Vol. 69: 129–136, 2006 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published April 6

Low impact of infectious hypodermal and hematopoietic necrosis virus (IHHNV) on growth and reproductive performance of *Penaeus monodon*

Boonsirm Withyachumnarnkul[1, 2,*], Kanokporn Chayaburakul[3], Supak Lao-Aroon[4], Pornthep Plodpai[4], Kallaya Sritunyalucksana[2], Gary Nash[5]

[1]Department of Anatomy, and [2]Centex Shrimp, Chalerm Prakiat Building, 4th Floor, Faculty of Science, Mahidol University, Rama 6 Rd, Bangkok 10400, Thailand

[3]Anatomy Unit, Faculty of Science, Rangsit University, Muang-Ake, Pathumtani 12000, Thailand

[4]Shrimp Culture Research Center, Charoen Pokphand Foods Company (Public), Nakorn Srithamaraj 80170, Thailand

[5]National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathumtani 12000, Thailand

ABSTRACT: No controlled studies on the effect of infectous hypodermal and necrosis virus (IHHNV) on *Penaeus monodon* have been previously reported. Here we describe domesticated *P. monodon* that became positive for IHHNV and other viruses at variable levels of prevalence during cultivation in 16 open-air, earthen ponds. These were stocked with domesticated postlarvae (PL) that tested negative for 7 shrimp viruses including IHHNV at 6% prevalence in 3 checks using polymerase chain reaction (PCR) methods. These PL were derived from domesticated female broodstock that individually tested negative for the same viruses. At 4 mo of culture, the shrimp in some ponds without obvious mortality tested positive by PCR methods for IHHNV and 3 other viruses at variable levels of maximum estimated prevalence (MEP). Stained tissue sections showed no lesions typical of IHHNV, but *in situ* hybridization tests with an IHHNV-specific DNA probe were positive. There was no significant difference in mean body weight (i.e. ca. 25 g) between shrimp groups positive or negative for IHHNV. Similar results were obtained with IHHNV negative and positive adults at 1 yr. Adults that individually tested negative for all 7 viruses and some that tested lightly positive for IHHNV were bred for the next generation. There were no significant differences in the number of eggs (>600 000) and nauplii (ca. 300 000) produced by females negative and positive for IHHNV. From these females, 11/49 (22%) IHHNV PCR-positive PL batches were obtained from PCR-negative spawners, while 8/11 (73%) were obtained from IHHNV PCR-positive spawners. The results suggested that IHHNV infection can be transmitted vertically but does not seriously retard growth of *P. monodon* or affect fecundity of lightly infected broodstock.

KEY WORDS: *Penaeus monodon* · Domestication · Broodstock · IHHNV · PCR · Reproduction · Growth

INTRODUCTION

Infectious hypodermal and hematopoietic necrosis virus (IHHNV) is an icosahedral, non-enveloped DNA virus, 22 nm in diameter (Bonami et al. 1990) known to infect several penaeid shrimp, including *Penaeus monodon*, *P. vannamei* and *P. stylirostris*. The infection is usually fatal in *P. stylirostris*, and also causes deformity, and slow growth known as runt deformity syndrome (RDS) in *P. vannamei* (Bell & Lightner 1984, 1987, Brock & Lightner 1990). However, problems with IHHNV-infected *P. monodon* are rare with only 1 report claiming RDS (Primavera & Quinitio 2000). Under light microscopy (LM) and by *in situ* hybridization (ISH) with a specific DNA probe for IHHNV, a positive reaction was demonstrated in 20% of *P. monodon* juve-

*Email: boonsirm@yahoo.com

niles that had been found to be positive for IHHNV infection using a polymerase chain reaction (PCR) assay (Charyaburakul et al. 2005). The positive ISH reaction was found in connective tissue in several organs, including the hepatopancreas and muscle, and in functional cells of the antennal gland, gills, hematopoietic tissue, and fixed phagocytes of the heart. Viral particles of 22 nm, presumed to be IHHNV, were also demonstrated in the lymphoid organ of the IHHNV PCR-positive *P. monodon*. Despite extensive presence of the virus, *P. monodon* juveniles did not show any signs of RDS.

Because of the apparent lack of serious effects of IHHNV on *Penaeus monodon*, most research activity on shrimp viruses in Thailand has focused on yellow head virus (YHV) (Chantanachookin et al. 1993) and white spot syndrome virus (WSSV) (Wongteerasupaya et al. 1995), which have caused serious production losses. In an effort to solve these problems, a small program was started in 1996 to develop domesticated stocks of *P. monodon* that were free of YHV and WSSV (Withyachumnarnkul et al. 1998, 2001). These stocks have now been propagated in captivity for 8 generations, but because of budgetary limitations the rearing of the stocks from postlarvae (PL) to adults has been carried out in outdoor ponds where there has been a continued risk of horizontal disease transfer. This activity has provided the opportunity to examine successive shrimp generations in greater detail than was previously possible, particularly with respect to disease issues.

This report summarizes observations on domesticated *Penaeus monodon* that became infected with IHHNV and other viruses during rearing in outdoor, earthen ponds in Thailand. The period of study covered generations F5 to F6, and included stages from PL to sexually mature adults and their subsequent larval offspring. Special attention was paid to the effects of IHHNV on growth rate and reproductive function.

MATERIALS AND METHODS

Penaeus monodon PL at the PL15 stage were stocked in 16 earthen ponds (0.2 ha) at an average density of 30 ind. m^{-2}. They were generated from a total of 68 F4 and F5 broodstocks that individually tested negative for WSSV, YHV, IHHNV, gill-associated virus (GAV), hepatopancreatic parvovirus (HPV), monodon baculovirus (MBV), and Taura syndrome virus (TSV), using PCR assays or reverse transcriptase PCR (RT-PCR) assays as appropriate. The PL were also monitored at the nauplius, PL5 and PL10 stages to determine freedom from these viruses at 6% prevalence (i.e. samples of 50 from populations over 50 000; Cameron 2002). Although the standard terminology for epidemiology texts (Cameron 2002) refers to 'freedom from disease' at various levels of prevalence, we have elected to refer to the results of our tests as an 'undetected agent' (UN) at various levels of prevalence. The shrimp were cultured in a close cultivation system designed to prevent the entry of pathogen carriers and to allow water quality monitoring and management (Withyachumnarnkul et al. 2001). The ponds were aerated to ensure dissolved oxygen (DO) of at least 4 ppm at 06:00 h. The shrimp were fed with commercial pellets.

After 4 mo of culture, the shrimp were gently harvested by pond drainage and the top 40%, based on size, were restocked in 16 newly prepared ponds at a density of 10 ind. m^{-2}. At 8 mo (i.e. 4 mo later), the shrimp were harvested again in the same manner and the top 40% were restocked in 16 new ponds, but at 2 to 5 ind. m^{-2} for further cultivation. Average body weight (BW), average daily growth (ADG), and survival rate were determined using approximately 100 shrimp from each pond at 4 mo, 8 mo and 1 yr. After 1 yr the shrimp were shifted to a hatchery for breeding.

At 4 mo, shrimp samples were taken in various numbers from the ca. 17 000 harvested from each pond and tested individually for WSSV, IHHNV, MBV, and HPV by PCR, and for YHV, GAV and TSV by RT-PCR. For MBV and HPV assays, DNA templates were derived from extracts of hepatopancreatic tissue, but templates for other PCR and RT-PCR reactions were derived from the last pair of swimming legs. To compare growth rate between normal and IHHNV-infected *Penaeus monodon* having different severities of infection, 100 shrimp were randomly sampled from the harvested shrimp from 1 pond where only IHHNV was detected (Pond E2). Shrimp that tested negative for IHHNV, as well as all 6 other viruses, were designated 'normal' shrimp. Shrimp infected with IHHNV alone were further subdivided into lightly infected, moderately infected, and severely infected groups according to the criteria outlined in the instruction manual accompanying the IQ2000™ kit (Farming Intelligene Technology). They were individually weighed.

The PCR tests for WSSV and IHHNV, and the RT-PCR tests for YHV, GAV and TSV were carried out according to the instructions accompanying commercial IQ2000™ kits. The IQ2000™ kit for YHV-GAV is designed to distinguish between 2 viral types in the yellow head virus complex referred to as YHV and GAV (Walker et al. 2001). However, the GAV-specific band obtained with the IQ2000™ kit in Thailand has been previously reported to be derived from a type of YHV that differs from, but is closely related to, GAV (Soowanayan et al. 2003). Despite this difference we

refer here to the GAV-like virus as GAV. PCR methods for MBV and HPV were carried out according to a protocol described previously (Chayaburakul et al. 2004a). Determination of infection prevalence was based on Cameron (2002).

Additionally, at 4 mo, 10 normal (see criteria above) shrimp and 10 shrimp positive for IHHNV by PCR at moderate to severe levels of infection were selected from the harvested shrimp. They were then injected with Davidson's fixative at several sites in the cephalothorax before immersion in Davidson's fixative and processed for tissue sectioning as previously described (Bell & Lightner 1988, Pantoja & Lightner 1999). Sections of 4 and 5 µm were used for hematoxylin and eosin (H&E) staining, and for ISH, respectively. The 5 µm sections were placed on positively charged microscope slides (Fisher Scientific) and deparaffinized by heating for 30 to 45 min at 65°C, followed by ISH using the standard protocol recommended in the IHHNV-*In Situ* ShrimProbe™ kit (DiagXotics). Each slide was examined using normal bright field LM for cells displaying a dark blue to dark purple precipitate, indicating hybridization to homologous IHHNV DNA.

When the shrimp reached 1 yr of age, 71 individuals were sampled by trapping from a population of approximately 4000 and placed in a hatchery for breeding. Their BW and orbital lengths were recorded. Endopodites and exopodites of the fifth swimming legs were then individually cut and subjected to viral assays by PCR and RT-PCR as described above. PCR for MBV and HPV was carried out 1 mo later using hepatopancreatic tissue after the hatchery phase was completed. Broodstock were unilaterally eyestalk-ablated and allowed to spawn, and total numbers of eggs and nauplii produced by individual spawners were recorded during 1 mo in the hatchery. Any brooders testing positive for any virus except light IHHNV were killed together with their larvae, and excluded from the data set. The shrimp may have spawned only once or more than once. Altogether, 60 female broodstock, either free of all the tested viruses (49) or lightly positive for IHHNV (11), were analyzed for reproductive performance including the average numbers of eggs and nauplii produced. Nauplii produced from individual broodstock specimens were reared in separate tanks until they reached PL Stage 5, at which time they were again tested for IHHNV infection status by PCR. Except for batches of nauplii included in the IHHNV study, any batches that became positive for any of the other screened viruses were destroyed.

After 1 mo (i.e. at the end of the hatchery phase), the broodstock were sacrificed and lymphoid organs, ovaries, heart and hepatopancreas were removed for use in viral detection assays by PCR, RT-PCR, and histology by H&E staining and ISH to confirm their infection status.

All histograms in the figures are expressed as means ± SD bars. Statistical comparisons were made using the Kruskal-Wallis 1-way analysis of variance (ANOVA) on ranks. A value of $p < 0.05$ was considered to indicate a significant difference.

RESULTS

At the end of 4 mo cultivation, the domesticated shrimp stocked at 30 ind. m^{-2} showed a mean survival of approximately 70%. Only 2 out of 16 ponds gave negative results for all 7 viruses tested and only 5 gave negative results for IHHNV. The remaining ponds showed variable prevalences of infection by IHHNV and other viruses (Table 1). No positive test results were obtained for GAV, YHV, or WSSV. When the whole shrimp-population (16 × 17 000) is taken into account, a negative result with a sample size of 457 would indicate that these 3 viruses were not present at 1% prevalence with 95% confidence (Cameron 2002).

At 4 mo, the mean BW (±SE) for all 16 ponds was 28 ± 2 g, and ADG was 0.24 ± 0.08 g d^{-1} (Table 1). The BW at 8 mo and 1 yr were 66 g (ADG, 0.26 g d^{-1}) and 92 g (ADG, 0.25 g d^{-1}), respectively, with survival for each of the three 4 mo cultivation periods being more than 70%. There was no significant difference ($p > 0.05$) in mean BW between ponds where no IHHNV was detected (29 ± 11 g for n = 5) and in ponds where only IHHNV was detected (29 ± 4 g for n = 3) — or any IHHNV was detected (28 ± 8 g for n = 11). Because the sample sizes for some of the ponds were small and therefore only relatively high levels of IHHNV prevalence would be detected, we carried out another comparison excluding Pond B4 (where only 5 shrimp were sampled) and divided the remaining ponds into 2 groups: 1 group (6 ponds) with low IHHNV prevalence (undetected at 26% or maximum estimated prevalence, MEP, at 11% or less), and another group (9 ponds) with high IHHNV prevalence (MEP 51 to 100%). The mean weights (±SE) were 32 ± 5 g and 26 ± 2 g and the difference was not significant ($p = 0.27$), although the power of the test was low, which suggests caution in the interpretation of these results. None of the IHHNV-positive shrimp had abnormal body shapes, bent rostrums, or narrow 6th abdominal segments as reported in IHHNV-infected *Penaeus vannamei* (Lightner 1996). Since the ponds were stocked with different shrimp families, the pond-to-pond variation could have arisen from differences in genetic traits in addition to differences in disease status and pond environments.

Table 1. *Penaeus monodon*. Maximum estimated prevalence (MEP) of viral infections, body weight (BW) and average daily growth (ADG) on a pond-by-pond basis for shrimp reared in individual earthen ponds for 4 mo after stocking at a density of 30 ind. m^{-2} (except for Pond E6). The number of shrimp (n) randomly sampled from each pond containing approximately 17 000 individuals varied from 10 to 80 (Pond B4 only 5) so that prevalences marked as undetected (UN) for a virus mean UN at 4% prevalence (80 specimens), 26% (10 specimens) and 46% (5 specimens) with 95% confidence. MEP was calculated based on each pond's sample size (Cameron 2002). IHHNV: infectious hypodermal and hematopoietic necrosis virus; HPV: hepatopancreatic parvovirus; MBV: monodon baculovirus; TSV: Taura syndrome virus. ND: not done

Pond	n	MEP for various viruses				BW (g)	ADG
		IHHNV	HPV	MBV	TSV	(means ± SD)	(g d^{-1})
D13	10	UN	UN	UN	UN	46 ± 2	0.39
D14	10	UN	UN	UN	UN	33 ± 6	0.27
B4	5	UN	81	UN	UN	30 ± 6	0.25
E6[a]	14	UN	73	93	UN	20 ± 0	0.17
D9	10	UN	96	60	UN	18 ± 3	0.15
E2	77	51	UN	UN	UN	26 ± 9	0.22
B7	37	100	UN	UN	UN	28 ± 7	0.24
E7	10	84	UN	UN	UN	33 ± 7	0.27
D10	10	60	UN	UN	39	39 ± 10	0.33
D8	35	100	UN	UN	16	29 ± 9	0.25
E1	10	100	100	91	UN	27 ± 4	0.23
E3	40	11	11	49	11	44 ± 8	0.39
B8	14	89	89	UN	ND	19 ± 5	0.16
B3	83	7	10	UN	ND	30 ± 4	0.21
D12	30	100	14	UN	ND	17 ± 5	0.14
B5	62	61	64	4	ND	18 ± 4	0.15
Total or mean	457	69 ± 35[b]	60 ± 38[b]	52 ± 38[b]	22 ± 15[b]	28 ± 2[c]	0.24 ± 0.08
Ponds infected	–	11/16	9/16	5/16	3/12	–	–
% infected	–	69	56	31	25	–	–

[a]Stocking density was 154 ind. m^{-2}
[b]Mean of infected ponds ± SD
[c]Mean ± SE

Using PCR and RT-PCR methods as appropriate: HPV (9 ponds or 56%), MBV (5 ponds or 31%) and TSV (3 ponds or 19%) were also detected in the 4 mo old shrimp (Table 1), but the proportion of ponds affected was lower than that for IHHNV (11 ponds or 79%). Only 4 ponds were positive for a single virus (3 with IHHNV and 1 with HPV), while most HPV and all MBV occurred in combination with each other or with IHHNV. Seven ponds were infected with 2 viruses, 2 with 3 viruses, and 1 pond (E3) with 4. In summary, 88% of the ponds were infected with at least 1 virus and about 63% with 2 or more. Surprisingly, 1 of the triple infection ponds (E1) had very high prevalences for all 3 viruses (IHHNV, HPV, and MBV) but a mean body weight (27 g) was far from the lowest (17 g) and close to the overall mean (28 g).

When the BW of the individual shrimp from Pond E2 that were positive for only IHHNV (at light, moderate, and severe levels of infection) was compared to that of the shrimp negative for all viruses tested, there was no statistically significant difference among the 4 groups (Fig. 1). Since this was a comparison based on individual shrimp infection status, it was different from the pond prevalence comparisons that were done based on the data in Table 1. However, the trends were similar, showing no differences associated with IHHNV test status.

Using LM with an H&E stain, tissues of IHHNV PCR-positive shrimp showed no Cowdry Type-A inclusions normally observed in IHHNV-infected *Penaeus vannamei* and *P. stylirostris* (Bell & Lightner et al. 1984, 1987). Nor was any necrosis or inflammation observed. Using ISH, only 2 out of 10 IHHNV PCR-positive *P. monodon* gave positive reactions. Tissues that were positive included the antennal gland, gills, hematopoietic tissue, subcuticular epithelium, the hepatopancreas, muscle, hemocytes, and lymphoid organs. In the antennal gland the positive reaction was found in the tubule epithelium (Fig. 2a). Some positive hemocytes and connective tissue cells in the hemal sinus between antennal tubules were also observed. In the gills and hematopoietic tissue positive reactions were located in the functional cells (Fig. 2b,c), whereas positive reactions in the muscle were localized more in the connective tissue between muscle fibers (Fig. 2d). No positive ISH reactions were seen in IHHNV negative shrimp.

Similar to the shrimp at 4 mo, those at 1 yr showed the presence of IHHNV, HPV, and MBV but at about half the MEP level (Table 2). In addition, GAV (none at 4 mo) and TSV (relatively low prevalence at 4 mo) were also found at high prevalence. Three of the viruses (IHHNV, GAV, and TSV) were found in both the ovary and lymphoid organ. IHHNV was also detected at high prevalence in the heart. Prevalence of HPV remained higher than MBV, as at 4 mo. The shrimp were infected by single, double, triple, or quadruple viruses. One shrimp out of 71 showed a positive reaction for YHV without signs and symptoms of yellow head disease. It is possible that the shrimp were tolerant to the virus, or that the YHV detected was a non-virulent type (Wijegoonawardane et al. 2004). Sequencing of the PCR product would be necessary to answer this question.

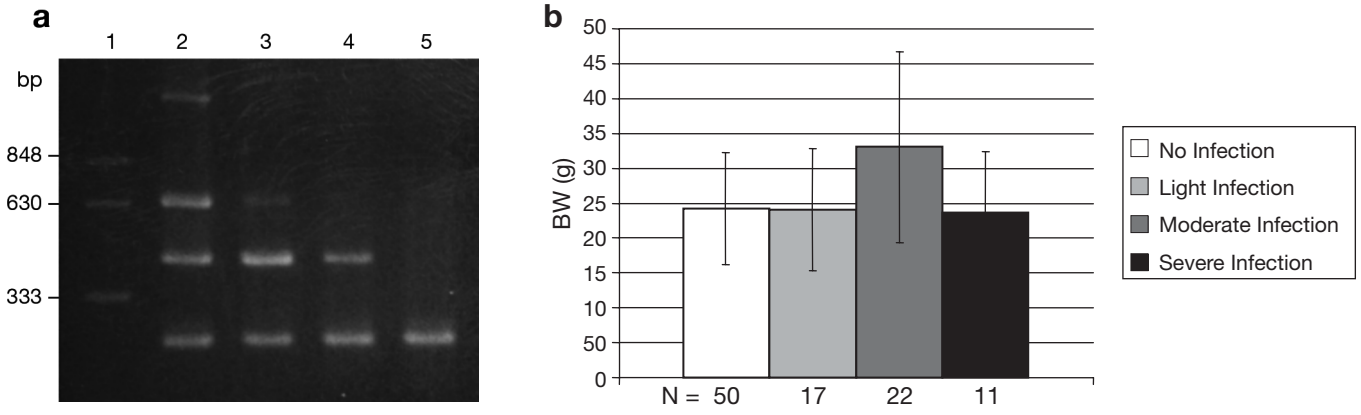

Fig. 1. *Penaeus monodon.* Relationship between severity of IHHNV infection and size. (a) Bands of PCR products in agarose gel electrophoresis of IHHNV-infected tissues with different severities of infection. (b) Body weights (BW) of shrimp randomly sampled at 4 mo in culture from a 0.2 ha earthen pond. No significant differences were observed, regardless of the IHHNV infection status or infection severity; error bars: ±SD

The mean BW of shrimp infected by IHHNV alone (86.7 ± 8.4 g, n = 8) was not significantly different from that of virus-free shrimp (92 ± 10.7 g, n = 26). Most of the shrimp had orbital lengths between 20 and 22 cm, and were very similar in size (coefficient of variation or CV <10%) (Fig. 3). After spawning, the numbers of eggs (600 000) and nauplii (300 000) per spawner were also comparable. From 11 female broodstocks lightly infected with IHHNV: 8 (73%) resulted in PL5 batches that were IHHNV PCR-positive, while 49 females that were PCR-negative for IHHNV resulted in 11 (23%) PL5 batches that tested positive.

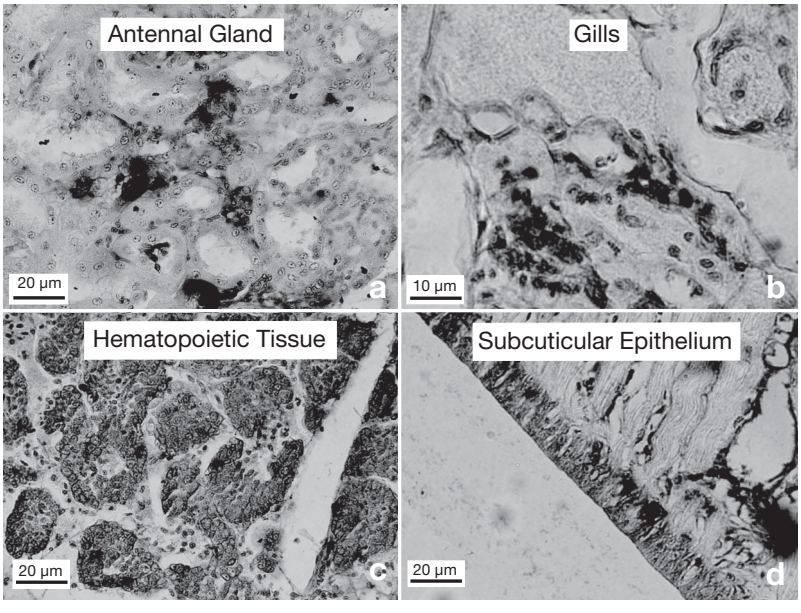

Fig. 2. *Penaeus monodon.* Photomicrograph showing *in situ* hybridization-positive reactions specific for IHHNV in the (a) antennal gland, (b) gills, (c) hematopoietic tissues and (d) subcuticular epithelium of IHHNV-infected shrimp at 4 mo in earthen pond culture

When broodstock tissues were examined by LM, the H&E stained sections appeared normal except for spheroids in the lymphoid organ and mild bacterial infections in the hepatopancreas of some specimens (not shown). In the ovaries, several resorptive oocytes were observed, and this feature was found in both IHHNV-infected and non-infected individuals to about the same degree. Using ISH, IHHNV-positive reactions were observed in tissues similar to those observed in the 4 mo old juvenile shrimp, but with less intensity and covering less area (Fig. 4). In the lymphoid organ (Fig. 4b), some of the positive staining was observed in the nucleoplasm, and some in the interstitial space. Gills and ovaries showed light positive reactions (Fig. 4a,c) and, in the ovary, this was occasionally observed in the nucleus of immature oocytes (Fig. 4d). Some positive reactions were observed in the cytoplasm of resorptive oocytes, and some in the ovarian capsule. The external epidermis and epidermis of the stomach and hindgut, nerve cords, nerve ganglia, connective tissue, striated muscle and heart muscle showed no reactions. None of the tissues from PCR-negative broodstock showed positive reactions (not shown).

DISCUSSION

In this study, the BW of IHHNV-infected *Penaeus monodon* at 4 mo in culture showed no significant difference from the non-infected shrimp (Fig. 1). Since these shrimp were sampled before selection for onward culti-

Table 2. *Penaeus monodon.* Results for PCR or RT-PCR detection of viruses in female broodstock specimens at 1 yr of age. The total population was approximately 4000 and the number of shrimp sampled was 71. Thus, failure to detect any virus would indicate absence at the level of 5% prevalence with 95% confidence. As in Table 1, maximum estimated prevalence (MEP) was calculated based on Cameron (2002). GAV: gill-associated virus. Other virus names as in Table 1

Type of infection	n	%	Virus					
			IHHNV	HPV	MBV	TSV	GAV	YHV
Free	26	36.6						
Single	7	9.9					+	
	6	8.5		+				
	8	11.3	+					
	3	4.2			+			
	2	2.8				+		
Dual	1	1.4	+		+			
	1	1.4	+			+		
	3	4.2				+	+	
	1	1.4		+		+		
	3	4.2	+				+	
Triple	3	4.2	+			+	+	
	2	2.8	+	+			+	
	1	1.4	+	+		+		
	1	1.4			+	+	+	
	1	1.4	+		+		+	
Quadruple	1	1.4	+	+		+	+	
	1	1.4		+		+	+	+
Total	71	100	21	12	6	15	22	1
% infected	–	–	29.6	16.9	8.5	21.1	31.0	1.4
MEP	–	–	39	25	15	30	41	6

vation, they represented an original unselected population. Since the standard deviations for BW were high and the power of the statistical test for the high and low IHHNV prevalence groups was low, it is possible that significant differences might be detected if the number of shrimp and ponds sampled could be increased. On the other hand, a comparison of individual shrimp with different severities of IHHNV infection (Fig. 1) and a comparison of IHHNV-positive and IHHNV-negative adults at 1 yr also showed no significant differences in size. Altogether, the limited data from this study suggests, at least, that IHHNV does not have a great effect on the growth rate of the *P. monodon*. This was previously suggested by Flegel et al. (2004).

The high MEP of IHHNV, HPV, MBV and TSV infections detected in the shrimp at 4 mo could have resulted either from vertical transmission of pathogens undetected in the broodstock and PL, or horizontal transmission which occurred after the ponds were stocked. However, the broodstock that generated the PL tested negative for these viruses, as did the PL in 3 tests at different times before stocking. This would suggest that horizontal transmission from the environment was the most likely source of the viruses. On the other hand, the PL tests targeted 6% prevalence and it is conceivable that the ponds became infected via the PL. The apparent decrease in MEP for IHHNV, HPV and MBV infections at 1 yr, compared to that at 4 mo, may have resulted either from shrimp mortality or from culling, since sub-samples of the largest individuals were selected at each of the 3 pond changes undertaken during the course of the study. Our data and samples were not sufficient to distinguish between these possibilities.

In contrast to IHHNV, HPV and MBV, the MEP for TSV remained at about the same level as at 4 mo, while GAV and YHV (previously undetected) became detectable. The MEP for GAV, in particular, was surprisingly high. Although YHV has been reported to cause high mortality (Chantanachookin et al. 1993) and TSV moderate mortality and/or morbidity in *Penaeus monodon* (Ruangsri et al. 2004), no unusual mortality was observed in our ponds. The absence of GAV and YHV at 4 mo and their appearance at 1 yr could have resulted from sampling errors, from horizontal transmission during the interval, or from tissue tropism. For the latter, for example, each tissue sample at 4 mo comprised the last swimming leg, while at 1 yr, they comprised the lymphoid organs and ovarian tissues. It is known that cryptic infections of GAV and YHV often give positive ISH and

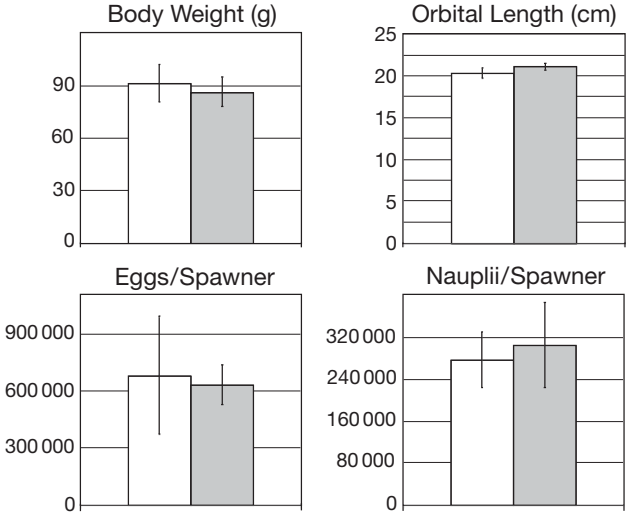

Fig. 3. *Penaeus monodon.* Body weights, orbital lengths, and the production of eggs and nauplii per female spawner of domesticated broodstocks that were virus-free (open bar, n = 49), and IHHNV-infected (gray bar, n = 11). No significant differences were found; error bars: ±SD

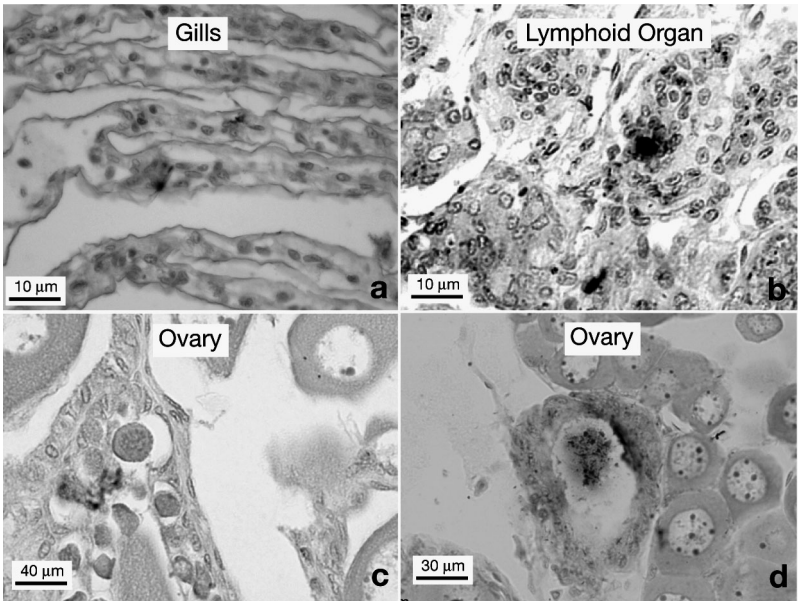

Fig. 4. *Penaeus monodon.* Photomicrograph showing *in situ* hybridization-positive reactions specific for IHHNV in the (a) gills, (b) lymphoid organ and (c, d) ovary of IHHNV-infected broodstock

immunohistochemical reactions only in limited tissues such as the lymphoid organ (Soowannayan et al. 2003, Spann et al. 2003, Longyant et al. 2005). Since no disease outbreaks occurred in the ponds, it is likely that carrier states of GAV and YHV would not be detected from swimming legs.

In past commercial hatchery practice in Thailand, it has generally been recommended that broodstock moderately or severely infected with IHHNV be discarded. In this study, we found that light IHHNV infections in *Penaeus monodon* broodstock did not decrease egg and nauplius production or negatively affect the growth of their offspring. The fact that some positive ISH reactions were localized in oocytes suggested that severe IHHNV infections might possibly cause a decrease in shrimp fecundity, but no evidence for this was found. In any case, the presence of IHHNV in the ovary suggests that IHHNV can be vertically transmitted and this was supported by the fact that most PL batches from IHHNV-positive broodstock harbored the virus. The results agree with those for IHHNV infection in *P. vannamei* where vertical transmission has also been proposed (Motte et al. 2003). A relatively low level of ISH-positive reaction in the tissue of the broodstock, compared to that of the 4 mo shrimp, is most likely due to the selection of the light positive PCR shrimp for the study. The fact that more than 20% of PL batches derived from the IHHNV PCR-negative *P. monodon* females were PCR-positive for IHHNV was probably due to false negative PCR results from very lightly infected female brooders or due to the presence of IHHNV in the sperm of the males with which they mated. This phenomenon has previously been reported for transmission of YHV in *P. monodon* (Cowley et al. 2002).

The lack of an IHHNV effect on *Penaeus monodon* fecundity is similar to the situation wtih *P. stylirostris* where IHHNV infection did not affect broodstock fecundity in terms of daily percent maturation, daily percent copulation and mean numbers of eggs and naulpii produced per spawn (Aguirre-Hinojosa et al. 2000). By contrast, IHHNV infection did cause low survival in *P. stylirostris* PL and juveniles but not with *P. monodon*. The possibility that the IHHNV we detected differed from that which causes RDS in *P. vannamei* is unlikely. A previous study of IHHNV isolates from Thailand revealed very little difference in DNA sequences from region to region (98 to 100% identity) (Charyaburakul et al. 2004b). Similar findings have been reported for IHHNV from Hawaii, the Americas, Southeast Asia, and Africa (Tang & Lightner 2002, Tang et al. 2003).

Although the focus of this manuscript was on IHHNV, it was striking to find that many of the domesticated shrimp carried dual to multiple infections after a long period of cultivation in outdoor ponds. These results are very similar to those previously reported for *Penaeus monodon* from normal outdoor production ponds in Thailand (Chayaburakul et al. 2004a, Flegel et al. 2004). Thus, it appears that the possibility of horizontal viral transfer during long-term outdoor cultivation is high and that domesticated broodstock for a breeding program would best be kept in indoor facilities if their viral-free status is to be maintained.

Acknowledgements. This study was funded by a Mahidol University Grant provided to Centex Shrimp, Faculty of Science, Mahidol University during the interval 2003 to 2004 and by a Golden Jubilee Grant from the Thailand Research Fund No. PHD/0086/2543.

LITERATURE CITED

Aguirre-Hinojosa E, Garza-Aguirre M del C, Castrejon-Lopez RJ, Murguia-Lopez LMCA (2000) Aquacultural potential of the blueshrimp *Penaeus stylirostris* from the Upper Gulf of California, infected with the IHHNV. In: Resumenes VII Congreso de la Asociacion de Investigadores del Mar de Cortes y I Simposium Internacional sobre el Mar de Cortes, lr-097

Bell TA, Lightner DV (1984) IHHN virus: infectivity and pathogenicity studies in *Penaeus stylirostr*is and *Penaeus vannamei*. Aquaculture 38:185–194

Bell TA, Lightner DV (1987) IHHN disease of *Penaeus stylirostris*: effects of shrimp size on disease expression. J Fish Dis 10:165–170

Bell TA, Lightner DV (1988) A handbook of normal penaeid shrimp histology. World Aquaculture Society, Baton Rouge, LA

Bonami JR, Trumper B, Mari J, Brehelin M, Lightner DV (1990) Purification and characterization of the infectious hypodermal and haematopoietic necrosis virus of penaeid shrimps. J Gen Virol 71:2657–2664

Brock JR, Lightner DV (1990) Diseases of Crustacea. Diseases caused by microorganisms. In: Kinne O (ed) Diseases of marine animals, Vol III. Biologische Anstalt Helgoland, Hamburg, p 245–349

Cameron A (2002) Survey toolbox for aquatic animal diseases; a practical manual and software package. Australian Centre for International Agricultural Research, Canberra

Chantanachookin C, Boonyaratanapalin S, Kasornchandra J, Direkbusarakom S, Ekpanithanpong U, Supamataya K, Siurairatana S, Flegel TW (1993) Histology and ultrastructure reveal a new granulosis-like virus in *Penaeus monodon* affected by 'yellow-head' disease. Dis Aquat Org 17:145–157

Chayaburakul K, Nash G, Pratanpipat P, Sriurairatana S, Withyachumnarnkul B (2004a) Multiple pathogens found in growth-retarded black tiger shrimp *Penaeus monodon* cultivated in Thailand. Dis Aquat Org 60:89–96

Chayaburakul K, Sriurairatana S, Flegel TW, Tang KFJ, Poulos BT, Lightner DV, Withyachumnarnkul B (2004b) Variation in infectious hypodermal and hematopoietic necrosis virus isolates from different areas in Thailand. In: The Abstracts of the 7th Asian Fisheries Forum 04, the Triennial Meeting of the Asian Fisheries Society, Penang, Malaysia, 30 November – 4 December, 2004, p 212

Chayaburakul K, Lightner DV, Sriurairattana S, Tang KF, Withyachumnarnkul B (2005) Different responses to infectious hypodermal and hematopoietic necrosis virus (IHHNV) in *Penaeus monodon* and *Penaeus vannamei*. Dis Aquat Org 67:191–200

Cowley JA, Hall MR, Cadogan LC, Spann KM, Walker PJ (2002) Vertical transmission of gill-associated virus (GAV) in the black tiger prawn *Penaeus monodon*. Dis Aquat Org 50:95–104

Flegel TW, Nielsen L, Thamavit V, Kongtim, Pasharawipas T (2004) Presence of multiple viruses in non-diseased, cultivated shrimp at harvest. Aquaculture 240:55–68

Lightner DV (1996) A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp. The World Aquaculture Society, Baton Rouge, LA

Longyant S, Sithigorngul P, Chaivisuthangkura P, Rukpratanporn S, Sithigorngul W, Menasveta P (2005) Differences in susceptibility of palaemonid shrimp species to yellow head virus (YHV) infection. Dis Aquat Org 64:5–12

Motte E, Yugcha E, Luzardo J, Castro F and 12 others (2003) Prevention of IHHNV vertical transmission in the white shrimp *Litopenaeus vannamei*. Aquaculture 219:57–70

Pantoja CR, Lightner DV (1999) Prevalence and geographic distribution of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in wild blue shrimp *Penaeus stylirostris* from the Gulf of California, Mexico. J Aquat Anim Health 11:23–34

Primavera JH, Quinitio ET (2000) Runt-deformity syndrome in cultured giant tiger prawn *Penaeus monodon*. J Crustac Biol 20:796–802

Ruangsri J, Kiriratnikom S, Sukrakanchana N, Arunrat S, Sukkasame N, Klowkliang T, Kasornchandra J, Supamattaya K (2004) Prevalence of Taura Syndrome virus (TSV) and infectious hypodermal and haematopoietic necrosis virus (IHHNV) in *Penaeus vannamei* and other aquatic species native to Thailand. In: Abstracts of the 7th Asian Fisheries Forum 04, the Triennial Meeting of the Asian Fisheries Society, Penang, Malaysia, 30 November – 4 December, 2004, p 211

Soowannayan C, Flegel TW, Sithigorngul P, Slater J and 6 others (2003) Detection and differentiation of yellow head complex viruses using monoclonal antibodies. Dis Aquat Org 57:193–200

Spann KM, McCulloch RJ, Cowley JA, East IJ, Walker PJ (2003) Detection of gill-associated virus (GAV) by *in situ* hybridization during acute and chronic infections of *Penaeus monodon* and *P. esculentus* shrimp. Dis Aquat Org 56:1–10

Tang KF, Lightner DV (2002) Low sequence variation among isolates of infectious hypodermal and hematopoietic necrosis virus (IHHNV) originating from Hawaii and the Americas. Dis Aquat Org 10:93–97

Tang KF, Poulos BT, Wang J, Redman RM, Shih HH, Lightner DV (2003) geographic variations among infectious hypodermal and hematopoietic necrosis virus (IHHNV) isolates and characteristics of their infection. Dis Aquat Org 53: 91–99

Walker PJ, Cowley JA, Spann KM, Hodgson RAJ, Hall MR, Withyachumanarnkul B (2001) Yellow head complex viruses: transmission cycles and topographical distribution in the Asia-Pacific region. In: Browdy CL, Jory DE (eds) The new wave. Proceedings of the special session on sustainable shrimp culture, Aquaculture 2001. World Aquaculture Society, Baton Rouge, LA, p 292–302

Wijegoonawardane P, Cowley JA, Kiatpathomchai W, Nielsen L, Walker PJ (2004) Phylogenetic analysis and evidence of genetic recombination among six genotypes of yellow head complex viruses from *Penaeus monodon*. In: Abstracts of the 7th Asian Fisheries Forum 04, the Triennial Meeting of the Asian Fisheries Society, Penang, Malaysia, 30 November – 4 December, 2004, p 210

Withyachumnarnkul B, Boonsaeng V, Flegel TW, Panyim S, Wongteerasupaya C (1998) Domestication and selective breeding of *Penaeus monodon* in Thailand. In: Flegel TW (ed) Advances in shrimp biotechnology. Proceedings of the Special Session on Shrimp Biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok, p 73–80

Withyachumnarnkul B, Plodphai P, Nash G, Fegan D (2001) Growth rate and reproductive performance of F4 domesticated *Penaeus monodon* broodstock. In: Proceedings of the 3rd National Symposium of Marine Shrimp. National Center for Genetic Engineering and Biotechnology, Bangkok, p 33–40

Wongteerasupaya C, Vickers JE, Sriurairatana S, Nash GL and 6 others (1995) A non-occluded, systemic baculovirus that occurs in the cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. Dis Aquat Org 21:69–77

Editorial responsibility: Timothy Flegel, Bangkok, Thailand

Submitted: May 26, 2005; Accepted: November 7, 2005
Proofs received from author(s): March 7, 2006