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

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Abstract

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

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

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

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

Introduction

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

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

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

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

Materials and Methods

Sources of Penaeus vannamei *and Gross Observations*

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

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

Table 1. Investigations carried out during the study period.

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

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

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

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

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

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

Water Quality

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

Haemolymph Clotting Time

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

Bacteriology

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

Histopathology

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

Molecular Biology

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

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

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

Statistical Analysis

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

Results

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

Shrimp Samples from Perak State

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

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

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

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

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

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

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

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

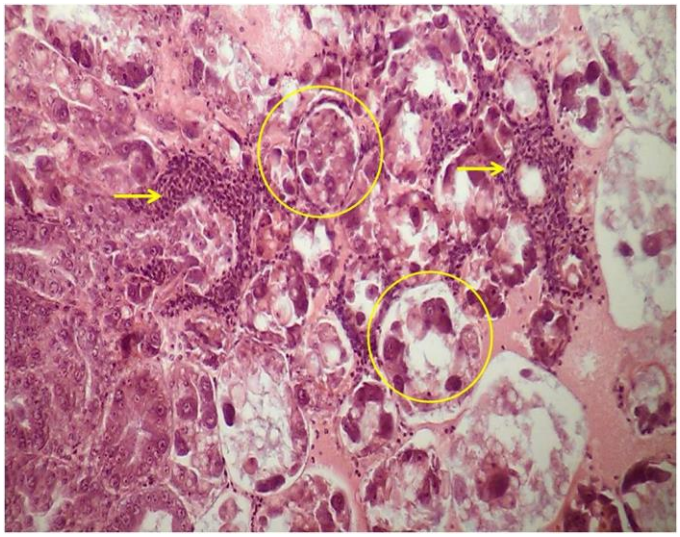


Fig. 2. Proximal hepatopancreas with no B, F or R cells, sloughing (in circle) and necrosis of hepatopancreatic cells with some haemocyte-encapsulated necrotic tubules (arrows).

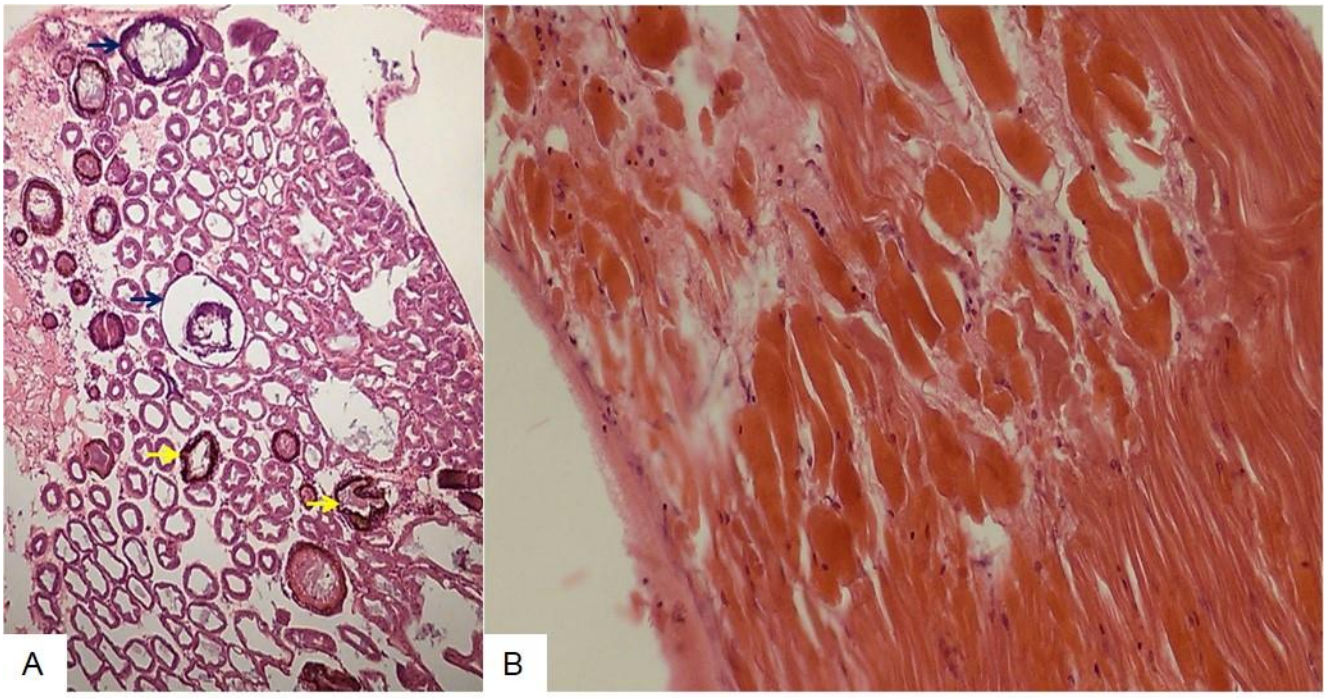
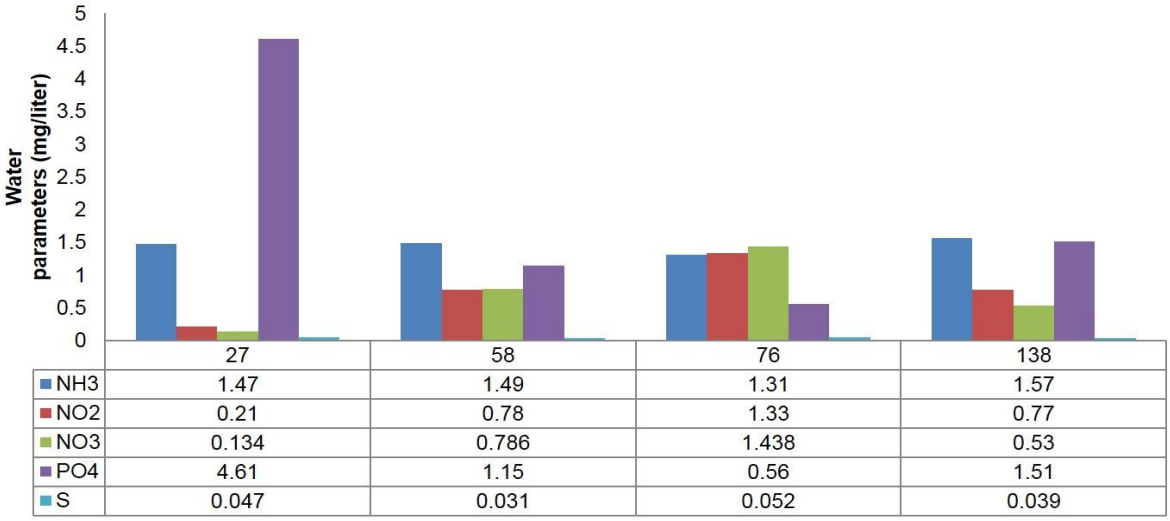


Fig. 3. (A). Hepatopancreatic cells with multifocal septic (black arrow) and melanized hepatopancreatic tubules with haemocyte encapsulation (yellow arrows) and (B). A focal acute necrosis with no obvious agent associated with the lesions in muscle.

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

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

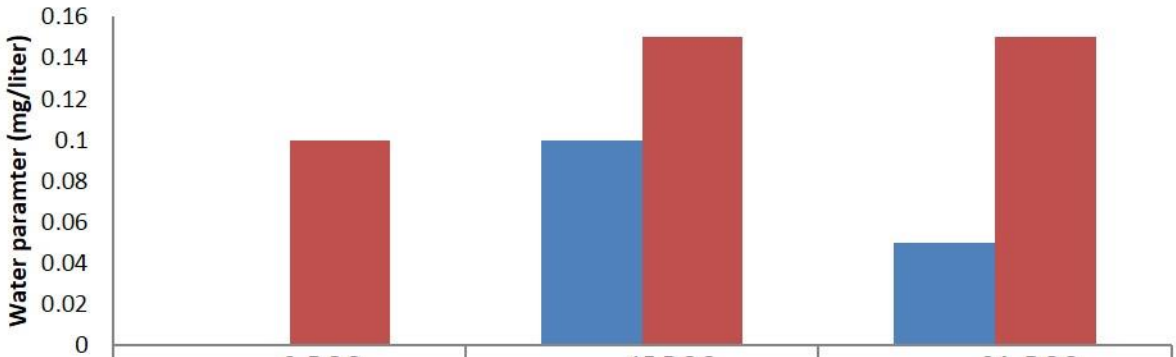


	27	58	76	138
NH3	1.47	1.49	1.31	1.57
NO2	0.21	0.78	1.33	0.77
NO3	0.134	0.786	1.438	0.53
PO4	4.61	1.15	0.56	1.51
S	0.047	0.031	0.052	0.039

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

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

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



	8 DOC	15 DOC	24 DOC
NH3	0	0.1	0.05
NO2	0.1	0.15	0.15

Fig. 5. Chemical parameters of water quality in a pond cultivating *Penaeus vannamei* in Perak.

Shrimp Sampling in Pahang and Penang States

Samples from Pahang and Penang were processed similar to samples from Perak. All shrimp samples (100 %) showed whitish patches in the abdominal segments, and 80 % had pale hepatopancreas, soft body and empty gut. All samples came from ponds with a history of mortality at 50 DOC except those from the hatchery at Balik Pulau, Penang. Haemolymph clotting time in 50 and 60 % of the shrimp from Pahang and Penang, respectively, exceeded 1.5 min, showing that they were under stress (Table 3). No farm tested positive for IHHNV infection. Pahang samples showed bacterial infection (*Vibrio* spp. and *Photobacterium damsela*). Samples from Pahang and Penang showed similar pathology in the hepatopancreas typical of developing EMS. However, in samples from Pahang, haemocytic infiltration and necrosis in the abdominal muscles were observed. This pathology is similar to lesions observed in IMNV or *Penaeus vannamei* nodavirus (PvNv). However, tests by IQ Plus for IMNV and PvNvin tissues obtained from live samples were negative (Table 4).

Discussion

The haemolymph clotting time test indicated 85, 50 and 64 % of the sampled shrimp were under stress in Perak, Pahang and Penang, respectively. Such stress could be triggered by disease occurrence, drastic changes in water quality parameters, poor diet or improper management. All of these factors, especially poor water quality, were present in all the investigated ponds. Stress due to consistent exposure to a high ammonium level during culture could be a contributing factor. Salinity, pH and dissolved oxygen (DO) levels were within acceptable ranges for shrimp culture, except for ammonium, nitrite and phosphate (Cheng et al. 2003). Throughout the cross-sectional study, the mean of ammonium concentrations ranged from 1.2–2.0 ppm, levels that were higher than the optimal range for cultured shrimp. Chien (1992) reported that ammonia is toxic to shrimp at high concentration, and Kasnir et al. (2014) highlighted that most shrimp can tolerate ammonia at a concentration of <0.1 $mg.L^{-1}$ NH_3–N. Data from one farm showed that at 27 DOC, rearing water for smaller shrimp has lower ammonium levels compared to that for larger shrimp at 58, 76 or 138 DOC, respectively. In another study, data from five farms registered higher ammonia levels ranging from 1.2–2.0 ppm, irrespective of the DOC at 32, 33, 42, 58 and 68. The increase in NH_4^+-N concentrations at certain times over the cultivation period could be due to the increased size of shrimp and the feeding rates (Guerrero-Galván et al. 1999).

At shorter DOC or smaller size, shrimp exposed to higher ammonium levels may not be able to tolerate such stress, which could reduce their immunity to infection. We believe that larger shrimp or those cultured for a longer period (i.e. > 40 DOC) are able to cope well compared with those at a shorter DOC (i.e. < 40). Prolonged exposure to stress due to a high concentration of ammonium could inhibit shrimp growth, as it could cause deterioration of the hepatopancreas and subsequently lead to increased susceptibility to EMS or AHPND.

Besides being associated with prolonged stress due to high concentrations of ammonium, EMS also could have another toxic etiology. Lightner et al. (2012) highlighted that degenerative pathology of the hepatopancreas is frequently a result of toxin. However, laboratory experiments conducted by Lightner et al. (2012) on crustacide and commercial feeds did not produce a consistent result similar to EMS pathology. The present study showed the presence of PSP toxin in infected shrimp; however, the concentration of toxin was lower than the human lethal dose of 2 mg (Hwang et al. 1992). The presence of PSP toxin in organs indicates that the affected shrimp ingested the toxin through food web transfer. The possibility of the toxin being present in commercial feeds, in common bacteria in the environment or in plankton could thus not be ruled out. In another study, Furio et al. (2012) showed that among several PSP-causative species of *Alexandrium*, *A. minutum* was found in low-salinity brackish environments in Viet Nam, Thailand, the Philippines and Malaysia. We believe that the presence of PSP toxin in EMS-affected shrimp could come the ingestion of diatoms, dinoflagellates or other micro-organisms in the water.

As the affected shrimp were under stress, we believe that they were more susceptible to all kinds of common pathogens, and particularly susceptible to multiple bacterial infections by members of the *Vibrio* group. Under these conditions, the shrimp were also exposed to other living organisms, including the dinoflagellate, which showed some PSP toxin. There is also the possibility that the toxin seen in affected shrimp showing acute pathology of the hepatopancreas could be protein based, as that toxin can be detected by the ELISA method used in the present study.

During the investigations conducted during Phases I and II (see Table 1), we observed that all samples tested for IMNV were negative, indicating that IMNV was not the cause of mortality despite the appearance of whitish abdominal muscles. According to the farm operators, most of the PL used originated from SPF broodstocks, and the affected shrimp tested negative for IMNV and TSV before being stocked into the ponds. Most of the samples also tested negative for IHHNV, with only seven of 64 samples from Perak being positive. IHHNV infection is known to cause "runt deformity syndrome", irregular and reduced growth, and cuticular deformities in *P. vannamei* (Kalagayan et al. 1991; Brock and Main 1994; Lightner 1996). During the investigation period, a single positive case of IHHNV in the affected shrimp would not have caused high mortality in cultivated *P. vannamei*. Vibriosis was recorded in samples from Perak and Pahang, but not in Penang. *Vibrio* species are part of the natural microflora of wild and cultured shrimp (Sinderman 1990) and become opportunistic pathogens when the natural defense mechanisms of shrimp are suppressed (Brock and Lightner 1990).

Mortalities due to vibriosis also occur when shrimp are stressed by factors such as poor water quality, crowding, high water temperature, low DO and low water exchange (Lewis 1973; Lightner and Lewis 1975; Brock and Lightner 1990). Vibrios are among the most important bacterial pathogens found in cultured shrimp, and they are responsible for a number of diseases

where mortalities may be up to 100 % (Jayasinghe et al. 2008). Shrimp-pathogenic vibrios are mainly *V. harveyi*, *V. fluvialis*, *V. parahaemolyticus*, *V. damsela* and *V. vulnificus* (Chythanya and Karunasagar 2002). The present study revealed that multiple bacterial infection consistently showed the presence the of *V. parahaemolyticus* in affected the shrimp.

Histopathological analysis showed a typical histopathology of AHPND in the hepatopancreatic tubules. Karyomegaly, sloughing of epithelial cells from hepatopancreatic tubules, multifocal septic tubules and melanized hepatopancreatic tubules with haemocyte encapsulation were seen in some specimens, suggesting the acute and terminal stages of AHPND. This provides evidence that the disease outbreaks in shrimp ponds in Perak, Pahang and Penang were due to AHPND.

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