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Pond sludge and increased pH cause early mortality syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND) in cultured white shrimp

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Abstract

Microcosm experiments simulating the occurrence of early mortality syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND) in white shrimp production ponds were performed in 30-L aquariums. Healthy white shrimp, *Litopenaeus vannamei*, were reared in aquariums containing EMS/AHPND-free hatchery or pond water. Raw pond sludge, collected from shrimp ponds where EMS/AHPND had occurred, was added to some test aquariums, while others were treated with sterilized pond sludge. In some aquariums, water pH was increased from 7.5 to 8.8. Microcosms with stable pH (around 7.5) and/or autoclaved sludge served as controls. The combination of raw sludge and increased pH induced EMS/AHPND and killed white shrimp, whereas raw sludge/stable pH and autoclaved sludge/increased pH combinations did not affect healthy shrimp. Thus, EMS/AHPND outbreaks are due not only to the causative agent but also to environmental stresses such as pH fluctuation. These findings contribute to improved management in shrimp production farms.

Keywords: White shrimp, *Litopenaeus vannamei*, EMS, AHPND, pH fluctuation, Environmental stress

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Introduction

White shrimp (*Litopenaeus vannamei*) have been reared in shrimp farms in Southeast Asia since the beginning of the 21st century. Production of this species has increased dramatically since 2006, replacing that of black tiger shrimp (*Penaeus monodon*). Benefits of white shrimp for aquaculture include their rapid growth, strong appetite, and tolerance to high density rearing, all related to their high swimming ability (Zhang et al., 2007; Yu et al., 2010). White shrimp utilize the entire water column (a three-dimensional space), while black tiger shrimp live in the bottom of the pond (a two-dimensional space). Thus, the white shrimp can be housed at higher density than black tiger shrimp. Furthermore, taste of the both species is similar (Benjakul et al., 2008), although the precise chemical composition of their meat differs (Striket et al., 2007). For these reasons white shrimp is considered an ideal species for aquaculture (Thitamadee et al., 2016). However, white shrimp aquaculture in Asian countries has faced serious problems since 2009 due to the early mortality syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND) (Lightner et al., 2012; Tran et al., 2013; Joshi et al., 2014). Within a month of release into an infected pond, shrimp develop opaque and whitish muscle tissue, and start dying; the hepatopancreas of affected shrimp appears paler and smaller than that of healthy shrimp (Lightner et al., 2012; Network of Aquaculture Centers in Asia-Pacific or NACA, 2012, 2014). The causative agent of EMS/AHPND is *Vibrio parahaemolyticus* (Tran et al., 2013; Soto-Rodriguez et al., 2015), an opportunistic pathogen, and the gene responsible for its toxicity has been sequenced (Kondo et al., 2014). However, the presence of the pathogen alone does not guarantee infection (Akazawa and Eguchi, 2013); environmental factors are also involved in the occurrence of EMS/AHPND (Gilles and Haffner, 2000).

The present study simulated the infectious process of EMS/AHPND in real production ponds using microcosm experiments, to isolate the combination of environmental stress/source of infection that results in shrimp mortality. Water pH variation was used as the environmental stress (Akazawa and Eguchi, 2013) and sludge from ponds where EMS/AHPND occurred was used as the source of infection. Under natural conditions, pond pH varies throughout the day due to respiration and photosynthesis (Wurts and Durborow, 1992; Tacon et al., 2002). White shrimp often peck at bottom sludge during feeding, so this is the likely the route of infection for EMS/AHPND.

The results presented here indicate that both environmental stress and the presence of the causative agent are important for EMS/AHPND outbreaks in culture ponds. The presented data will, therefore, be useful for improving pond management in white shrimp culture.

Materials and Methods

Sludge from shrimp ponds

Raw sludge was collected from shrimp ponds (50 × 100 m, 1.5 m deep) along the east coast of the Peninsular Malaysia, where EMS/AHPND was reported. The collected sludge was used in experiments in 2011.

Experimental shrimps

Healthy *L. vannamei* (average weight 3 g) were obtained from a commercial farm on the east coast of the Peninsular Malaysia, and acclimated in the laboratory for two weeks before being used in experiments. Stocking density was 133 shrimps/m^2.

Aquarium preparation

All the experiments were carried out in 30-L aquariums using hatchery and pond seawater. Hatchery water is commonly used in indoor tanks. It was first sterilized with 30-ppm chlorine gas and then filtered through sand and through a 10-µm-mesh cartridge. This treated water was then neutralized by aeration. Pond seawater was collected from outdoor shrimp ponds where EMS/AHPND is not known to have ever occurred. Salinity and temperature of both waters were around 35 psu and 28°C, respectively.

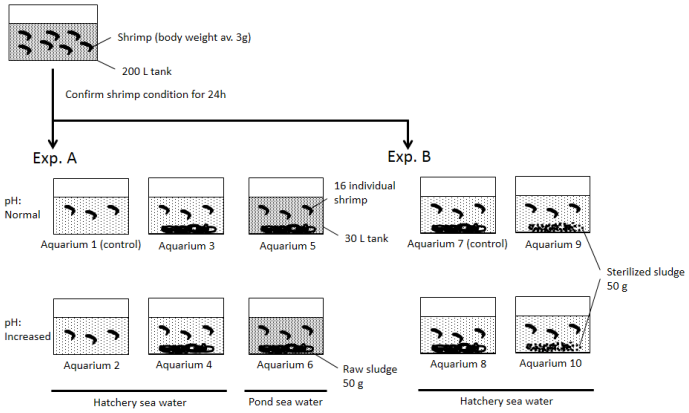

Figure 1. Outline of Experiments A and B

Experiment A: pH fluctuation stress and pond sludge effect

The outline of this experiment is illustrated in Figure 1, and experimental conditions were as follows: Aquarium 1, hatchery seawater without sludge at stable pH; Aquarium 2, hatchery seawater without sludge at high pH; Aquarium 3, hatchery seawater with raw sludge at stable pH; Aquarium 4, hatchery seawater with raw sludge at high pH; Aquarium 5, pond seawater with raw sludge at stable pH; Aquarium 6, pond seawater with raw sludge at high pH. All the test and control groups included 16 shrimp specimens per 30 L-aquarium (except for the third trial, see below), and two (Aquariums 3 and 4) or three (Aquariums 1, 2, 5, and 6) replicates were used per test. The third trial was conducted using 11 shrimp specimens per 30 L-aquarium. Shrimp density (i.e., 11 or 16 shrimps/30 L) was adjusted to the level observed in shrimp production ponds. Shrimp were introduced into aquariums with aeration and their status was monitored for 24 h. After this period, a 50 g sample of raw sludge, collected from production ponds where EMS/AHPND occurred, was slowly and gently added to Aquariums 3, 4, 5, and 6. Sludge concentration (i.e., 50 g/30 L) was similar to that observed in shrimp production ponds. Aquariums with added sludge were left to settle for 4 h and no abnormal shrimp behavior was observed during this period. Hydrated lime solution [0.1% (w/v)] was then gently added to Aquariums 2, 4, and 6 to increase pH from 7.5 to 8.8, and this solution was added whenever pH decreased. The pH conditions during the experiment are shown in Figure 2, and pH was monitored using a hand-held pH meter (Laquaact D-71, HORIBA). Survival rate of tested shrimp was checked after 72 h (first and second trials) or 120 h (third trial) rearing. Ecdysis frequency, and swimming and feeding behaviors were also observed. Shrimp were fed daily at 8:00, 12:00, and 16:00, which are the feeding times at shrimp production ponds. The feeding rate (ca. 2.4 g/day/30 L-aquarium) was also identical to that of production ponds.

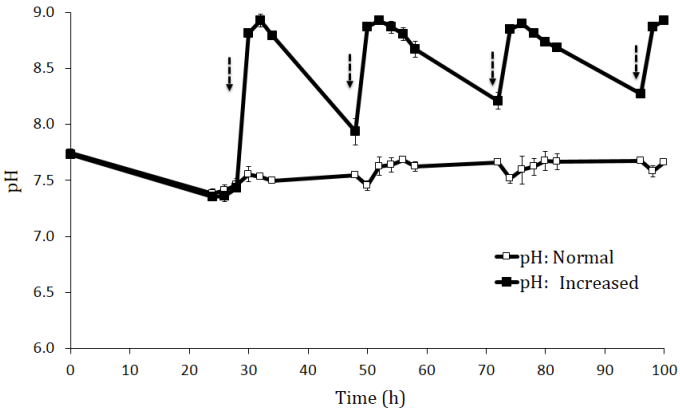

Figure 2. pH fluctuations in Experiment A. Arrows indicate the timings of the addition of hydrated lime solution. Bars at each point indicate standard deviations

Experiment B: Autoclaved sludge effect

Raw and autoclaved sludge samples were examined to confirm that the causative agent of EMS/AHPND was only present in sludge. Experimental procedures and conditions were similar to those described for *Experiment A*, using four aquariums: Aquarium 7 (normal pH, raw sludge), Aquarium 8 (increased pH, raw sludge), Aquarium 9 (normal pH, autoclaved sludge), and Aquarium 10 (increased pH, autoclaved sludge). Each 30 L-aquarium contained 16 shrimp specimens. The pH conditions in *Experiment B* were the same as in *Experiment A* (Figure 2).

Disease confirmation

Shrimp were observed in aquariums every 2 h between 8:00 and 18:00 during the rearing period. Dead shrimp showing clinical signs of EMS/AHPND, including pale-to-white hepatopancreas (HP) or significant atrophy of the HP, were considered to have died due to EMS/AHPND (NACA, 2012, 2014; Thitamadee et al., 2016). To rule out other causes of death, infections by nine known viruses (WSSV, White Spot Syndrome Virus; IHHNV, Infectious Hypodermal and Hematopoietic Necrosis Virus; TSV, Taura Syndrome Virus;

IMNV, Infectious Myonecrosis Virus; YHV, Yellow Head Virus; GAV, Gill Associated Virus; PvNV, *Penaeus vannamei* Nodavirus; HPV, Hepatopancreatic Parvovirus; and MBV, Monodon Baculovirus) and by NHPB (Necrotizing Hepatopancreatitis Bacterium) were checked by polymerase chain reaction (PCR) using a commercial probe kit (IQ2000, GeneReach Biotechnology), and following the manufacturer's instructions.

Results

Experiment A

When shrimp were reared in hatchery water and in the absence of pond sludge, increasing pH did not induce EMS/AHPND (Table 1, Aquariums 1 and 2). Although one of the 11 shrimp specimens died in Aquarium 2 after 120 h (Table 1, 3rd trial, 90.9%), this dead shrimp did not show the typical symptoms of EMS/AHPND (i.e., pale-to-white HP). No infection by WSSV, IHHNV, TSV, IMNV, YHV, GAV, PvNV, HPV, MBV, and NHPB was detected throughout the experiment.

Table 1. Survival rate (%) of shrimp after 72 hr (1st and 2nd trials) and 120 hr (3rd-trial) rearing in Aquariums 1-6

	1st-trial	2nd-trial	3rd-trial
Aquarium 1	100.0	100.0	100.0
Aquarium 2	100.0	100.0	90.9
Aquarium 3	ND	100.0	100.0
Aquarium 4	ND	87.5	63.6
Aquarium 5	100.0	100.0	100.0
Aquarium 6	25.0	37.5	27.3

In hatchery water to which raw sludge was added, the survival rate of healthy shrimp at increased pH (Aquarium 4) was 87.5% after 72 h and 63.6% after 120 h. All dead shrimp specimens showed the typical EMS/AHPND symptoms. Under stable pH (around 7.5), one shrimp died, even in the presence of raw sludge collected from production ponds where EMS/AHPND occurred.

In pond water with stable pH, no shrimp died, even when raw sludge was added (Table 1, Aquarium 5). However, the combination of raw sludge and increased pH killed more than 60% of the tested shrimps and all dead shrimp showed the typical EMS/AHPND symptoms.

Experiment B

Adding autoclaved sludge to pond water had no effect on shrimp survival, irrespective of pH (Figure 3). After 120 h, under increased pH and after the addition of autoclaved sludge, a few shrimp specimens died (Aquarium 10, Figure 3), but they did not show EMS/AHPND symptoms. The combination of increased pH and raw sludge addition killed all shrimp in Aquarium 8, and all the dead shrimp showed the typical EMS/AHPND symptoms. No infection by WSSV, IHHNV, TSV, IMNV, YHV, GAV, PvNV, HPV, MBV, and NHPB was detected throughout the experiment.

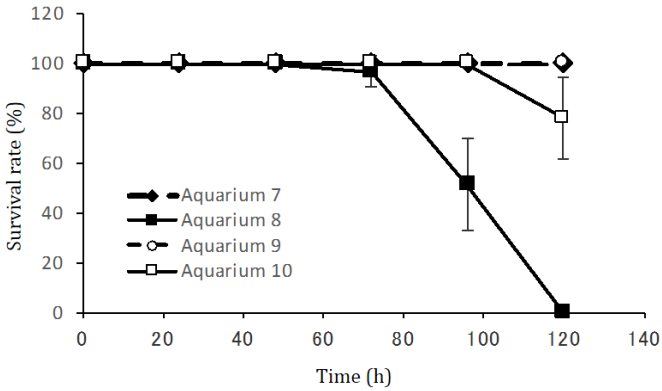

Figure 3. Survival rate (%) of shrimp in Experiment B. Bars at each point indicate standard deviations

Discussion

Since the initial EMS/AHPND outbreak in China in 2009, this disease has spread throughout Southeast Asia and even reached Mexico in 2013 (Nunan et al., 2014; Soto-Rodriguez et al., 2015). Tran et al. (2013) reported that *V. parahaemolyticus* was the causative agent of EMS/AHPND. Comparative genome analysis allowed obtaining the unique sequences of this bacterium from shrimp showing EMS/AHPND symptoms (Kondo et al., 2014; Yang et al., 2014; Lee et al., 2015). The data revealed that when this opportunistic marine pathogen acquired a unique EMS/AHPND-associated plasmid, it became highly virulent. An accurate and rapid diagnostic method was also developed (Kondo et al., 2014).

In the present study, we focused on the ecology of the pathogen in shrimp production facilities to determine how to improve management at these sites to avoid or reduce EMS/AHPND outbreaks. Because experiments were carried out in 2011, before PCR probes to detect *V. parahaemolyticus* were developed, we confirmed the occurrence of EMS/AHPND based on the display of the typical symptoms of this disease (i.e., pale-to-white HP; NACA, 2012, 2014; Thitamadee et al., 2016).

White shrimp are voracious feeders, and even consume sedimented feces and leftover feed, and grow faster than black tiger shrimp, which is an advantage in aquaculture (Smith et al., 2002; Tacon et al., 2002). In the present study, we hypothesized that the strong appetite of *L. vannamei* might be a disadvantage because it leads them to consume raw sludge and thereby acquire EMS/AHPND. However, when sludge was added to aquariums but there was no environmental stress such as pH increase, and EMS/AHPND did not occur. Thus, both infectious raw sludge and an environmental stress bring about an EMS/AHPND outbreak. In aquaculture ponds, pH fluctuates daily and often reaches 9 during daytime due to photosynthesis (Wurts and Durborrow, 1992; Tacon et al., 2002). After sunset, as dissolved oxygen is consumed and CO_2 is produced by respiration of all plants and animals, pH decreases. In the studied shrimp ponds, pH was higher in the afternoon than in the morning, and the highest value was 9.18 (data not

shown). Our results showed that this environmental stress occurring daily in shrimp production ponds was required to induce EMS/AHPND. In the present study, we focused on increased pH as an environmental stress. However, it is possible that other environmental stresses such as variations in temperature and salinity also induce the occurrence of EMS/AHPND (Moullac and Haffner, 2000).

Li and Chen (2008) studied the immune response of white shrimp and their susceptibility to a related pathogen, *Vibrio alginolyticus*, under low and high pH stress. According to their report, shrimp showed significantly decreased phenol oxidase activity, respiratory burst, phagocytic activity, and clearance efficiency against *V. alginolyticus* over 6–72 h, when pH was changed from 8.2 to either 6.5 or 10.1. Thus, pH stress seems to weaken the immune system of white shrimp. Indeed, our observations of reduced appetite and increased ecdysis in the experimental groups under pH stress suggested that the shrimp were stressed.

Wu et al. (2001) reported that the high mortality observed in another species of shrimp, *Penaeus japonicus*, infected by a penaeid rod-shaped DNA virus was due to horizontal transmission of the virus through cannibalism and the waterborne route. Cannibalism was also likely to spread EMS/AHPND in the present study.

The two types of rearing water used in the present study, hatchery and pond water, are used in commercial shrimp production sites. Shrimp mortalities were relatively lower (12.5% and 36.4%, Table 1) in hatchery water sterilized with chlorine and neutralized with aeration than in pond water (75.0%, 62.5%, and 72.7%, Table 1). Bacterial counts by thiosulfate-citrate-bile salts-sucrose (TCBS) agar were higher in pond water (10–100 colony forming units/mL) than in hatchery water (below the detection limit; data not shown), and these counts reflect the amount of *Vibrio* spp. Thus, *Vibrio* spp. appear to grow better in pond water than in hatchery water, although this tendency needs to be further clarified in future studies.

Conclusions

The present research clarified two points in the mechanism of the onset of EMS/AHPND infection. One is both the pathogen and an environmental stress, such as pH change, cause the onset of EMS/AHPND. The second is that the pathogen is likely to be acquired from the sludge within shrimp culturing ponds. Therefore, as a preventive countermeasure for EMS/AHPND infection, we suggest constantly monitoring environmental changes in ponds, avoiding excess feeding, and reducing sludge accumulation at the bottom of ponds. Increasing the amount of water exchange in the ponds may also work effectively to keep a stable and healthy environment.

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