

Presence of Viral Pathogens amongst Wild *Penaeus monodon* in Thailand

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Abstract

An epizootiological study of 230 wild *Penaeus monodon* collected from a set net in the seashore of Klong Khone, Samut Songkhram, Thailand was conducted from December 2012 to November 2013. Each month approximately 20 wild *P. monodon* were tested for the following five pathogenic shrimp viruses: yellow head virus (YHV), white spot syndrome virus, infectious hypodermal and hematopoietic necrosis virus, taura syndrome virus, and infectious myonecrosis virus (IMNV). Among all *P. monodon* individuals, 45.7% were positive for YHV, which causes yellow head disease that is commonly encountered in Thai shrimp cultivation. Overall, pathogenic viruses were detected in 80.9% of all the shrimp; however, IMNV, which has yet to be identified in Thailand, was not detected in any shrimp. This finding suggests that there is a link between the prevalence of pathogenic viruses in wild shrimp and the outbreaks of viral diseases in farmed shrimp.

Discipline: Fisheries

Additional key words: IHHNV, infection, WSSV, YHV

Introduction

Disease has been a prominent issue throughout the history of intensive shrimp culture in Thailand. Farming of the giant tiger prawn (*Penaeus monodon*) became mainstream in Thailand in 1985 (Flegel et al. 1995, Flegel 1997). Yellow head disease (YHD) caused by the yellow head virus (YHV) was initially detected in *P. monodon* aquaculture in 1989 (Boonyaratpalin et al. 1993, Chantanachookin et al. 1993). As a measure to prevent the disease from spreading via culture water, many *P. monodon* farmers changed from open to closed culture systems in 1992 (Kasai et al. 2005). However, this change failed to prevent the spread of YHV and white spot syndrome virus (WSSV). Mass mortalities attributed to YHD were reported in 1995 and to white spot disease (WSD) in 1996 (Flegel 1997). Soon

afterwards, inland shrimp farming began to expand widely and rapidly (Flaherty et al. 2000). Retarded growth amongst *P. monodon* was reported in 2001-2002 (Chayaburakul et al. 2004). Despite a large-scale transition away from farming *P. monodon* to farming Pacific white shrimp (*Litopenaeus vannamei*) in the early 2000s (Wyban 2007), YHD continued to occur in *L. vannamei* ponds located in inland areas (Senapin et al. 2010). A newly emerging disease, acute hepatopancreatic necrosis disease (AHPND) that is caused by *Vibrio parahaemolyticus*, began to inhibit *L. vannamei* production in Thailand, resulting in great losses (Flegel 2012, Tran 2013).

Wild *P. monodon* from coastal areas should be used in juvenile production to protect against the introduction of invasive alien viruses from foreign countries, and to preserve genetic diversity. Monitoring wild shrimp for pathogenic

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viruses is important to prevent future disease outbreaks in shrimp farms. The only epizootiological studies regarding wild *P. monodon* are those conducted by de la Pena et al. (2007) in the Philippines and Withyachumnarnkul et al. (2006) in Thailand.

This study aims to provide information for supporting the sustainable aquaculture of *P. monodon* in Thailand, and suggests protective measures against viral outbreaks. Over the course of one year, we monitored wild shrimp on a monthly basis for YHV, WSSV, and infectious myonecrosis virus (IMNV), which are the most serious viral pathogens in terms of overall production losses in Southeast Asian countries (Flegel 2012). Moreover, we checked for infectious hypodermal and hematopoietic necrosis virus (IHHNV), which has spread widely across wild and farmed shrimp in other parts of the world, and taura syndrome virus (TSV), a pathogen that is transferred via shrimp importation to Southeast Asian countries.

Materials and methods

1. Shrimp samples

Approximately 20 wild *P. monodon* individuals were collected at each new moon from December 2012 to November 2013. All the shrimp were collected from a 2-m deep-set net that was placed in a 4-m deep mangrove channel near Klong Khone, Samut Songkhram in central Thailand (Fig. 1). Upon collection, each shrimp was individually wrapped to avoid contamination and transported to the laboratory on ice. Females were checked for maturity using an LED light. Every shrimp was measured for its wet weight and then stored at -80°C .

2. Detection of viruses

For RNA viruses (YHV, IMNV and TSV), total RNA was extracted from the gills (100 mg) using TRIzol (Invi-

trogen), and cDNA was synthesized from approximately 1 μg of total RNA using random primers and a RevertAid First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol. For DNA viruses (IHHNV and WSSV), DNA was obtained by homogenizing 10 mg of gill tissue using the DNeasy Blood & Tissue Kit (QIAGEN). cDNA and DNA were subjected to virus diagnostic polymerase chain reaction (PCR). The primer sets used included those previously described for YHV (De La Rosa-Vélez et al. 2006). 146F and 146R were recommended by OIE for the 1st step of WSSV and those designed from the first step PCR products for nested PCR, due to visible non-specific bands by existing nested primers. Primers for IHHNV, TSV, and IMNV were designed based on the universal regions of multiple alignments of capsid protein sequences on 7, 4, and 6 published GenBank sequences, respectively. PCR and nested PCR for the five viruses using the respective primer set (Table 1) were conducted on the wild *P. monodon*. PCR amplification was conducted in 20 μl using EmeraldAmp PCR Master Mix (TaKaRa) as per the manufacturer's instructions. The mixture was heated at 94°C for 3 min. and DNA was amplified by 30 cycles of $95^{\circ}\text{C}/30\text{ sec.}$, $55^{\circ}\text{C}/30\text{ sec.}$, and $72^{\circ}\text{C}/30\text{ sec.}$, followed by $72^{\circ}\text{C}/3\text{ min.}$ The first PCR reactions were diluted 1:100, followed by 25 cycles of nested PCR. Other conditions of PCR reaction were implemented as described in the 1st PCR. The PCR products were sequenced and the virus identities confirmed.

Results

Only healthy shrimp (total, $n=230$; $n=106$ females and $n=124$ males) were collected from the set net. No mature females were collected. The sizes of the shrimp collected varied. On average, the females (51.6 g; range, 14.5-117.6 g) were consistently larger than the males (40.0 g; range, 12.8-80.5 g) throughout the study (Fig. 2). The number of shrimp collected each month varied; in some months, less than 20 shrimps were collected (December, $n=14$; April, $n=18$; and August, $n=18$).

TSV and IMNV were not detected in any of the shrimp collected throughout the year (Fig. 3). YHV infections were identified in 50% of females and 42% of males, accounting for 46% of all the shrimp tested. WSSV was identified in 30% of females and 23% of males, accounting for 26% of all the shrimp. IHHNV was found in approximately 47% of the individuals tested, with females accounting for 53% and males 43%. The infection rates of YHV, WSSV, and IHHNV were slightly higher in females than in males. In the first PCR, shrimp that were positive for YHV or WSSV infection accounted for less than 20% of all the infected shrimp, while those that were positive for IHHNV exceeded 40% (Fig. 3). Smaller females (less than 30 g) and larger males (more than 60 g) were not infected



Fig. 1. Map of Thailand showing the location of the trap net.

Table 1. Primer sequences used for PCR detection of five viruses

	1st PCR 5'→3'	Products (bp)	nested PCR 5'→3'	Products (bp)
YHV	CGTATTGCATCGAACGTCCTG CAAGATCACTAATAACGCCTGATGC	885	CGGGGTTACCCGCTTATATT GCCTGAGGTGAAGTCCATGT	400
WSSV	ACTACTAACTTCAGCCTATCTAG TAATGCGGGTGTAATGTTCTTACGA	1447	GAGCACAATGAACGCTCAAA GAGATGGCTTCGAAAACCTCG	501
IHHNV	ATCAAGTGGACCAGACCCA TGGGAGTCGACTTCTAGGT	811	ACAAGAAGATACTTCGAATT TAGTTGATTATGTAGTCCAGT	333
IMNV	CGGTTACAGGGAATTGAAACACA AGTCCCATCATATAACTGGCCA	1262	TGGTTAATGCTCAAAAAGACATC TCAGGAATACCTAAGTTTGCA	1000
TSV	TAGCACAGCGACCATATATG AATTTCACTGGAGCTTTGGA	1063	TTGCAGATATTCCAGTGAGT ACCCAAGGATACATGACAAT	439

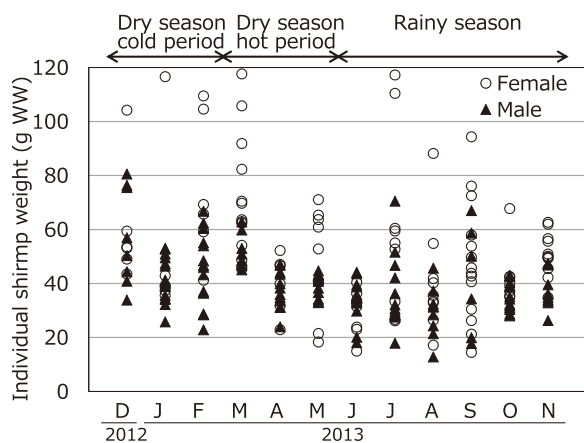


Fig. 2. Shrimp samples collected over a 12-month period at the Klong Khone site. WW: Wet weight.

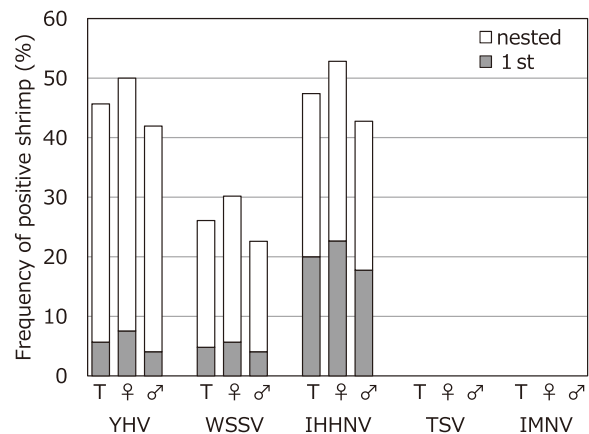


Fig. 3. Prevalence of YHV, WSSV, and IHHNV in captured shrimp by nested PCR.

T: prevalence in captured shrimp, ♀: female prevalence, ♂: male prevalence

First: Positive in the first PCR step. Nested: Positive only in the second PCR step.

with WSSV, whereas YHV and IHHNV infections were identified in all sizes of shrimp (Fig. 4). In both males and females, individuals weighing more than 70 g were infected with one virus or another (Table 2).

YHV infections were identified during every month except May. The infection rates exceeded 50% in April, July, August, and September (Fig. 5). WSSV infections were only identified in some months, with higher infections in May, October, and November (Fig. 5). IHHNV infections were identified throughout the year, and higher infection rates were confirmed during the months of August, September, December, and January (Fig. 5).

Viral infections were identified in 80% of the sample population. Of these individuals, approximately 47% exhibited single infections, 28% exhibited dual infections,

and 5% exhibited triple infections. Non- and single-infection rates were higher in males, whereas females revealed a higher percentage of dual or triple infections (Table 2). Figure 6 shows data regarding coinfections. In both males and females, coinfection with YHV and IHHNV was most frequent. Triple infection was not observed in individuals weighing more than 80 g (Fig. 7). Triple-infected individuals were prevalent from September to November (Fig. 8).

More individuals were uninfected in May and June (Fig. 8). Non-infected individuals were small in size and weighed less than 70 g (Table 2).

Discussion

YHV infections were found to be prominent in wild *P. monodon* captured in the Klong Khone coastal area. YHD caused by YHV is prevalent in shrimp farms throughout Thailand. IMNV was not detected at any time of the year during this study, and the prevalence of IMN has not oc-

curred in the area. Pathogenic viral infection rates of wild *P. monodon* were lower in the dry season. Shrimp aquaculturists say that outbreaks of diseases in shrimp farms during Thailand's dry season are lower than in the rainy season. These findings suggest a close link between the outbreak of viral diseases in farmed shrimp and pathogenic viral infections in wild shrimp. The pathogenic viruses WSSV

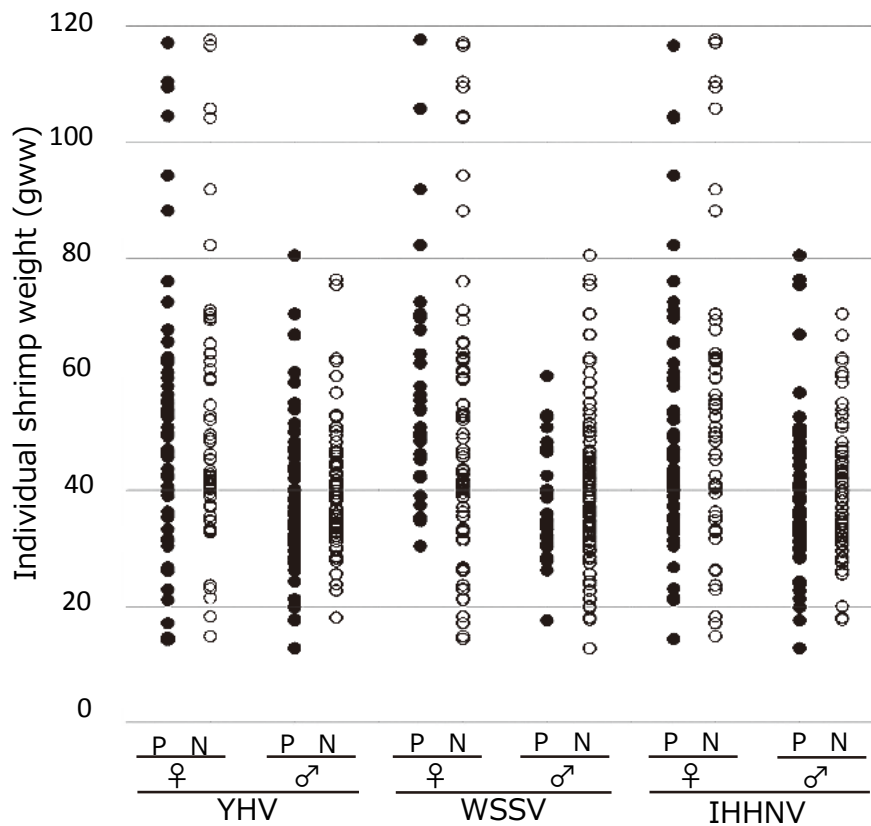


Fig. 4. Shrimp infection status of YHV, WSSV, and IHNV. The same individuals are plotted in the YHV, WSSV, and IHNV columns.
P: Positive, N: Negative

Table 2. Incidence of viral infection by shrimp weight

shrimp weight (g)	Female				Male			
	triple	dual	single	negative	triple	dual	single	negative
70-120	1 (6)	4 (25)	11 (69)	0 (0)	0 (0)	1 (25)	3 (75)	0(0)
50-70	3 (10)	11 (37)	11 (37)	5 (16)	0 (0)	3 (17)	12 (67)	3(16)
40-50	1 (4)	11 (42)	10 (39)	4 (15)	1 (3)	7 (21)	14 (41)	12(35)
30-40	3 (14)	5 (24)	9 (43)	4 (19)	2 (4)	13 (28)	22 (47)	10(21)
10-30	0 (0)	4 (31)	6 (38)	3 (31)	1 (5)	6 (29)	11 (52)	3(14)
Total	8 (8)	35 (33)	47 (44)	16 (15)	4 (3)	30 (24)	62 (50)	28 (23)

Number of infected shrimp (percentage for captured shrimp)

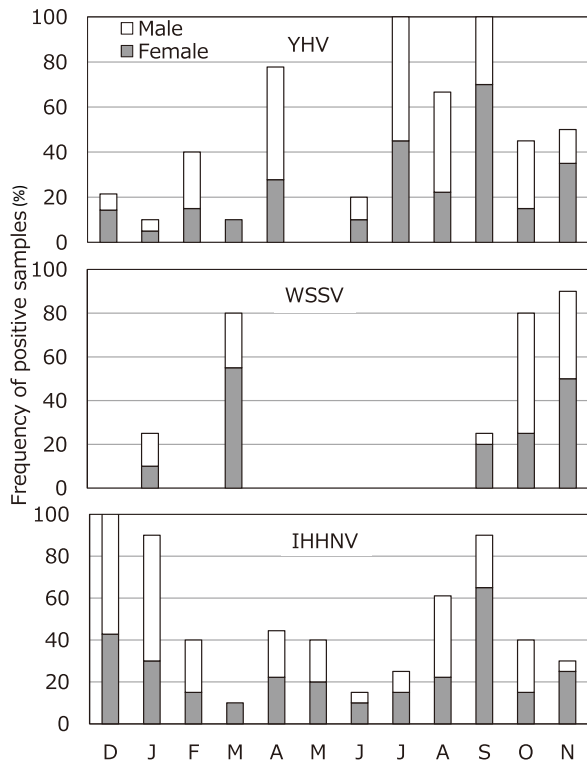


Fig. 5. Seasonal prevalence of YHV, WSSV, and IHNV in captured shrimp.

and IHNV, which seriously impact shrimp farming in Mexico, are reported to concur with other viruses detected in wild shrimp (Macias-Rodriguez et al. 2014). Gill-associated virus inflicted damage in Australian shrimp farms in 1996, and was found at high rates in broodstock captured in the wild from 1997 to 1998 (Cowley et al. 2000). In Japan, WSSV from imported juvenile shrimps has spread since 1993 and was detected in wild *M. japonicus* that were collected in Japanese coastal waters in 1996-1998 (Mushiake et al. 1998). The vertical infection of broodstock is assumed to result in transmission of the virus through offspring into shrimp farms and then back into the wild.

In this study, the incidence of IHNV infection was as high as that of YHV. More individuals had a large amount of IHNV (First PCR, positive) than any other infection (Fig. 3), though the sensitivity of detection for each virus was similar (data not shown). This observation suggests that IHNV has low virulence for *P. monodon*, resulting in their survival despite strong infection. The low impact of IHNV for *P. monodon* was reported by Withyachumnarnkul et al. (2006). In Northeast Mexico, 52% of *L. vannamei* broodstock were IHNV positive, as are most wild Western blue shrimp (*P. stylirostris*) in California bays (Mendoza-Cano et al. 2014). Strong infection has also been detected

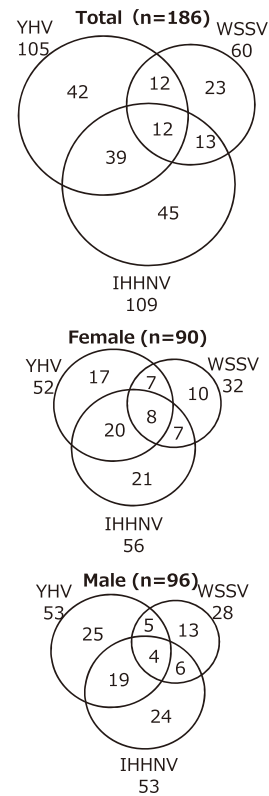


Fig. 6. Details of coinfection. Each number indicates the number of infected shrimp.

in wild *L. vannamei* in Ecuador (Motte et al. 2003). These reports suggest that IHNV infection has spread throughout the wild Penaeidae family. Although reported evidence of the spread of IHNV in wild *P. monodon* is scarce, our results show IHNV infection in wild *P. monodon*. The presence of IHNV in wild *P. monodon* should be noted when using them as broodstock because viruses easily mutate and acquire pathogenicity.

Dual and triple infections were identified in approximately 30% and 5% of all the shrimp tested, respectively. Shrimp that were first PCR positive for one virus or another included 25% of single-, 40% of dual-, and 50% of triple-infected shrimp (data not shown). The percentage of strong infection increased in line with a higher degree of coinfection. In a study by Flegel et al. (2004), 40 *P. monodon* individuals collected from each of the six cultivation ponds in Chumpol, Southern Thailand were tested for hepatopancreatic parvovirus (HPV), Monodon baculovirus (MBV), WSSV, and IHNV. Approximately 29% exhibited dual, 34% exhibited triple, and 10% exhibited quadruple infections. In 2001-2002, 32 shrimp farms in Thailand were tested, with half revealing dual infection of MBV and HPV or IHNV, and approximately 40% revealing triple infections of MBV, HPV, and IHNV (Chayaburakul et

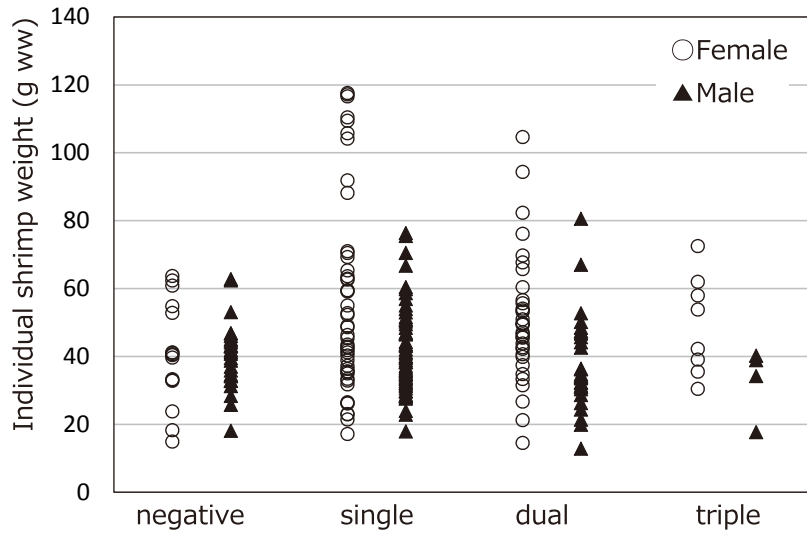


Fig. 7. Shrimp size associated with negative, single, dual, and triple infections.

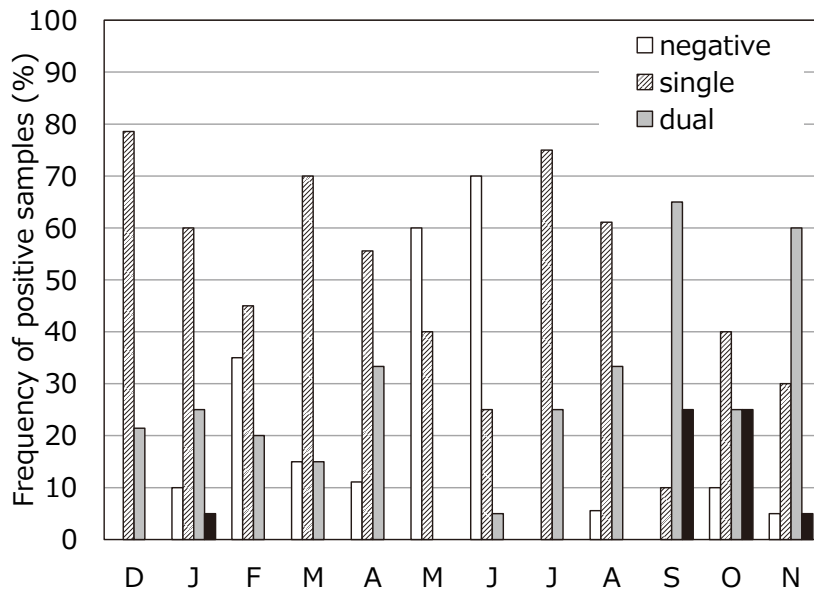


Fig. 8. Seasonal changes in the prevalence of coinfection.

al. 2004). The studies above were conducted in cultivation farms, and while MBV and HPV have not caused any devastation in Thailand in recent years, the reported infection rates would have been higher if these viruses had been included in this study.

From the standpoints of genetic diversity and the prevention of alien pathogens, wild shrimp captured in coastal areas where they are indigenous are ideal for use as broodstock. Inbreeding has had a negative impact on

Chinese white shrimp (*Fenneropenaeus chinensis*), and genetic diversity is reportedly necessary for successful reproduction (Luo et al. 2014). In this study, approximately 20% of wild *P. monodon* demonstrated no infection by any of the three viruses; this fraction increased during May and June. Furthermore, non-infected individuals weighed less than 70 g, regardless of sex. Thus, it is possible to select non-infected males and females from the wild. The SPF candidate population of wild shrimp should be reared

in primary quarantine until maturity. A commonly used technique in Southeast Asia is the artificial insemination of male spermatophores into female receptacles. It is possible to manually mate a selected, non-infected population to reproduce juveniles. As viral transmission through cultivation water is considered unlikely (Hamano et al. 2015), we believe that blocking vertical transmission will prevent the spread of viral diseases in shrimp cultivation ponds with a high degree of probability.

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