAACTGGAACAGTTCTTCAGACAAATCAAGACCCTAAACCCAC |
| IHHNV-BIP | ACGAGGAAGACAACTCTCAAACTGTTATCCACGCAGACCTTAG |
| IHHNV-F3 | TCTCCAAGCCTTCTCACC |
| IHHNV-B3 | TCCCTCTCGAATTCCCAG |
| | |
| TSV-FIP | AGTTCATCTCAATGCCAGGAAATGAAGACATCAATTATTCGACGC |
| TSV-BIP | GCAGTCTGAAGCTCGAGCTATTGTTATTCACATTTCTGGGGTT |
| TSV-F3 | TGGAATAAGATGAATGCTAAGC |
| TSV-B3 | GACTCAGAACGGAAAGCC |

### *Optimization of reaction time and temperature*

The real-time RT-LAMP was carried out in a total volume of 25 μL of reaction mixture using Loopamp DNA and RNA Amplification Kit (Eiken Chemical, Japan) according to the manufacturer's instructions for analysis of DNA and RNA viruses respectively. Briefly, the specified amount of target nucleic acid was mixed with 1 μL (40 pmol) of each -FIP and -BIP, 1 μL (5 pmol) of -F3 and -B3 primers, 12.5 μL of Reaction Mix (2×), 1 μL Enzyme Mix containing *Bst* DNA polymerase and distilled water used to make up to 25 μL. AMV reverse transcriptase was used in the case of YHV and TSV. The reaction temperature (60, 63 and 65 ºC) was optimized using Loopamp Real Time Turbidimeter LA-200C (Teramecs, Japan). Real-time monitoring was performed every 6 sec using spectrophotometric analysis by recording the optical density (OD) at 650 nm. Each assay was carried out three times.

### *Determination of sensitivity of real-time RT-LAMP assay*

Ten-fold serial dilutions ($10^{-1}$ to $10^{-9}$) of nucleic acids extracted from various shrimp viral diseases were used as templates for RT-LAMP under the optimized conditions. RT-LAMP reaction was performed using the Loopamp real-time turbidimeter.

***Specificity of real-time RT-LAMP detection***

The specificity of the RT-LAMP method was evaluated using different sources of DNA/cDNA templates prepared from YHV-, PRDV-, IHHNV- and TSV-infected shrimp and healthy shrimp. Each assay was carried out in duplicate.

***Quantitative real-time RT-LAMP***

To determine the quantity of unknown nucleic acid using the RT-LAMP assay, the specific target fragments of each viral disease were cloned into plasmid. The amplified PCR product was cloned into pGEM-T Easy Vectors (Promega, USA) according to the manufacturer's instructions. Quantitation of the constructed plasmid (using pGEM vector) was achieved using the NanoDrop spectrophotometer, and ten-fold serial dilutions ($10^1$ to $10^9$) were made to evaluate the real-time LAMP assay. The copy numbers of the plasmid DNA were calculated based on the molecular weight and Avogadro's number, and a standard curve was constructed. The standard curve of the specific virus was generated each time during the analysis of samples. The reaction setup was the same as that optimized above, and the reactions were carried out in the Loopamp real-time turbidimeter.

## Results

***Optimization of real-time RT-LAMP assay conditions for PRDV, YHV, IHHNV & TSV detection***

RT-LAMP was performed using DNA/RNA as a template in order to determine the optimal temperature and reaction time for various shrimp viral pathogen. Out of the three different temperatures (60, 63 and 65 ºC), the best results were obtained at 63 ºC. The most rapid amplification was achieved at this temperature, requiring less than 20 min for the initiation of amplification as determined by a change in the turbidity by magnesium pyrophosphate (Fig. 1). Amplification was efficient at all temperatures tested; however, 63 ºC for 60 min of reaction time was selected as optimal conditions for further experiment.

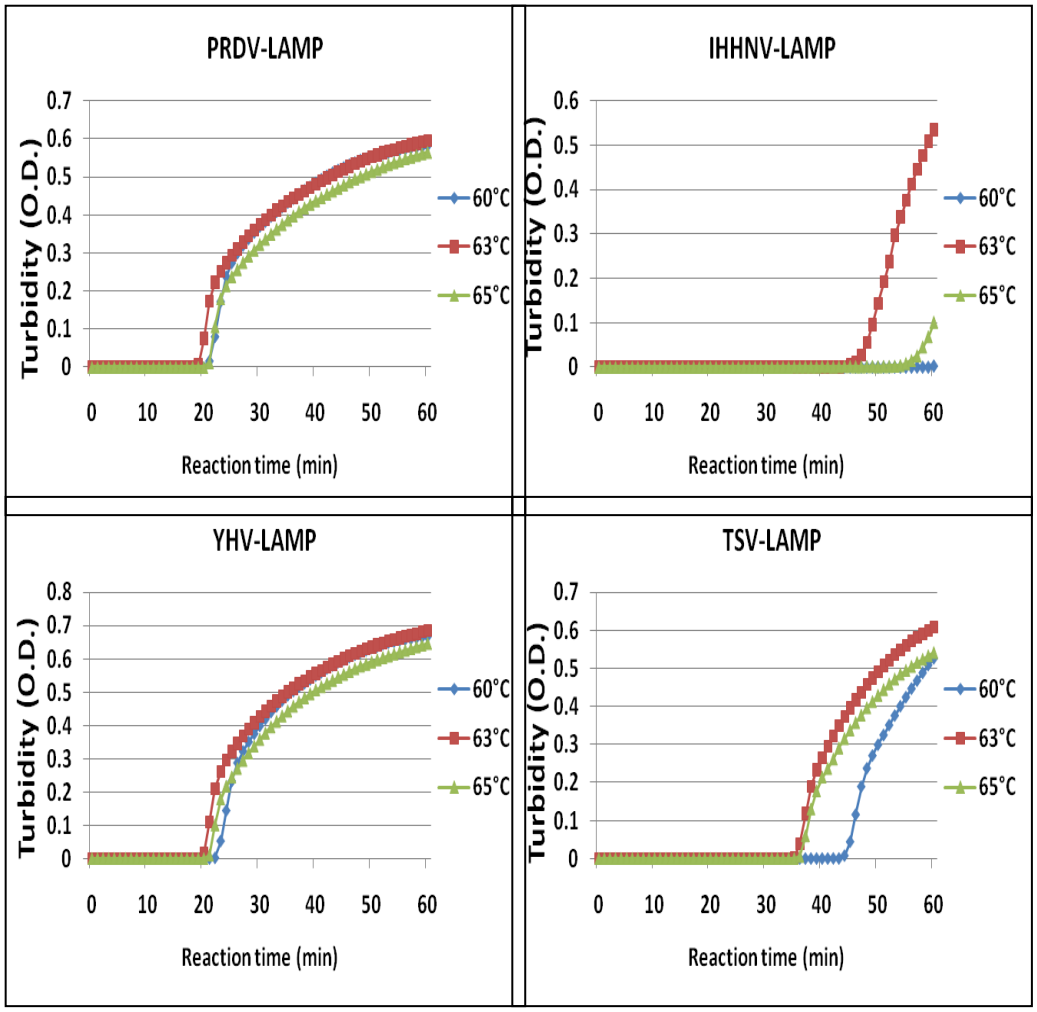


**Fig. 1.** Optimization of the reaction temperature for RT-LAMP assay of shrimp viral pathogens (PRDV, YHV, IHHNV and TSV) performed at 60, 63 and 65 ºC.

### *Specificity of RT-LAMP detection*

Cross-reactivity analysis was performed to examine the specificity of real-time RT-LAMP assay. DNA/cDNA of other shrimp viral disease viruses (PRDV, YHV, IHHNV and TSV) and healthy shrimp were used to determine the specificity of each viral diagnostic assay using RT-LAMP assay. As shown in Fig. 2, the RT-LAMP assay was highly specific to each virus without any cross-reaction with other shrimp viral pathogens.

***Quantitative detection using RT-LAMP***

For quantitative detection of samples of unknown concentrations, a standard curve was generated using the turbidity time (Tt) plotted against the log of the initial template using serially diluted, $10^1$ to $10^9$ copies·µL$^{-1}$ of plasmids with inserts of concern viral target DNAs (Fig. 3). High correlation coefficient values ($R^2$ = 0.99, $R^2$ = 0.99, $R^2$ = 0.98 and $R^2$ = 0.99 respectively for PRDV, YHV, IHHNV and TSV) were obtained for each viral pathogen by using real-time LAMP assay (Fig. 4). Ten-fold dilutions were used to generate standard curves for each pathogen to run in parallel with unknown samples for quantitative and diagnostic analysis.

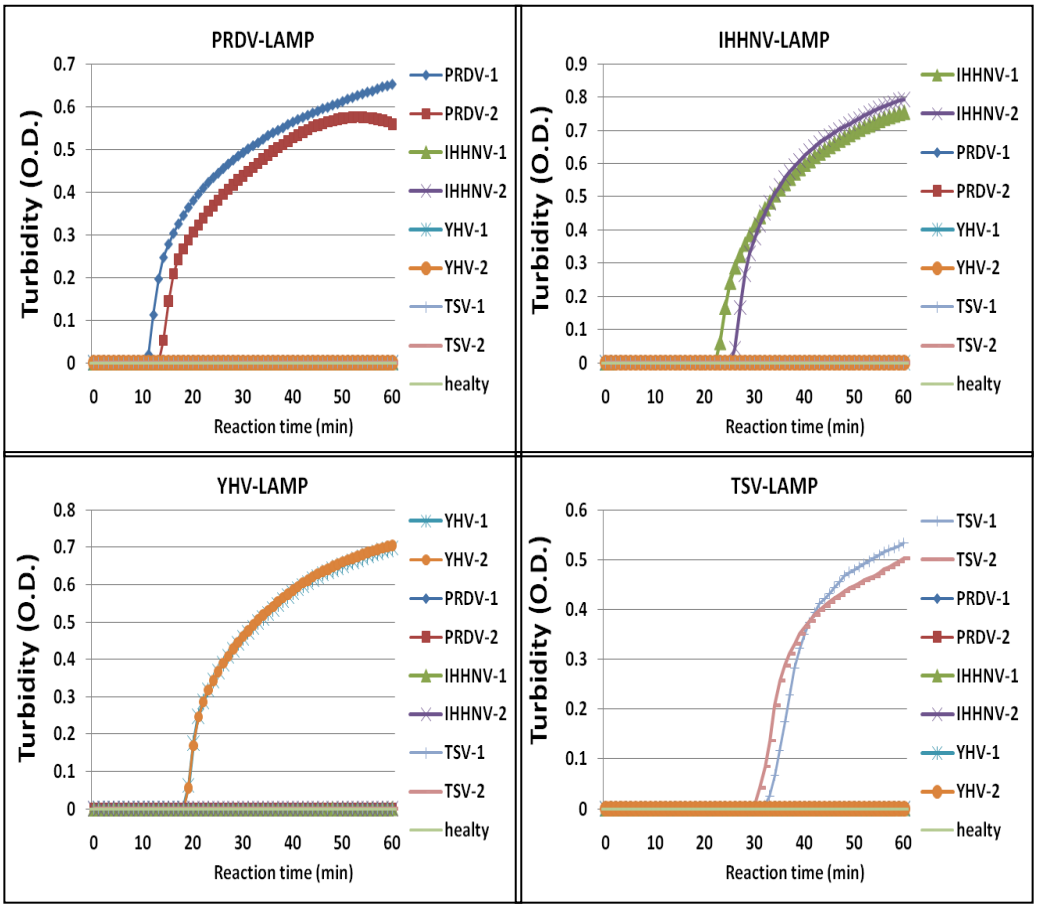


**Fig. 2.** Specificity of the RT-LAMP assay. Results of cross-reaction analysis with templates of individual shrimp pathogen with other major shrimp viruses (PRDV, YHV, IHHNV and TSV-DNA/cDNA) and with a healthy shrimp DNA template are shown.

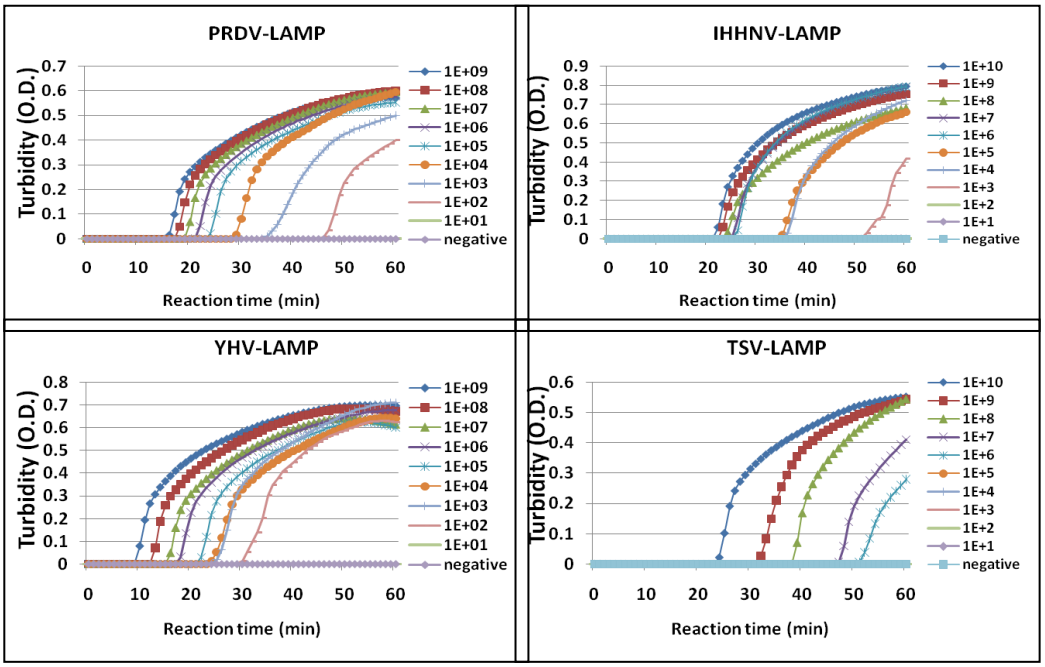


**Fig. 3.** Real – time amplification of shrimp viral pathogens (PRDV, YHV, IHHNV and TSV) using RT-LAMP assay. Plasmid standards corresponding to target gene of each shrimp viral pathogen at concentrations of $10^1$ to $10^9$ copies·µL$^{-1}$ (time is shown on the X-axis and OD at 650 nm on the Y-axis).

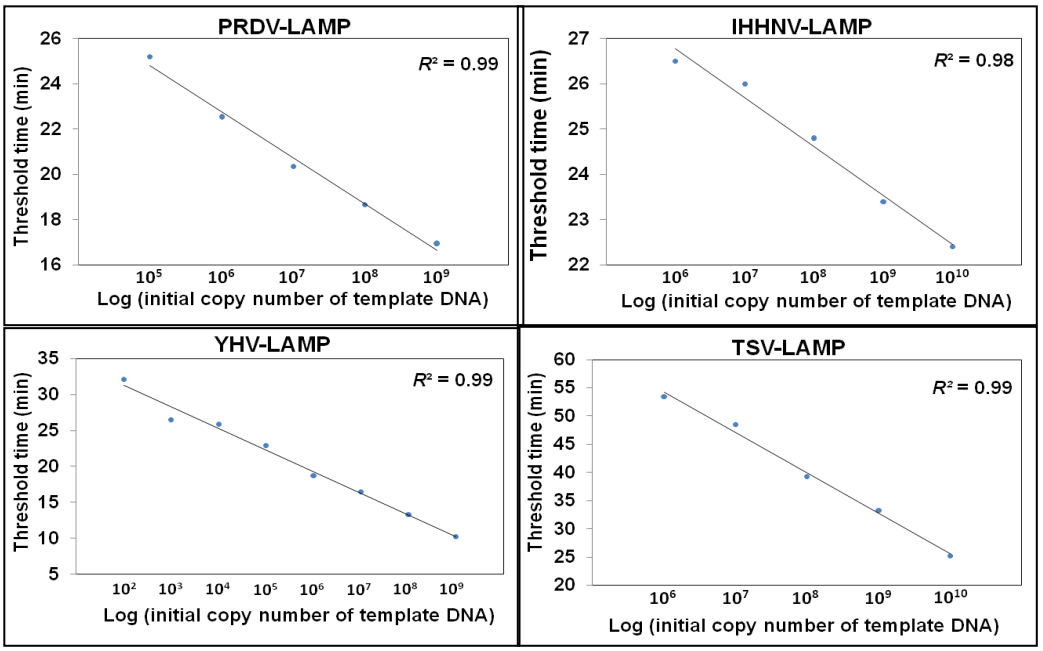


**Fig. 4.** Real – time amplification of shrimp viral pathogens (PRDV, YHV, IHHNV and TSV) using RT-LAMP assay. Standard curves generated from plasmid standards corresponding to target gene of each shrimp viral pathogens.

## Discussion

Recent outbreaks of IHHNV, YHV and TSV virus in the Asia-Pacific area and other regions have caused severe economic loss to shrimp farmers; however, these viral disease outbreaks have not been reported in Japan. Therefore, these diseases have been designated as "a specified disease" using Japanese law, where if the disease is detected in Japan, all shrimp must be destroyed to prevent wide spread dissemination. Therefore, it is essential to develop an efficient method for field surveillance for these pathogens which has high specificity and sensitivity with a short reaction time, as well as the ability to quantify the viral load. A recent outbreak of YHV in the Asia-Pacific and other regions has led to severe economic loss to shrimp farmers (Cowley and Walker 2002) because there was no user-friendly detection assay available.

Here we demonstrate a new diagnostic method for the quantitative detection of shrimp viral pathogens (PRDV, YNV, IHHNV, TSV) using the real-time LAMP method. Early detection of shrimp viral pathogens is important in the shrimp industry for effective health management and preventive measures. Previously, various nucleic acid and protein based assays (Sritunyalucksana et al. 2006) have been employed for the detection of WSSV and other shrimp viral pathogens in the shrimp culture sector. All of these assays can determine only the presence of viral pathogen; however, they do not determine the number of virus particles present. Quantification of WSSV can be achieved by real-time PCR assays using Taqman (Sritunyalucksana et al. 2006) and SYBR chemistry (Khadijah et al. 2003; Yuan et al. 2007).

Various conventional diagnostic methods for WSSV have been developed and reported by several researchers worldwide with different sensitivity. Sensitivity of the normal qualitative LAMP assay of WSSV was almost equal to the RT-LAMP assay, whereas it lacks quantitative measurement (Kono et al. 2004). Different PCR-based assays such as one step PCR, nested PCR, real-time PCR have the sensitivity limit of up to 1000 copies, 50 copies and five copies, respectively (Sritunyalucksana et al. 2006). In contrast, the RT-LAMP method is found to be a cost-effective quantitative assay for shrimp viral disease diagnosis. As all conventional methods were developed based on a specific genome, the chance of non-specificity is found to be much less. Real-time RT-PCR assays have been developed for laboratory diagnosis of shrimp viruses; however, these techniques have the intrinsic disadvantage of requiring both high-precision instrument, high costs for amplification and a complex method for the detection of amplified products and technically qualified persons.

The increased need for an inexpensive method to quantify shrimp viral pathogens led us to develop the RT-LAMP method. Also the cost of the LoopAmp

turbidimeter is low, whereas the real-time PCR assays require fluorogenic primers and probes using an expensive fluorometer (Parida et al. 2004). The sensitive RT-LAMP assay has been successfully applied to detect many human pathogenic RNA viruses, resulting in rapid and simple diagnostic measures. The rapid simple detection and quantification of shrimp viral pathogens using RT-LAMP take less time when compared to other PCR and real-time PCR methods. The optimum conditions for the real-time LAMP reaction were 63 $^{o}$C for 60 min. Higher temperatures can support the rigorous binding of primer and target template in the LAMP reaction than at lower temperatures (Teng et al. 2007), leading to amplicons consisting of concatemer hairpin repeats (Cai et al. 2008). Using the optimized conditions to perform the assay in a short period of time (<60 min), the turbidity caused by the magnesium pyrophosphate can be visualized without an instrument (Sun et al. 2006). This assay will provide a practical tool in the field for quantitative detection of viral infection in cultured shrimp, even at the early stages.

Each virus-specific standard curve was generated using 10-fold dilutions of $10^1$ – $10^9$ copies·$\mu L^{-1}$ of purified plasmids and the reactions were run in duplicate. The mean $T_t$ for the plasmid standards was generated using the specific software provided with the Loopamp real-time turbidimeter. Standard curve equations were calculated using regression analysis, which compared the average Tt to the standard copy number. We obtained a high correlation coefficient (greater than or equal to $R^2$ = 0.988 for each shrimp viral pathogens) for the unknown quantity DNA templates. Cross reactivity analysis showed the primers used were specific to each viral pathogen and did not amplify for other shrimp viral pathogens and healthy shrimp cDNA/DNA templates. Sensitivity analysis showed the assay for each shrimp viral pathogen is detectable up to 100 copies of the template DNA, which is more sensitive than the earlier-developed LAMP and PCR based methods. Furthermore, the gradual decrease of turbidity in the reaction was also clearly observed by the naked eye.

The RT-LAMP assay is an alternate method to conventional PCR and the LAMP assay (Sun et al. 2006) and in addition, it quantifies the shrimp viral nucleic acid templates. This gives a triplex amplification synchronization on one target gene (Cai et al. 2008). The RT-LAMP assay allows positive samples to become clouded (turbid) and can be viewed visually, eliminating electrophoresis for further confirmation (Mori et al. 2004). The sensitivity of the loop primer was demonstrated in a previous report (Nagamine et al. 2002). The real-time quantitative LAMP assay can be used for gene expression analysis, as the reaction is performed under isothermal conditions and a relatively low temperature where the reverse transcriptase can efficiently work. Thus, this assay has the potential to simplify quantitative gene expression analysis.

Therefore, we believe the genome specific RT-LAMP assay will be routinely used as a comprehensive shrimp viral detection system in most field diagnostic laboratories because of its speed, simplicity, specificity and lower cost. We are considering further studies using RT-LAMP with the fluorescence probe, SYBR-I, as the intercalation dye to increase the LAMP sensitivity to quantify very low numbers of virus. The RT-LAMP assay would be a promising technology for shrimp viral pathogen detection, which contributes to better shrimp health management and disease surveillance in shrimp hatcheries and culture ponds for prevention of disease outbreak.

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# Omics' studies for genetic improvement of shrimp in China

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## Abstract

This paper mainly summarizes the progress of the research group in omics research on shrimp at the Institute of Oceanology, Chinese Academy of Sciences. With the increasing requirements for genetic improvement of shrimp in order to satisfy the sustainable development of the shrimp industry, we have undertaken research on the omics of shrimp, including transcriptomics, proteomics and genomics, which can provide important information or a platform for the further genetic improvement of shrimp.

## Introduction

Production of shrimp aquaculture in China has increased very quickly since the end of the 20th century. However, sustainable development of the shrimp aquaculture industry has been facing difficult issues, including genetic improvement, disease control and remediation of polluted water environment. Therefore, development of knowledge-based technologies is urgently needed. Omics study can provide significant information about the genetic background, immune mechanisms and genetic markers of shrimp. Over the last decade, we have undertaken some studies on the omics of the Chinese shrimp, *Fenneropenaeus chinensis* and the Pacific white shrimp, *Litopenaeus vannamei* on DNA, RNA and protein levels. In this paper, we describe the major progress made in this area from our institute.

### *EST sequencing and data mining*

Chinese shrimp, *F. chinensis*, is one of the most important mariculture species in China. However, its culture industry has been seriously affected by outbreaks of white spot syndrome virus (WSSV) since 1993. The mortality rate of WSSV-infected shrimp has been almost 100% in 3 to 10 days post-infection. Not much information about shrimp genetics and immunity was available at the time of its appearance.

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Expressed sequence tags (EST) sequencing from cDNA libraries has been proven as an efficient approach to identify new genes with large scale sequencing as the most direct and effective approach to get detailed genetic information of the shrimp. EST data is very useful in genome annotation, comparative genomics, genetic mapping and marker-assisted selection for genetic breeding. Therefore in 2000, we constructed four cDNA libraries from different tissues, including cephalothorax, hemocytes, eyestalk and ovary. In total, 16000 ESTs were sequenced (Table 1).

**Table 1**. Sequenced EST numbers from different cDNA library

| Tissues for cDNA library | cephalothorax | hemocytes | ovary | eyestalk |
|---|---|---|---|---|
| Sequenced EST numbers | 10446 | 3483 | 1517 | 1283 |

Through bioinformatic analysis, information on a number of functional genes was obtained. For example, the ESTs from the cephalothorax were clustered into 1399 contigs and 1720 singletons which represent about 3120 unique genes (Xiang et al. 2008). Among the discovered unique genes, 1373 EST clusters are known genes and 1747 EST clusters may represent novel genes that do not match any known genes from other species in the NCBI data bank. In order to find more information about the immune related genes, Shen et al. (2004) analyzed the ESTs from cephalothorax and found 47 contigs and 34 singletons responding to 428 ESTs which showed similarity to known immunity-related proteins. Considering the function of hemocytes in the immunity of shrimp, more immune-related genes were identified through bio-informatic analysis from the cDNA library of hemocytes (Dong and Xiang 2007). Among 2371 ESTs from the hemocytes cDNA library, 34 genes including 177 ESTs have been identified and these genes might be involved in defense or immune functions in shrimp, based on current knowledge. These genes are classified into five categories according to their putative functions in the shrimp immune system: 13 genes are different types of antimicrobial peptides (AMP, penaeidin, antilipopolysaccharide factor, etc.), 11 genes belong to prophenoloxidase system (prophenoloxidase, serine proteinase, serine proteinase inhibitor, etc.), five genes have high homology with clotting protein (lectin, transglutaminase, etc), three genes may be involved in inter-cell signal communication (peroxinectin, integrin etc.) and two genes have been identified to be chaperone proteins (Hsc70, thioredoxin peroxidase etc.). The aforementioned EST sequences information will enrich our understanding of the immune genes of *F. chinensis* and also help our research on the genetic improvement of shrimp.

In addition to ascertaining information on functional genes, EST sequence analysis can also help to obtain genetic markers such as microsatellite markers. Among the 10443 ESTs from the cephalothorax library, 229 SSRs (simple sequence repeats) were identified (Wang et al. 2005) and these could be developed into markers for pedigree analysis and genetic map construction to enhance the genetic breeding of shrimp.

***Construction of cDNA microarray and its application***

The microarray technique has been proven to be a powerful tool to investigate the expression of thousands of genes in a single hybridization. In order to understand the interaction between shrimp and pathogens such as bacteria or virus, a cDNA microarray including 3136 spots was constructed in *F. chinensis* based on a large batch of the above ESTs. We used a microarray technology to study differentially expressed genes in WSSV-infected shrimp. Gene expression patterns in the cephalothorax of shrimp injected with WSSV 6 h after and moribund shrimp naturally infected by WSSV were analyzed. A total of 105 elements on the arrays showed a similar regulation pattern in artificially infected shrimp and naturally infected moribund shrimp; parts of the results were confirmed by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The up-regulated expression of immune-related genes including heat shock proteins (HSP70 and HSP90), trehalose-phosphate synthase (TPS) and ubiquitin C were observed when shrimp were challenged with WSSV. Genes including myosin LC2, ATP synthase A chain and arginine kinase were found to be down-regulated after WSSV infection (Wang et al. 2006).

To compare the gene expression pattern of shrimp to different pathogens such as bacteria and virus, we used microarray technique and analyzed the gene expression profiles of shrimp when they were challenged by WSSV and heat-inactivated *Vibrio anguillarum*, respectively. At 6 h post challenge (HPC), a total of 806 clones showed differential expression profile in WSSV-challenged samples but not in *Vibrio*-challenged samples. A total of 155 clones showed differential expression in the *Vibrio* challenged samples but not in WSSV-challenged samples. In total, 188 clones showed differential expression in both six and 12 HPC-WSSV and *Vibrio* samples. Most of the differentially expressed genes (185/188) were down-regulated in the samples of 12 h post WSSV challenge, whereas they were up-regulated in the samples at six and 12 h post *Vibrio* challenge and 6 h post WSSV challenge (Wang et al. 2008). The data will provide great insight for us in understanding the immune mechanism of shrimp responding to WSSV or *Vibrio*.

***Proteomic approach***

Proteins are vital parts of living organisms as they are the main components of the physiological metabolic pathways of cells. Proteomics is the large-scale study of proteins, particularly their structures and functions. The expression profile at protein level can reflect the physiological state of animals. The outbreak of shrimp disease is usually accompanied by environmental stress such as a variation in temperature and/or dissolved oxygen. Hypoxia not only affects the growth of shrimp by reducing their molting frequency and retarding growth, but it also increases the susceptibility of shrimp to infectious disease. To better understand the mechanism of response to hypoxic stress in *F. chinensis*, a proteome research platform was developed in our laboratory and differentially expressed proteins of hepatopancreas in adult Chinese shrimp (between the control and hypoxia stressed groups) were screened. By a two-dimensional gel electrophoresis (2-DE) analysis, 67 spots showed obvious changes after hypoxia. Using LC-ESI-MS/MS, 51 spots representing 33 proteins were identified (Jiang et al. 2009). This study was the first analysis of differentially expressed proteins in the hepatopancreas of shrimp after hypoxia and it provides new insight for further study in hypoxic stress response of shrimp at the protein level.

In order to gain an understanding of the function of the shrimp lymphoid organ at the protein level, we analyzed the proteome of the lymphoid organ in healthy *F. chinensis* through a 2-DE analysis based proteomic approach. A total of 95 spots representing 75 protein entries were identified by liquid chromatography tandem mass spectrometry (LCeMS/MS) with both an online and in-house database. Among them, approximately 36% of proteins related to cytoskeleton are noticeable. Subsequently, a comparative proteomic approach was employed to investigate the differentially expressed proteins in lymphoid organ of *V. anguillarum*-challenged *F. chinensis*. At 24 h post-injection (hpi), 17 differentially expressed protein spots were successfully identified, including four up-regulated protein spots (representing four proteins: cathepsin L, protein similar to squid CG16901-PC, protein kinase C and protein similar to T-complex Chaperonin 5 CG8439-PA), and 13 down-regulated protein spots (representing nine proteins: actin, beta-actin, cytoplasmic actin CyII, alpha tubulin, beta tubulin, protein similar to proteasome delta, vacuolar ATP synthase subunit B, elongation factor 2, carboxypeptidase B) (Zhang et al. 2010). This data may help us to understand the function of the lymphoid organ and the molecular immune mechanism of shrimp in response to pathogen infection.

***Immune related genes***

Based on the information obtained through EST analysis, cDNA microarray hybridization and proteomic analysis, we have focused on several categories of genes involved in the immune functions of shrimp.

## Identification of important genes involved in humoral immune pathways

***(1) Pattern recognition receptors (PRRs),***

PRRs are proteins expressed by cells of the innate immune system to identify pathogen-associated molecular patterns (PAMPs), which are associated with microbial pathogens or cellular stress. Therefore, PRRs play very important roles in innate immunity. We have identified a series of PRRs in *F. chinensis*, including lectin, toll, LGBP and tetraspanin which are key factors to recognizing different pathogens and triggering a series of immune reactions.

Lectin is regarded as a molecule potentially involved in immune recognition and phagocytosis through opsonization in crustaceans. Knowledge on lectin at a molecular level would help us to understand its regulation mechanism in crustacean immune systems. A novel C-type lectin gene (*Fclectin*) was cloned from hemocytes of *F. chinensis* by 3' and 5' rapid amplification of cDNA ends (RACE) PCR. The deduced amino acid sequence contains a putative signal peptide of 19 amino acids and two carbohydrate recognition domains/C-type lectin-like domains (CRD1 and CRD2). Expression profiles of *Fclectin* gene were greatly modified after bacteria, LPS or WSSV challenge (Liu et al. 2007). This data indicated that C-type lectin might play an important role in the recognition of different pathogens in innate immunity of shrimp and would therefore be helpful in controlling shrimp disease.

Toll-like receptors (TLRs) are a class of proteins that play a key role in the innate immune system. They are single, membrane-spanning, non-catalytic receptors that recognize structurally conserved molecules derived from microbes. Once these microbes have breached physical barriers such as the skin or intestinal tract mucosa, they are recognized by TLRs and immune cell responses are activated. In *F. chinensis*, we cloned the full-length cDNA of Toll (FcToll), which encodes a putative protein of 931 amino acids. The predicted protein consists of an extracellular domain with a potential signal peptide, 16 leucine-rich repeats (LRR), two LRR-C-terminal (LRR-CT) motifs and two LRR-N-terminal (LRR-NT) motifs, followed by a transmembrane segment of 23 amino acids and a cytoplasmic Toll/Interleukin-1R (TIR) domain of 139

residues. Genomic structure of FcToll gene contains five exons and four introns. Phylogenetic analysis revealed that it belongs to the insect-type invertebrate Toll family. Transcripts of FcToll gene were constitutively expressed in various tissues, with predominant levels in the lymphoid organ. Real-time PCR assays demonstrated that expression patterns of FcToll were distinctly modulated after bacterial or viral stimulation with significant enhancement after 5 h post-*V. anguillarum* challenge but markedly reduced levels immediately after WSSV exposure (Yang et al. 2008). These results suggest that FcToll might be involved in innate host defense especially against the pathogen *V. anguillarum.*

The full length cDNA of lipopolysaccharide and ß-1,3-glucan binding protein (LGBP), a pattern recognition protein (PRP) was cloned from *F. chinensis* using homology cloning and RACE techniques. Two putative integrin binding motifs, RGD (Arg-Gly-Asp) and a potential recognition motif for ß-1,3-linkage of polysaccharides were observed in the deduced amino acid sequence. The cloned LGBP gene was mainly expressed in hemocytes and hepatopancreas. A significant enhancement of LGBP transcription appeared at 6 h post-injection in response to bacterial infection. When shrimp were challenged by bacteria (*V. anguillarum* and *Staphylococcus aureus*), the transcript of LGBP increased significantly at 6 h post challenge which indicated that LGBP plays an important role in the recognition to bacteria pathogen (Liu et al. 2009).

Besides the aforementioned three types of PRRs, a potential new type of PRRs, tetraspanins/TM4SF was focused. The tetraspanins/TM4SF is a widely expressed superfamily in eukaryotic organisms. Three members of the tetraspanins/TM4SF superfamily were cloned from *F. chinensis*. The deduced amino acid sequences of the three proteins have typical motifs of the tetraspanins/TM4SF superfamily. Phylogenetic analysis of the proteins together with the known tetraspanins of invertebrates and vertebrates revealed that they belong to different tetraspanin subfamilies: CD9, CD63 and tetraspanin-3. CD9, CD63 and tetraspanin-3 showed apparently different tissue distributions in shrimp. The CD9 gene (*FcCD9*) was specifically expressed in hepatopancreas. The highest expression of the CD63 gene (*FcCD63*) was detected in nerves, epidermis and hearts; low expression was detected in hemocytes, ovaries, gills, hepatopancreas and stomachs, and no expression was detected in intestines, muscles and lymphoid organs. Compared with *FcCD9* and *FcCD63*, the tetraspanin-3 gene (*FcTetraspanin-3*) was more broadly expressed and its highest expression was detected in the intestine. All three tetraspanins were markedly up-regulated in the live WSSV-challenged shrimp tissues (Wang et al. 2010). Additionally, dsRNA interference was utilized to examine the functional role of *FcTetraspanin-3* in response to WSSV infection. Results found a decrease of the viral copy number in the tetraspanin knockdown shrimp. These results suggested that *FcTetraspanin-3* might play an

important role in response to WSSV infection and the large extracellular loop (LEL) of tetraspanin might mediate the entry of WSSV (unpublished data). These data suggested that the three cloned members of TM4SF superfamily in Chinese shrimp may play a key role in the route of WSSV infection.

***(2) Antimicrobial peptides (AMP)***

Antimicrobial peptides (also called host defense peptides) are an evolutionarily conserved component of the innate immune response and are found among all classes of life. Antimicrobial peptides are a unique and diverse group of molecules that are divided into subgroups based on their amino acid composition and structure. Since the first antimicrobial peptide was identified in *Litopenaeus vannamei* in 1987, different groups of antimicrobial peptides including penaeidin, anti-lipopolysaccharride factor (ALF), crustin and lysozyme were found in shrimp. In *F. chinensis*, we have identified the genes encoding different antimicrobial peptides, penaeidin (Kang et al. 2004), ALF (Liu et al. 2005) and crustin (Zhang et al. 2007a; Liu et al. 2008). The recombinant protein of penaeidin (Li et al. 2005) and crustin (Zhang et al. 2007a,b) in *Escherichia coli* or yeast showed apparent antimicrobial activity. Lysozyme activity changed after a bacteria challenge showed that lysozme is also an important antimicrobial effector in shrimp (Yao et al. 2008). In summary, antimicrobial peptides should play crucial roles in a shrimp's immunity to bacterial infection.

***(3) Transcription Factors***

Rel/NF-kB transcription factors play key roles in regulating the AMP gene expression in Toll and Imd pathways. In shrimp, different types of AMP were reported. In order to know how the transcription of AMP was regulated in shrimp, we identified two different Rel/NF-kB transcription factors including relish and dorsal. Relish homolog (FcRelish) was cloned from *F. chinensis*. The full length cDNA of FcRelish consists of 2,157 bp, including 1,512 bp open reading frame, encoding 504 amino acids. FcRelish had the highest expression levels in the hemocytes and lymphoid organ. Both *V. anguillarium* and *Micrococcus lysodeikticus* stimulation to shrimp can affect the transcription profile of FcRelish. Silencing of FcRelish through dsRNA interference can greatly change the transcription profile of AMP (Li et al. 2009b). Furthermore, another transcription factor, Dorsal homolog (FcDorsal), was cloned from *F. chinensis*. The full length cDNA of FcDorsal consists of 1627 bp, revealing a 1071 bp open reading frame encoding 357amino acids. Spatial expression profiles showed that FcDorsal had the highest expression level in the hemocytes and lymphoid organ (Oka). The expression profiles in the hemocytes and lymphoid organ were apparently modulated when shrimp were stimulated by bacteria or WSSV. Both Gram-positive (G+) bacteria (*M. lysodeikticus*) and Gram-negative (G−) bacteria (*V. anguillarium)* injection to shrimp

caused the up-regulation of FcDorsal at the transcription level. DsRNA approach was used to study the function of FcDorsal and the data showed that FcDorsal was related to the transcription of penaeidin 5 in shrimp (Li et al. 2010). Through comparison of the expression profiles between FcDorsal and FcRelish in shrimp responsive to WSSV challenge, we speculate that FcDorsal and FcRelish might play different roles in shrimp immunity.

***Antioxidant enzymes***

The main function of antioxidant enzymes is neutralizing free radicals. When the organism encounters pathogen infection or environmental stress, its NADPH-oxidase will activate, in turn enhancing the glycolytic reactions that will increase the consumption of oxygen and induce the production of a mass of reactive oxygen species (ROS) such as superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^-$). Though ROS can kill foreign invaders, the mass accumulation of these reactive molecules in animals will cause serious host cell damage. So the rapid elimination of these excessive ROS is essential for the proper functioning of cells and the survival of organisms. To protect against toxicity and eliminate ROS, organisms have evolved protective enzymatic systems including superoxide dismutase, catalase and many kinds of peroxidases. In *F. chinensis*, we identify several oxidant enzymes including mMnSOD (Zhang et al. 2007c), peroxiredoxin (Zhang et al. 2007d) and catalase (Zhang et al. 2008) that are closely related to the immune response of shrimp to pathogens.

***Chaperone proteins***

When studying the differential expressions of genes or proteins of shrimp after pathogen infection or stress through cDNA microarray or proteomic approach, we found that chaperone genes showed apparent variation at transcription or translation level. This indicates that chaperone proteins play a very important role in shrimp. We have cloned different chaperon protein genes including HSC70 (Jiao et al. 2004), HSP70 (Luan et al. 2010), HSP90 (Li et al. 2009a), glucose regulated protein 78 (GRP78) (Luan et al. 2009) and calreticulin (Luan et al. 2007). Their expression of different genes was analyzed. The data further indicated that chaperone proteins were essential for the immunity homeostasis in shrimp.

***Construction of genetic map***

A primary genetic linkage map is a prerequisite to detailed genetic studies in any organism. High-density linkage maps are necessary for efficient mapping of quantitative trait loci (QTLs) to complement marker-assisted selection (MAS) and for comparative genome mapping. Due to the worldwide importance of *L. vannamei* in

shrimp aquaculture, a genetic linkage map was constructed using amplified fragment length polymorphism (AFLP) and microsatellite markers. One hundred and eight select AFLP primer combinations and 30 polymorphic microsatellite markers produced 2071 markers that were polymorphic in either of the parents and segregated in the progeny. Of these segregating markers, 319 were mapped to 45 linkage groups of the female framework map covering a total of 4134.4 cM and 267 markers were assigned to 45 linkage groups of the male map covering a total of 3220.9 cM. A sex-linked microsatellite marker was mapped on the female map with 6.6 cM to sex and a LOD of 17.8. Two other microsatellite markers were also linked with both 8.6 cM to sex and LOD score of 14.3 and 16.4 (Zhang et al. 2007e). The genetic maps presented here will serve as a basis for the construction of a high-resolution genetic map, quantitative trait loci (QTLs) detection, marker-assisted selection (MAS) and comparative genome mapping.

### *Construction of BAC library and genome sequencing*

To facilitate gene cloning and characterization, genome analysis, physical mapping and molecular selective breeding of marine shrimp, we have developed the techniques to isolate high-quality megabase-sized DNA from hemocyte nuclear DNA of female shrimp and constructed a bacterial artificial chromosome (BAC) genomic library for the species. The library was constructed in the Hind III site of the vector pECBAC1 and it consists of 101760 clones arrayed in 265, 384-well microtiter plates; the average insert size is 101 kb and the genome is covered approximately fivefold. To characterize the library, 92160 clones were spotted onto high-density nylon filters for hybridization screening. A set of 18 pairs of overgo probes designed from eight cDNA sequences of *L. vannamei* genes were used in hybridization screening and 35 positive clones were identified (Zhang et al. 2010). These results suggest that the shrimp BAC libraries will provide a useful resource for screening of genomic regions of interest candidate genes, gene families or large-sized synthetic DNA regions. In addition, the libraries will promote future work on comparative genomics, physical mapping and large-scale genome sequencing in the species. Genetic information especially whole genome sequence is necessary to better understand and improve shrimp. However, the research on shrimp genome is still very limited; until Sept. 2010, there were only about 170000 EST sequences and a small quantity of genes of *L. vannamei* published in NCBI. Next-generation sequencing (NGS) technologies have been demonstrated as a feasible way to sequence large eukaryotic genomes with high-throughput and rapid, cost-effective de novo assembly and re-sequencing. Using NGS technologies, different insert-size pair-end and mate-pair libraries were constructed in *L. vannamei* and sequenced by Solexa. A total of 125-gigabase data were generated successfully with an average reads length 80 bp; another 390-megabase data were obtained by GS FLX

(454). With the Kmer frequency of Solexa data and C-value, we predicted that the genome size of *L. vannamei* was about 2.4-gigabase, suggesting that more than 51× coverage data of the genome size of *L. vannamei* has been obtained (Xiang , reported at PAG XIX, San Diego, USA 2011).

***Selective breeding***

Through seven generations of selective breeding in *L. vanamei*, a selected line called "KeHai No. 1" was obtained which showed an increase of 12-42% in growth rate when cultured at different densities. The selected line can achieve superior growth at higher culture density than unselected shrimp. During the selective breeding, microsatellite markers were used for pedigree identification which is a proven selective breeding strategy.

In summary, the discovery and research on a batch of immune related genes of shrimp provides a possible opportunity to develop disease control strategies and also provides a platform for marker selection for disease resistant shrimp. The construction of a genetic map, genome sequencing and the establishment of a selective line provide the basement for QTL mapping, marker assistant selective breeding and whole genome selective breeding. The improvement of shrimp is necessary to satisfy the requirements of the shrimp aquaculture industry.

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# An integrated approach to sustainable shrimp farming

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## Abstract

An estimated 3.4 million metric tons of farmed shrimp were produced globally in 2008 and crop value was estimated at more than US$14 billion. Despite the economic importance of farmed shrimp, the shrimp farming industry has been slow to adopt biosecurity and genetic improvement strategies which are prevalent in more mature meat-producing industries. However, this trend is changing rapidly. Historically, the giant tiger prawn, *Penaeus monodon* has been the most common shrimp species cultured in Asia. Now, most Asian shrimp farmers stock their ponds with non-indigenous Pacific white shrimp, *Litopenaeus vannamei*. There are a number of advantages in culturing *L. vannamei* over *P. monodon*, including the availability of healthy and domesticated stocks. Commercially available populations of specific pathogen free (SPF) *L. vannamei* exist in the Americas and Asia and these populations are free of such pathogens as white spot syndrome virus (WSSV) and Taura syndrome virus (TSV). Another significant advantage in culturing *L. vannamei* is the opportunity to benefit from selective breeding. Commercially available populations of this species have been bred for rapid growth and enhanced TSV resistance over multiple generations. Despite the benefits of culturing healthy and selectively bred *L. vannamei*, there are significant challenges. For example, the genetic potential of these shrimp cannot be fully realized if they are grown in environments where virulent pathogens exist. Farmers using selectively bred shrimp need to adopt cost-effective, biosecure strategies to mitigate the risk of pathogen introduction into their growout ponds. In addition, care must be taken by shrimp breeders to ensure that founder stocks come from genetically diverse populations in order to mitigate problems associated with inbreeding depression. The sustainability of the global shrimp farming industry will be predicated on the use of genetically diverse and selectively bred populations of SPF shrimp stocked in biosecure environments.

## Introduction

Historically, the giant tiger prawn, *P. monodon* has been the most common shrimp species cultured in Asia. However, about a decade ago, an increasing number of Asian farmers began stocking their ponds with non-indigenous Pacific white shrimp, *L. vannamei* and the dominance of *P. monodon* started to fade. In 2000, an estimated

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630984 metric tons (MT) of farmed *P. monodon* were produced globally, whereas only 145386 MT of farmed *L. vannamei* were produced during the same year (Fig. 1, FAO 2010). However, farmed *L. vannamei* production increased to 2259183 MT in 2008 and this represents a 1454% increase over eight years. During the same period, farmed production of *P. monodon* increased to 721867 MT representing only a 14% increase. This dramatic species shift occurred primarily in Asia where more *L. vannamei* are now produced than in the Western Hemisphere.

The historical dominance of *P. monodon* in Asia can be attributed to a number of factors including the availability of gravid female broodstock from local waters, rapid growth of juveniles in ponds and the ability of this species to grow to a relatively large size. However, over the past decade, the availability and quality of wild broodstock have declined and many Asian farmers have observed poor growth and survival of *P. monodon* in their ponds, resulting in decreased production and profitability. Poor pond performance was attributed, in part, to bacterial and viral pathogens which are ubiquitous in the major shrimp farming regions of Asia. Because of declining profit margins associated with culturing *P. monodon*, shrimp farmers in Asia began exploring other options including the culture of *L. vannamei*.

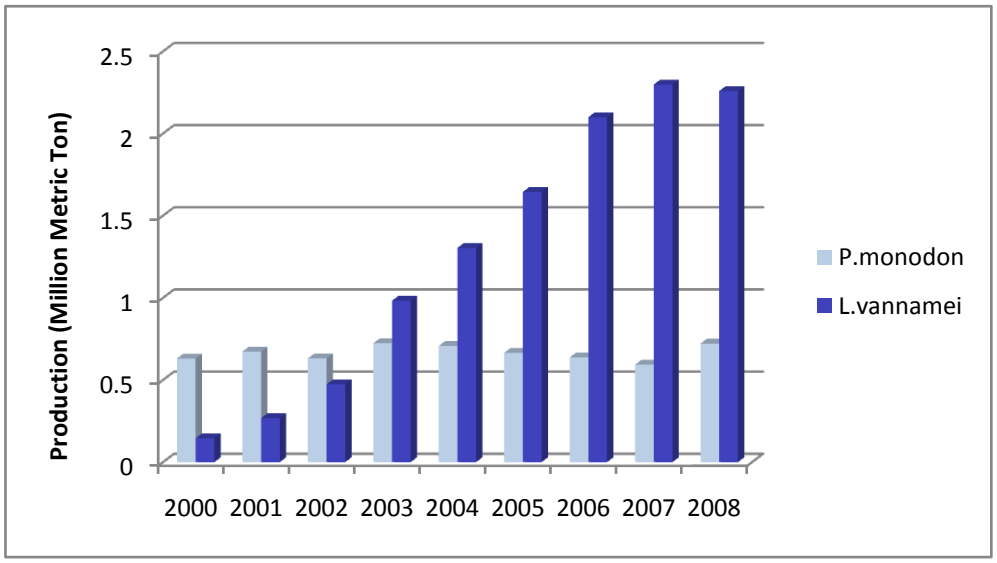


**Fig. 1**. Global production (metric tons) of farmed *P. monodon* and *L. vannamei* from 2000-2008.

### *Specific Pathogen Free Shrimp*

*Litopenaeus vannamei* is native to the Eastern Pacific Ocean, from Mexico to Northern Peru and has dominated shrimp farming in the Western Hemisphere for decades (Bailey-Brock and Moss 1992). This species initially was introduced into

Taiwan and China for commercial purposes in the late 1990s and its culture spread rapidly to Southeast Asia (Briggs et al. 2005). There are a number of advantages in culturing *L. vannamei* over *P. monodon*, including a lower dietary protein requirement, ease of captive reproduction and the commercial availability of healthy and domesticated stocks. This latter attribute is especially important in light of recent problems faced by *P. monodon* farmers.

Unlike many vannamei farmers, monodon farmers typically do not have access to an adequate and predictable supply of healthy and domesticated postlarvae to stock their ponds because postlarvae are either caught from the wild or produced from wild-caught broodstock. Wild shrimp often are infected with bacterial or viral pathogens which can be transmitted to non-infected shrimp. In contrast, commercially available populations of healthy and domesticated *L. vannamei* exist throughout the Americas and more recently in Asia, and these populations may be specific pathogen free (SPF). SPF shrimp are free of specific disease-causing agents (Lightner et al. 2009) and there are three essential criteria that need to be met for a pathogen to be considered for inclusion on an SPF list. These are: 1) the pathogen can be reliably diagnosed, 2) the pathogen can be physically excluded from a facility, and 3) the pathogen poses a significant threat to the industry. Although there is no internationally recognized SPF list used by the global shrimp farming industry, there are SPF *L. vannamei* suppliers who provide shrimp that are certifiably free of white spot syndrome virus (WSSV), yellow head virus (YHV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), Taura syndrome virus (TSV) and infectious myonecrosis virus (IMNV). These viral pathogens have cost the global shrimp farming industry billions of dollars in lost crops, jobs and export revenue over the past decade. The current working list of specific pathogens for SPF penaeid shrimp used by the U.S. Marine Shrimp Farming Program includes eight groups of virus, two prokaryotes and certain classes of parasitic protozoa (Table 1). It is important to note that this list is dynamic and will be revised and expanded as new pathogens are identified and more accurate disease diagnostic tools become available. The importance of using SPF shrimp cannot be understated as they offer clear advantages over diseased shrimp or shrimp with undetermined disease status (Lightner et al. 2009; Wyban 2009).

The first population of SPF *L. vannamei* was developed by the U.S. Marine Shrimp Farming Program in the early 1990s (Wyban et al. 1993; Lotz et al. 1995; Lightner et al. 2009). In general, to develop SPF stocks from the wild, shrimp are collected and transferred to a primary quarantine facility where they are analyzed for specifically listed pathogens using appropriate disease diagnostic tools (Fig. 2; Moss et al. 2001; Lightner et al. 2009). If shrimp test positive for any of the listed pathogens, they are destroyed in the primary quarantine facility. If shrimp test negative for specifically

**Table 1.** Recommended working list of specific pathogens for "SPF" penaeids in 2010-2011 (modified from Lightner et. al. 2009).

| Pathogen | Pathogen Type | Category |
|---|---|---|
| **VIRUSES** | | |
| TSV | Dicistrovirus (n.f.) | C-1* |
| WSSV | Nimavirus (n.f.) | C-1* |
| YHV. GAV. LOV | Ronivirus (n.f.) | C-1,2* |
| IHHNV | Parvovirus | C-2* |
| MBV. BP. BMN | Crustacean baculoviruses | C-2*** |
| IMNV | Totivirus | C-1,2* |
| HPV | Parvovirus | C-2 |
| PvNV | Nodavirus | C-3 |
| **PROCARYOTES** | | |
| NHP | Alpha proteobacteria | C-2* |
| RLB-MHD | MHD (rickettsial-like bacteria) | C-2** |
| **PROTOZOA** | | |
| Microsporidians | Microsporidia | C-2 |
| Haplosporidians | Haplosporidia | C-2 |
| Gregarines | Apicomplexa | C-3 |
| *OIE listed disease; **under study for OIE listing; ***formerly OIE listed | | |

listed pathogens after several successive screenings, they are transferred to a secondary quarantine facility where they are matured and spawned to produce an $F_1$ generation of captive shrimp. Representative shrimp from the $F_1$ generation are then analyzed for specifically listed pathogens and if they test negative after several successive screenings, they are transferred out of the secondary quarantine facility and can be included as part of the germplasm in a genetic nucleus (GN) used in a selective breeding program. Shrimp that are maintained in a highly biosecure GN (i.e. where there is a history of negative disease status documented through a surveillance program) may be designated as SPF (Lotz 1997). However, once shrimp leave such a facility, they no longer are referred to as SPF even though they may be free of specifically listed pathogens. The new designation is High Health (HH) shrimp and this indicates that

these shrimp are at greater risk of pathogen exposure and infection (Lotz 1997). If shrimp are transferred to a low-biosecurity shrimp farm, they have entered the Commodity Production (CP) stream which is most vulnerable to pathogen outbreaks and the shrimp are neither SPF nor HH. An important point is that the SPF designation refers to present pathogen status only and is a function of where the shrimp are maintained (i.e. the level of biosecurity and disease history of the facility).

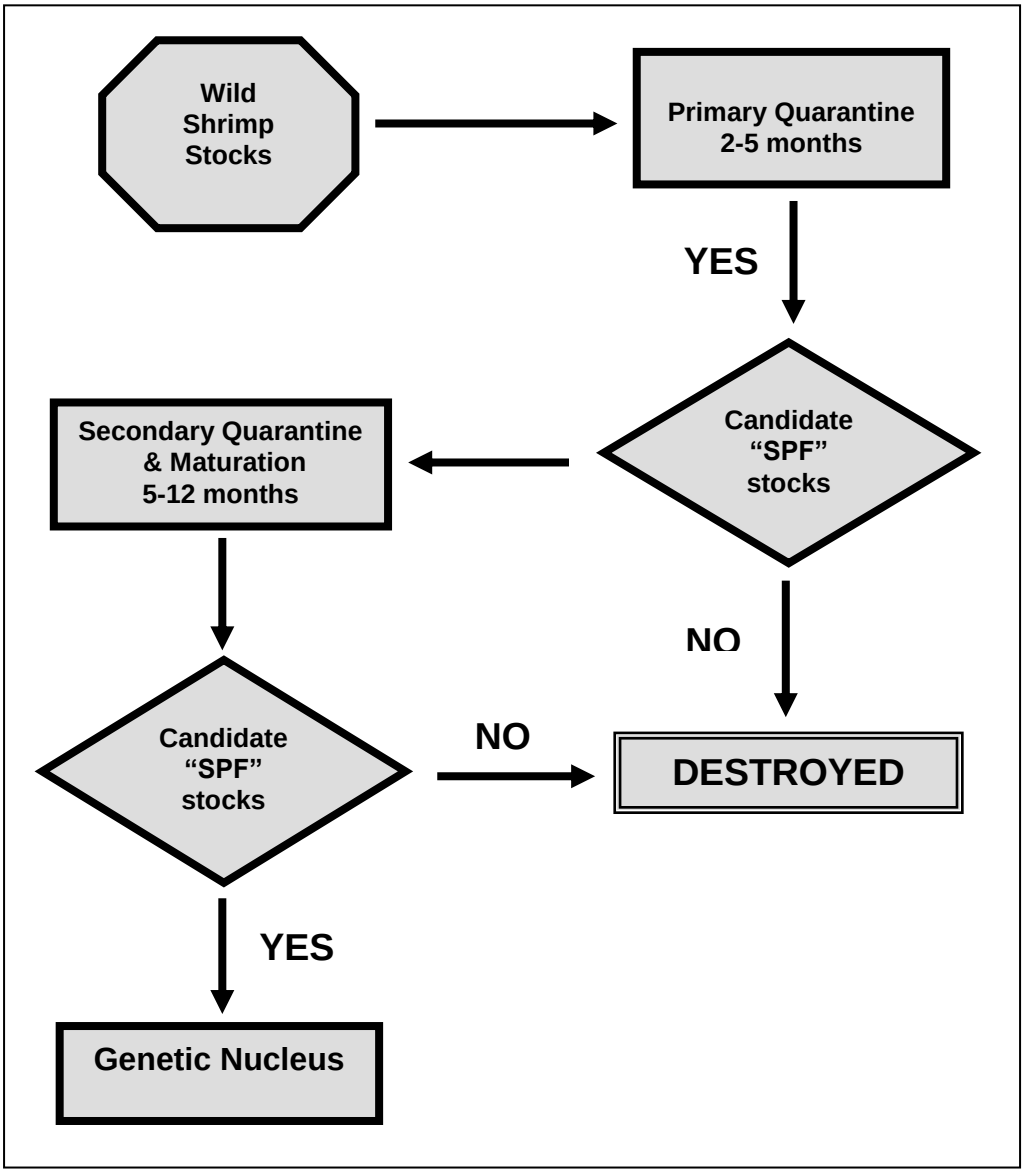


**Fig. 2**. Steps used to develop Specific Pathogen free (SPF) shrimp (see text for explanation; modified from Carr et al. 1994).

Although SPF shrimp are by definition, free of all specifically listed pathogens, SPF shrimp may not be disease free. They may, for example, be infected with a known pathogen which is not included on the SPF list of the shrimp supplier or they may be infected with an unknown pathogen that has not yet been described. Interestingly, although some bacteria of the genus *Vibrio* can cause significant disease problems and can be reliably diagnosed (two of the essential criteria that need to be met for a pathogen to be considered for inclusion on an SPF list), they cannot be included on an SPF list. This is because they cannot be physically excluded from a facility due to the fact that they are ubiquitous members of the shrimp's normal flora. Finally, SPF shrimp have no innate resistance to a particular pathogen nor are they innately susceptible. Disease resistance or susceptibility can be bred into a line of shrimp through selective breeding or other approaches but this has no bearing on SPF status. SPF status is not an indication of the shrimp's genotype nor is it a heritable trait.

***Selective Breeding***

Another significant advantage in culturing *L. vannamei* is the opportunity to benefit from selective breeding. There are a number of shrimp breeding programs that produce and distribute *L. vannamei* which have been selectively bred for rapid growth. Faster growth will either increase the number of crops per year, thereby increasing annual yield ($kg \cdot ha^{-1} yr^{-1}$) or increase the weight of shrimp at harvest resulting in higher prices per kg for the farmer. Selecting for faster growth also may improve other commercially important traits by indirect selection such as feed conversion efficiency (Goyard et al. 2002) and pond survival (Gitterle et al. 2005a).

Heritability estimates ($h^2$) for growth and size-related traits (i.e. weight, length, growth rate, etc.) have been reported in *L. vannamei* (Table 2; Carr et al. 1997; Argue et al. 2002; Pérez-Rostro and Ibarra 2003a, Gitterle et al. 2005a). In general, $h^2$ estimates for growth are considered moderate to high ($h^2 \geq 0.2$) and this trait has responded well to selection. For example, Argue et al. (2002) reported that selected *L. vannamei* were 21% and 23% larger at harvest than unselected control shrimp after one generation of selection when reared in a raceway and pond, respectively. More recently, Otoshi et al. (2009) reported that selectively bred *L. vannamei* stocked in a 75 $m^2$ raceway at a density of 408 $shrimp \cdot m^{-2}$ grew 1.88 $g \cdot wk^{-1}$ over a 74 days period. Such rapid growth at such a high stocking density was unachievable a decade ago but is now possible due to selective breeding for rapid growth over multiple generations.

**Table 2**. Heritability estimates ($h^2$ ± SE) for growth- and size-related traits in *L. vannamei*.

| Trait | $h^2$ ± SE | Reference |
|---|---|---|
| weight at ~ 11 g | 0.42 ± 0.15 | Carr et al. 1997 |
| weight at ~ 23 g | 0.84 ± 0.43 (raceway) | Argue et al. 2002 |
| | 1.19 ± 0.59 (pond) | |
| weight at 29 wk | 0.34 ± 0.18 | Pérez-Rostro and Ibarra (2003a) |
| total length at 29 wk | 0.28 ± 0.18 | Pérez-Rostro and Ibarra (2003a) |
| weight at ~ 20 g | 0.24 ± 0.05 (line 1) | Gitterle et al. 2005a |
| | 0.17 ± 0.04 (line 2) | |

Growth (and other commercially important traits) may be affected by the interaction between an organism's genotype and its environment (G×E interaction). If these interactions are significant, breeders may need to develop different lines of shrimp for each unique rearing environment. There is little published data on G×E interactions for growth in penaeid shrimp, although Gitterle et al. (2005a) reported a low genotype by test environment interaction for harvest weight in *L. vannamei* reared in ponds and tanks. Similarly, Pérez-Rostro and Ibarra (2003b) reported an insignificant G×E interaction for harvest size in *L. vannamei*. These data suggest that shrimp which grow well in one environment will also grow well in other environments. The lack of a significant G×E interaction precludes the need to develop multiple fast-growing shrimp lines for different rearing conditions. However, there may be a significant G×E interaction for growout survival and additional research is needed to explore this relationship.

In addition to selecting for growth, shrimp breeders have focused their efforts on developing families of *L. vannamei* with enhanced resistance to TSV and WSSV (Argue et al. 2002; Jiang et al. 2004; Gitterle et al. 2005b) because these two viruses have had a significant economic impact on the global shrimp farming industry. Selective breeding for TSV resistance began in the mid 1990s in response to a TSV epizootic in Ecuador and the subsequent spread of TSV throughout the Americas. This virus has now spread to and impacted major shrimp farming regions in Asia (Tu et al. 1999; Phalitakul et al. 2006). TSV can infect juvenile shrimp within 2 to 4 wk after stocking into nurseries or growout ponds and cumulative mortalities of unselected shrimp in TSV-infected ponds have been reported to be as high as 80–90% (Brock et al. 1997; Lightner et al. 1998).

Unlike growth, $h^2$ estimates for TSV resistance are considered low to moderate ($h^2 \leq 0.2$, Argue et al. 1999; Argue et al. 2002). However, despite low to moderate $h^2$,

significant improvements in TSV resistance have been made through selection. For example, Moss et al. (2011) reported selection responses of 10-20% per generation (expressed as the relative increase in survival when shrimp were exposed to TSV in a *per os* challenge) during the first several years of selection. There are now commercially available families of *L. vannamei* which exhibit >80% survival after TSV exposure and such a high level of survival was unimaginable when the initial TSV outbreak occurred in Ecuador in 1992.

The ability to improve TSV resistance by selection (despite low to moderate $h^2$) is attributed, in part, to high phenotypic/genotypic variation in TSV survival. This variation allows for a larger selection differential (and higher selection intensity) which increases the selection response. Argue et al. (2002) reported that TSV survival ranged from 15% to 94% among 80 *L. vannamei* families exposed to TSV in a *per os* laboratory-challenge test. Similarly, White et al. (2002) reported that TSV survival ranged from 0% to 100% among 176 families that were challenged. Although large variations in family survival after TSV exposure have been observed among populations of *L. vannamei*, the magnitude of this variation can decline as selection progresses. For example, whereas mean family survival increased from 44% to 84% after five generations of selection for TSV resistance among a population of *L. vannamei* families maintained at Oceanic Institute (OI, Waimanalo, Hawaii, USA), the coefficient of variation (CV) for TSV survival decreased from 43.3% and 13.6% (Fig. 3). This reduction in variability is expected as selection progresses and will result in progressively lower selection responses (Falconer and Mackay 1996).

In addition to developing families of shrimp with enhanced TSV resistance, shrimp breeders have explored the possibility of selecting shrimp for resistance to WSSV. Limited research suggests that $h^2$ estimates for WSSV resistance are lower than those reported for TSV. Published $h^2$ estimates for WSSV resistance in *L. vannamei* range from 0.00 to 0.21 and most estimates are <0.1 (Gitterle et al. 2005b, 2006a, 2006b). Thus, only small improvements in WSSV resistance have been made in *L. vannamei* through selection. For example, Gitterle et al. (2005b) reported a mean selection response of only 2.8% after one generation of selection for WSSV resistance in a population of *L. vannamei*. In contrast, Huang et al. (2010) recently reported producing families of *L. vannamei* with a mean survival of 22.7% to WSSV infection after three generations of selection. These researchers also reported that the relatively resistant families appeared to be able to inhibit WSSV replication in muscle tissue.

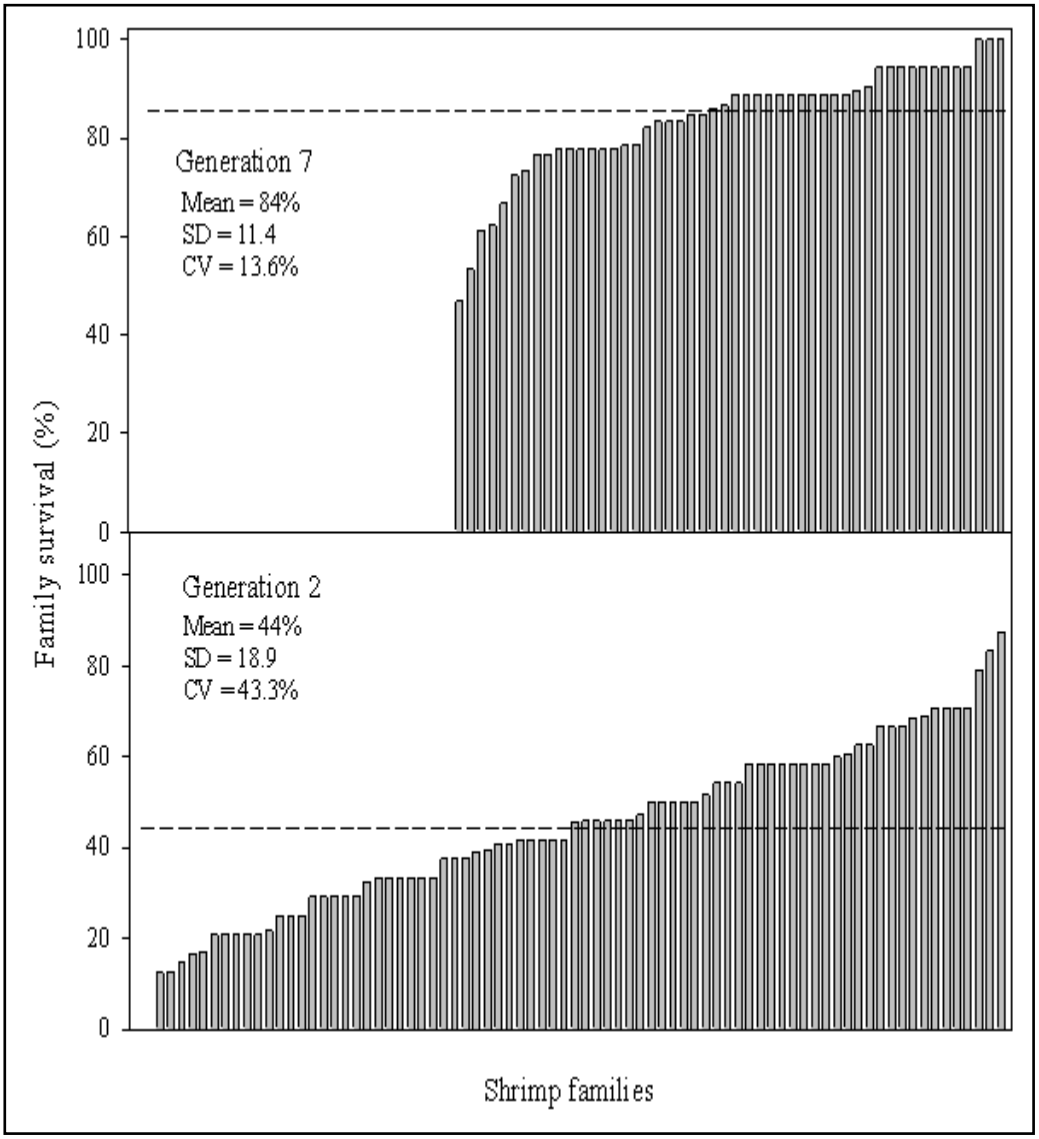


**Fig. 3.** Phenotypic variation in family survival for a population of shrimp at Oceanic Institute (Waimanalo, Hawaii, USA) after two and seven generations of selection for TSV resistance. Each bar represents a family mean, and the dashed line represents mean family survival for the entire population (mean of family means).

There are a number of other shrimp pathogens which negatively impact the global shrimp farming industry. However, these pathogens have received little or no attention from shrimp breeders to date. Breeding shrimp for disease resistance is a costly and lengthy process and is justified only if a pathogen has a significant economic impact on the industry and there are no cost-effective measures to prevent or treat infection. In addition, there must be sufficient additive genetic variation in resistance to the pathogen under selection (i.e. the trait must be heritable) for selection to work, and there must be reliable disease-challenge protocols developed if a family-based breeding

program is to be used to enhance disease resistance. Finally, it is important to note that each additional trait added to a selection program will result in slower progress (i.e. smaller selection response per generation) for all selected traits, even if the traits are positively correlated. This restriction puts an upper limit on the number of traits that can be reasonably selected for in a given shrimp line.

***Biofloc Technology***

Traditionally, penaeid shrimp have been cultured in coastal, earthen ponds where flow-through water exchange is used to maintain acceptable water quality (Hopkins et al. 1993). However, influent water can serve as a vector for virulent pathogens (Lotz 1997; Lotz and Lightner 2000) and pond effluent can adversely affect coastal water quality (Goldburg and Triplett 1997). Biofloc technology (BFT) offers an alternative approach to traditional shrimp farming methods. With BFT, water is remediated by an *in situ* microbial community that grows on suspended water-column particles and on biofilms attached to surfaces. This approach results in a dramatic reduction in water use which minimizes the opportunity for pathogen introduction into the shrimp culture environment. With this level of biosecurity, the genetic potential of selectively bred shrimp can be realized.

In addition to the biosecurity benefits, BFT-managed systems typically are stocked at super-intensive densities (>300 shrimp·$m^{-2}$) and these systems may be enclosed in greenhouse structures where optimal temperatures can be maintained. Super-intensive densities and temperature control allow for year-round production and requires a much smaller footprint. A BFT facility can be sited at inland locations away from sensitive coastal areas where multiple-use conflicts exist and at temperate latitudes closer to major markets, thereby reducing transport costs and the number of "food miles" (which is a measure of environmental impact).

Researchers at OI in Hawaii have conducted studies on BFT shrimp production systems since 1997 and numerous trials have been conducted in research-sized (33 $m^2$ - 75 $m^2$), greenhouse-covered raceways. Stocking densities have ranged from 100 to 700 shrimp·$m^{-2}$ and production of 8.9 kg·$m^{-2}$ was achieved in a 58.4 $m^2$ BFT system (Moss et al. 2005). Uncertainties remained regarding potential scale-up issues which may arise when transferring this technology from a research to a commercial-sized system. In an effort to address this concern, the first trial conducted at OI in a commercial-sized (337 $m^2$) system was completed in 2007 and the stocking density was increased to 828 shrimp·$m^{-2}$. Trial results included 18.3 g harvest weight, 67.9% survival, 1.5 g·$wk^{-1}$ growth rate and 2.1%·$day^{-1}$ water exchange rate. There were no significant scale-up issues encountered and production of 10.3 kg·$m^{-2}$ was achieved (Otoshi et al. 2007). Importantly, shrimp bred for rapid growth and high survival under conditions specific

to BFT (i.e. super-intensive densities, high water-column bacterial concentrations and high carbon dioxide and nitrite concentrations) were used in this trial, and these selectively bred shrimp likely played a significant role in achieving high production.

The two most recent growout trials using OI's selectively bred shrimp were conducted in a 75 $m^2$ BFT system stocked at densities of 363 and 300 shrimp·$m^{-2}$, respectively. For these trials, production was 5.8 and 5.3 kg·$m^{-2}$, survival was 90.3 and 87.1% and growth rate was 1.67 and 1.59 g·$wk^{-1}$, respectively. Water usage was 219 and 304 L·$kg^{-1}$ shrimp produced and water exchange was 0.3 and 0.6%·$day^{-1}$, respectively. Results from these trials demonstrate that the use of selectively bred shrimp and improved system design and management of the BFT system can result in excellent shrimp performance at high stocking densities.

Commercial production of shrimp requires consistency and predictability to adequately meet market demand. Results from the growout trials conducted at OI demonstrate that high shrimp production can be achieved consistently using BFT when stocked at densities ranging from 300 to 400 shrimp·$m^{-2}$. Furthermore, a trial conducted in 2007 demonstrated that stocking densities over 800 shrimp·$m^{-2}$ and production over 10 kg·$m^{-2}$ are possible in a commercial-scale system. Through continued research and development, a more comprehensive understanding of BFT system design and management has developed. Further improvements in production efficiencies (i.e. simplification of system design, improved management and continued selective breeding) likely will reduce production costs even further, thereby removing a significant barrier to the commercial viability of this alternative shrimp growout technology.

***An Integrated Approach***

The use of SPF, selectively bred *L. vannamei*, coupled with on-farm biosecurity protocols such as BFT, provides the best opportunity for increased production, profitability and sustainability for shrimp farmers. This strategy has been employed most pervasively in Thailand which ranks as one of the world's most important producers of farmed shrimp. In 2001, monodon farmers in Thailand began observing a phenomenon now referred to as monodon slow-growth syndrome (MSGS) which is characterized by abnormally slow growth rates and unusually high size variation at harvest. Although the etiology of MSGS remains unclear, its ramifications are unequivocal. MSGS in combination with other factors, rendered monodon farming in Thailand unprofitable and resulted in estimated financial losses of approximately US$310 million in 2002 (Chayaburakul et al. 2004). Since then, most Thai farmers have switched to culturing SPF, selectively bred *L. vannamei* and have enjoyed the

benefits of better production efficiencies and higher profits. Wyban (2009) recently reported that vannamei farmers in Thailand have profit margins two to three times greater than monodon farmers (Table 3) and that by 2006 *L. vannamei* represented over 98% of farmed shrimp entering the Thai shrimp auction. On a broader scale, current estimates suggest that *L. vannamei* represents 76% of farmed shrimp in Asia and this number is projected to increase.

**Table 3.** Production parameters and profits for a typical shrimp farm in Thailand culturing *P. monodon* and *L. vannamei*, respectively (modified from Wyban 2009).

| Parameter | *P. monodon* | *L. vannamei* | % Difference |
|---|---|---|---|
| Density ($PL \cdot m^{-2}$) | 45 | 160 | + 256 |
| Crop duration (days) | 125 | 112 | - 10 |
| Harvest size (g) | 25 | 23 | - 8 |
| Yield ($MT \cdot ha^{-1} crop^{-1}$) | 8 | 24 | + 200 |
| Crop value ($US\$ \cdot ha^{-1}$) | $45000 | $96000 | + 113 |
| Crop costs ($US\$ \cdot ha^{-1}$) | $32000 | $60000 | +87.5 |
| Production profit ($US\$ \cdot ha^{-1}$) | $13000 | $36000 | +177 |

Although the benefits associated with culturing SPF, selectively bred *L. vannamei* in Asia have been significant, the importation of this species has created some problems. As with the importation of any non-indigenous species, there are concerns about the introduction and spread of exotic pathogens. History is replete with examples of exotic introductions in aquaculture and the worldwide shipment of live and frozen shrimp has served as an important vector for the trans-global movement of virulent viral pathogens including the relatively recent introduction of IMNV from Brazil into Asia. Importing countries should continue to require foreign broodstock suppliers to provide documentation about the SPF status of their shrimp, although illegal smuggling of non-SPF *L. vannamei* likely will continue as well. In addition to ensuring the importation of healthy shrimp, care must be taken by importing countries to ensure that founder stocks come from genetically diverse populations of *L. vannamei*. If not, problems associated with inbreeding depression may arise, resulting in reduced production and profitability. The long-term success of the global shrimp farming industry will be predicated on the use of genetically diverse and selectively bred populations of SPF shrimp stocked into controlled and biosecure environments.

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