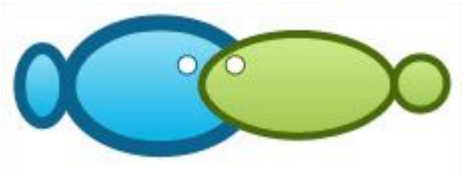

Immune responses and susceptibility to *Vibrio parahaemolyticus* colonization of juvenile *Penaeus vannamei* at increased water temperature

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Abstract. Outbreaks of acute hepatopancreatic necrosis disease (AHPND), which was reportedly caused by a strain of *Vibrio parahaemolyticus*, resulted in a significant decline in the world shrimp production of the previous years. Cultured shrimp are subjected to seasonal changes and changes due to culture management that can influence the physico-chemical quality of the rearing water. Elevated water temperature is assumed to have significant influence on the health status of crustaceans as well as on the pathogenesis of opportunistic pathogens such as *Vibrio* species. Hence, the present study evaluated the influence of abrupt exposure to higher temperature on selected immune parameters of Pacific white shrimp *Penaeus vannamei* as well as on the initial step in the pathogenesis of AHPND-causing strain *V. parahaemolyticus* which is colonization in the host's gut. Respiratory burst activity (RBA) and phenoloxidase (PO) activity of shrimp exposed to 29°C (control temperature), 32°C and 35°C were measured at 0, 24, 48, 72, and 96 hour post exposure (hpe). Results showed that RBA of shrimp transferred to 35°C significantly increased at 24 hours hpe and started to decline although comparable with the control at 48 to 96 hpe. Shrimp transferred to 32°C displayed higher PO activity than those in control throughout the 96 hour trial with significant increase at 48 hpe. No significant changes were observed in PO activity of shrimp transferred to 35°C. On the other hand shrimp kept at the test temperatures for 24, 48, 72 and 96 hours and exposed to *V. parahaemolyticus* for 24 hours were found to be colonized by the bacteria on the gut. Highest colonization was documented in shrimp exposed to 35°C for 96 hours which indicated that prolonged exposure to high temperature can cause shrimp to become more susceptible to pathogen invasion which may result in diseases.
Key Words: *Vibrio parahaemolyticus*, respiratory burst activity, phenoloxidase activity, gut colonization.

Introduction. Shrimp farming is an important economic activity in many tropical and subtropical countries including the Philippines. However, outbreaks of infectious diseases have always been the main problem faced by shrimp growers. The new disease known as Acute Hepatopancreatic Necrosis Disease (AHPND) or Early Mortality Syndrome (EMS) added to the growing number of recorded shrimp diseases. The disease originated in China in 2009 and spread in its neighboring countries like Vietnam in the same year, Malaysia, Thailand, and Mexico in 2011, 2012, and 2013 respectively. The disease was first recorded in the Philippines in 2015 (Dabu et al 2017). Countries hit by AHPND outbreaks suffered from huge production losses making the disease a major factor in the decline of world shrimp production in 2013. The disease can cause shrimp mortality up to 100% within the 20-30 days of culture in grow out ponds (Lightner et al 2012). A strain of *Vibrio parahaemolyticus* was identified as the causative agent of AHPND (Tran et al 2013).

Vibrio species are ubiquitous in the marine and brackish water environments, however, some species are considered as opportunistic pathogens of immune-compromised fish and shrimp. Studies on the pathogenesis of AHPND-causing *V. parahaemolyticus* revealed that the main route of infection is the gastrointestinal (GI)

tract (Tran et al 2013; Joshi et al 2014). The shrimp stomach serves as the establishment site at the same time initial multiplication site of bacterial pathogens that entered the shrimp thru infected feeds (de la Peňa et al 1995). Soonthornchai et al (2015) were able to document that when *P. monodon* was fed with diet infected with *Vibrio* species, the bacteria is capable of attaching to the stomach and increasing their number dramatically at 6 and 24 hours post infection respectively. These reports demonstrated the importance of the GI tract particularly the stomach in the initial processes of infection.

Cultured shrimp are subjected to seasonal changes and changes due to culture management that can influence the physico-chemical quality of the rearing water. The health status of the reared animal can be directly affected by changes in these physico-chemical parameters. Previous studies reported that variations in extrinsic factors such pH, salinity, oxygen and temperature could affect crustaceans' immune responses (Le Moullac & Haffner 2000). Among these factors, temperature is considered as the most important as it can influence other environmental parameters as well. In the tropics, particularly during summer months, surface temperature of shallow water bodies like the ponds can have a daily maximum of 30-35^{o}C and can occasionally increase up to 40^{o}C or above (Kutty 1987). Changes in water temperature were assumed to have significant impact on the health status and disease resistance of cultured animal. Variations in this parameter caused alterations in the several immunological tools used to evaluate the immune status of crustaceans (Le Moullac & Haffner 2000) as well as enhanced susceptibility of animals to pathogens (Cheng & Chen 1998; Jiravanichpaisal et al 2004; Cheng et al 2005). Water parameters including temperature also have a direct relationship with *V. parahaemolyticus* population (Zorriehzahra & Banaederakhshan 2015). In this context, the present study aims to characterize the effect of increased water temperature on the selected immune parameters of penaeid shrimp *Penaeus vannamei* as well as evaluate the ability of pathogenic *Vibrio* species to colonize and proliferate in the gut of shrimp exposed to increased water temperature.

Material and Method. The study was conducted at the facilities of the Institute of Aquaculture, College of Fisheries and Ocean Sciences, University of the Philippines Visayas from October to December, 2016.

Experimental animals. *P. vannamei* post larvae were obtained from Oversea Hatchery in Minglanilla, Cebu, Philippines and reared in laboratory conditions for two months. Shrimp were fed to satiation with commercially formulated diet (Vannamei Feed No. 3, OverSea Feed Corp.) two times daily. Rearing water was changed daily and water quality monitored regularly. Temperature and salinity were maintained at 28-29°C and 25 g L^{-1} respectively. Uniformly sized juveniles with an average body weight (ABW) of 10±0.05 grams were used in the experiment.

Immune responses. *P. vannamei* were transferred to 50 x 40 x 30 cm tanks with working volume of 20 L at a density of five shrimp per tank. Water parameters were kept constant in all tanks except for the water temperature which was pre-set to 29±0.5 (control/ambient temperature), 32±0.5 and 35±0.5°C using a thermostatted aquarium heater. Each test temperature was replicated four times and one (1) shrimp was sampled from each replicate at 0, 24, 48, 72 and 96 hours post exposure. Haemolymph was withdrawn from the animal's ventral sinus using a one (1) mL 25G syringe containing pre-cooled anticoagulant solution (450 mM NaCl, 10 mM KCl, 10 mM HEPES and 10 mM EDTA at pH 7.3). Extracted haemolymph was divided into two microcentrifuge tubes for the measurement of respiratory burst activity (RBA) and phenoloxidase activity (PO).

Respiratory burst activity (RBA). Respiratory burst activity was quantified using the reduction of nitroblue tetrazolium (NBT) to blue formazan as measure of superoxide anion (O_2^-) production according to Muñoz et al (2000) with modifications. Fifty microliter of 1:3 haemolymph-anticoagulant mixture was placed in the assigned well of a 96 well microtiter plate and incubated for 30 minutes at room temperature. The supernatant was

then discarded and replaced by 25 µL Modified Hank's Balanced Salt Solution Medium, 25 µL of elicitor and 25 µL NBT working Solution. After one (1) hour of incubation, the supernatant was removed and the haemocytes were fixed by adding 100 µL absolute methanol. After 10 minutes, methanol was discarded and haemocytes washed with 70% methanol twice and left to dry. The formazan deposits were solubilized by adding 60 µL 2 M Potassium Hydroxide (KOH) and 70 µL Dimethyl Sulfoxide (DMSO). Sixty microliter of 2 M KOH and 70 µL DMSO served as blank. The optical density (OD) was measured using a microplate reader (Dynamica Ledetech 96©) at 620 nm. Respiratory burst activity was expressed as OD 50 µL haemolymph^{-1}.

Phenoloxidase (PO) activity of haemocyte lysate supernatant. Haemocyte lysate supernatant (HLS) for the measurement of PO activity was prepared according to Sung et al (1998) with modification. Extracted haemolymph was centrifuged (Hettich Zentrifugen Universal 320R) at 300 x g for 10 minutes at 4°C for the collection of haemocytes. The settled cell pellets were washed with shrimp salt solution (SSS) and subjected to another centrifugation at 300 x g for 10 minutes at 4°C. After removal of supernatant, the haemocytes were reconstituted with 300 µL SSS, homogenized uniformly with disposable propylene pestles and centrifuge at 20,000 x g for 20 minutes at 4°C. The supernatant/haemocyte lysate was used for the PO assay. Protein concentration of the lysate was determined by the Bradford assay (Bradford 1976).

PO activity was measured based on the procedure presented by Hernandez-Lopez et al (1996) using L-DOPA (L-3,4-dihydroxyphenylalanine (L-dopa)) as substrate. Twenty-five µL HLS was incubated with 25 µL 1% trypsin for 30 minutes before addition of 25 µL 0.3 % L-DOPA. Change in OD per minute was measured at 492 nm (Dynamica Ledetech 96©). PO activity was expressed as change in OD minute^{-1} mg protein HLS^{-1} 0.001^{-1}.

Colonization of Vibrio parahaemolyticus on shrimp gut. AHPND positive *V. parahaemolyticus* strain provided by the UPV-DOST Shrimp Program was cultured overnight in nutrient agar (NA; Sharlau) supplemented with 2.0% sodium chloride (NaCl). The colonies were harvested using a sterile inoculating loop and suspended in sterile normal saline solution (NSS) to make a bacterial suspension equivalent to 1x10^{10} colony forming units (cfu) mL^{-1}. *P. vannamei* kept at 29±0.5 (control), 32±0.5 and 35±0.5°C for 0, 24, 48, 72 and 96 hours were bathed in a 10 L seawater containing 1x10^{6} cfu mL^{-1} *V. parahaemolyticus* for 24 hours. This bacterial suspension was prepared by diluting one (1) mL of the original suspension to 10 L using UV-treated seawater and bacterial density confirmed by plating on thiosulphate citrate bile sucrose agar (TCBS; Sharlau). Shrimp were also fed with commercial diet equivalent to 2% of biomass. Shrimp kept in the test temperature at 0, 24, 48, 72 and 96 hours bathed in one (1) mL NSS diluted to 10 L and fed with commercial diet served as the uninfected control.

The number of colonizing *V. parahaemolyticus* in the shrimp gut was determined by culturing on TCBS. The foregut (stomach) of shrimp from each temperature group at each period was aseptically dissected out and weighed. The stomach was then suspended in one (1) mL NSS and homogenized uniformly using disposable propylene pestles. The sample was subjected to a 10-fold serial dilution and 0.1 mL of the representative dilution was plated on TCBS agar in duplicate using the spread plate method. *V. parahaemolyticus* load (green to blue-green colonies on TCBS) was determined after 24 hours of incubation and expressed as cfu g^{-1} gut. The test was replicated four times for each test temperature and period.

Statistical analysis. Data for each sampling period were analysed by one-way analysis of variance (ANOVA) using SPSS ver. 16.0 with significance level set at 0.05. If the effects are significant, the means were subjected to post hoc analysis using Duncan Multiple Range Test (DMRT). Bacterial counts were log 10 transformed before analyses.

Results

Effect of increase temperature on the immune responses of P. vannamei. Mortality was not recorded when shrimp were exposed to higher temperature for 96 hours. RBA of shrimp haemocytes transferred to 32°C was comparable all throughout the trial to that of shrimp retained in 29°C (Figure 1). Meanwhile, RBA of shrimp transferred to 35°C significantly increased at 24 hours post exposure (hpe) and started to decline although comparable with the control at 48 to 96 hpe.

No significant change was noted in the protein content of the haemocyte lysate of all samples as determined by the Bradford assay. On the other hand, phenoloxidase activity varied between 108.61±14.00 to 114.70±17.00, 108.61±14.0 to 205.2±16.0 and 98.92±14.0 to 139.74±26 OD minute^{-1} mg protein HLS^{-1} 0.001^{-1} for 29°C (control), 32°C, and 35°C respectively. Shrimp transferred to 32°C displayed higher PO activity than those in control throughout the 96 hour trial with significant increase occurring at 48 hpe. No significant changes were observed in PO activity of shrimp transferred to 35°C (Figure 2).

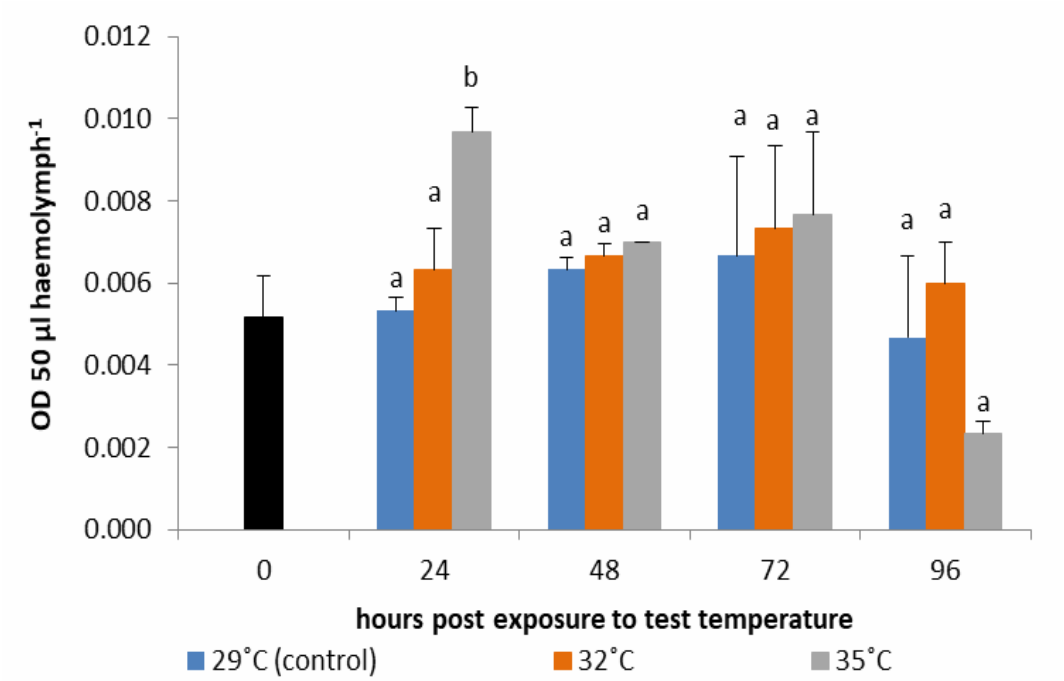

Figure 1. Respiratory burst activity of *Penaeus vannamei* exposed to increase temperature levels. Values are expressed as Mean ± SEM (n = 4 shrimp). Values with different labels during the same hour post exposure to test temperature are significantly different at P<0.05.

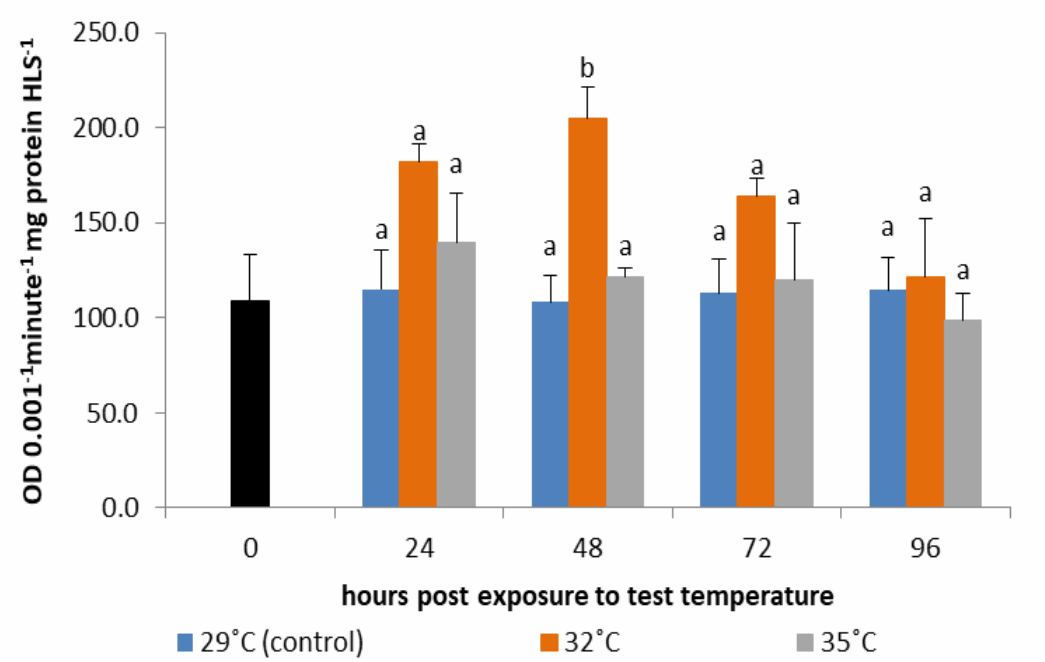

Figure 2. Phenoloxidase activity of *Penaeus vannamei* exposed to increase temperature levels. Values are expressed as Mean ±SEM (n=4 shrimp). Values with different labels during the same hour post exposure to test temperature are significantly different at P<0.05.

Colonization of V. parahaemolyticus on the gut of P. vannamei. Shrimp kept at the 29°C (control), 32°C, and 35°C for 24, 48, 72 and 96 hours not exposed to *V. parahaemolyticus* did not harbour the bacteria on its gut (data not shown). On the other hand, shrimp held at these temperatures and exposed to *V. parahaemolyticus* after 24, 48, 72 and 96 hours were found to be colonized by the bacteria on the gut (Table 1). Significantly higher *V. parahaemolyticus* counts were recorded in the shrimp exposed to the bacteria 96 hours after transfer to higher temperature.

Table 1

V. parahaemolyticus load in the foregut of *P. vannamei* exposed to increased water temperature (x10^6 cfu mL^{-1})

Temperature	*Hours post exposure to test temperature*			
	24	*48*	*72*	*96*
29˚C (control)	0.03±0.01	1.27±0.13	1.36±0.03	12.06±0.63[a]
32˚C	0.45±0.28	0.05±0.04	2.15±1.40	171.05±40.90[b]
35˚C	0.17±0.06	14.20±7.60	8.25±3.90	262.40±43.30[b]

Values with different labels during the same hour post exposure to test temperature are significantly different at $p < 0.05$.

Discussion. Temperature is considered as an important extrinsic factor affecting aquatic organisms. Aside from affecting other environmental variables such as salinity and oxygen, temperature was also reported to directly affect growth and survival of different aquatic species such as crustaceans (Chen et al 1995; Ponce-Palafox 1997; Hennig & Andreatta 1998). On the other hand, effects of thermal stress to the immune functions of different crustacean species were also previously investigated. A slow rise in temperature from 10 to 20°C significantly increases blood cell numbers of crab *Carcinus maenas* (Truscott & White 1990). According to Chisholm & Smith (1994), the lowest antibacterial activity of the haemocytes of *C. maenas* is when temperature is at its lowest and highest for the year. In addition, indicators of oxidative and metabolic stresses in common tropical freshwater crayfish *Cherax quadricarinatus* were found to be elevated at higher temperature levels (Bone et al 2015). In the present study, the effect of increased temperature to the respiratory burst and phenoloxidase activities of juvenile *P. vannamei*

was investigated. Increase in temperature affected both RBA and PO activities, however, significant changes were observed at different exposure time.

The result showed respiratory burst activity of shrimp significantly increased 24 hours post exposure to 35°C. In contrast, Cheng et al (2005) observed a significant decrease in RBA of *P. vannamei* transferred to 32°C from 28°C. RBA is a process that refers to the generation of reactive oxygen intermediates (ROIs) as a result of oxygen dependent defence mechanisms such as the elimination of internalized particles or microorganisms during phagocytosis (Rodriguez & Le Moullac 2000). RBA can be stimulated by foreign particles such as lipopolysaccharide (LPS) or yeast cell wall which lead to an increase oxygen consumption wherein the reduction by NADPH-oxidase generates superoxide anion and other reactive products (Muňoz et al 2000). Several studies however, reported that enhanced production of ROIs occurs as a response to stress caused by changes in some environmental factors. RBA of Taiwan abalone *Haliotis diversicolor supertexta* increased significantly after transfer to 32°C from 28°C (Cheng et al 2004). Toxicants such as ammonia, nitrite and copper sulphate were demonstrated to have increased the release of superoxide anions in *Macrobrachium nipponense* and *M. rosenbergii* (Cheng & Wang 2001; Wang et al 2004, 2005). Acidic and alkaline stresses were also found to induce production of reactive oxygen species (ROS) in *L. vannamei* (Wang et al 2012). Although reactive oxygen molecules are capable of killing invaders, the mass accumulation of these oxygen species in an organism can cause cell damage. Overproduction of ROS including superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen can lead to protein oxidation and DNA damage and can affect viability of cell due to membrane damage and enzyme inactivity (Nordberg & Arner 2001; Livingstone 2001). Eliminating excess reactive oxygen species is a responsibility of antioxidant enzymes including superoxide dismutases (SOD) that scavenges superoxide anions (Holmblad & Söderhäll 1999; Campa-Cordova et al 2002). An increase in RBA could also be due to decrease in activity of these enzymes that detoxifies superoxide radicals. Although this phenomenon was not demonstrated in the present study, Jia et al (2014) reported that SOD activity of *L. vannamei* reared in seawater was found to be relatively stable despite hyperthermal stress. This could also be a factor why RBA of shrimp exposed to higher temperature returned to normal after 48 hours onwards. Further study characterizing the relationship of RBA and SOD activity in shrimp under thermal stresses is recommended.

Phenoloxidase is an enzyme responsible for many processes such as melanisation, keratin hardening, and wound healing (Ashida & Söderhäll 1984; Rodriguez & Le Moullach 2000). High levels of PO activity occur as a mechanism to maintain resistance against infection and can be activated by invasive structural components such β-glucan (Le Moullach et al 1998; Le Moullac & Haffner 2000); however, PO activity can also change as a response to changes in environmental conditions. In the present study, although significant difference in PO activity between temperature groups was documented only at 48 hours, generally higher PO activity was observed in haemocytes lysate of shrimp transferred to higher temperature than those that remained in the reference temperature. Likewise, PO activity of juvenile *Holothuria scabra* also increased when temperature was elevated (Kamyab et al 2017). Higher PO activity was recorded in the cell free haemolymph from the crab *Carcinus aestuarii* kept at 4°C and 30°C than those held in control temperature (Matozzo et al 2011). However, the same study reported no significant change in the PO activity of haemocyte lysate from *C. aestuarii*. Fluctuating temperature can be a stimulating factor to activate proPO and increase phenoloxidase activity in shrimp haemocytes (Lu-Qing et al 2007). The increase in temperature might have triggered the degranulation of haemocytes that activated the proPO system. On the other hand, several studies reported that PO together with total haemocyte count have the tendency to decrease when an animal reared in optimal temperature was suddenly transferred to higher temperature (Smith & Chisholm 1992; Vargas-Albores et al 1998; Cheng et al 2004).

The present study also investigated the effect of exposure to higher temperature to the colonization of *V. parahaemolyticus* on the shrimp gut. The capacity to colonize the animal tissue is an important property of pathogenic microorganism as this can be an

initial step towards infection. Although *Vibrio* spp. are normal flora of shrimp digestive tract, *V. parahaemolyticus* load in the stomach of shrimp from unchallenged group remained below the detectable levels in this study. On the other hand results showed that all shrimp exposed to *V. parahaemolyticus* for 24 hours, regardless of temperature group, were colonized by the bacteria. Despite the presence of multiple defense mechanisms, bacterial pathogens have wide range of strategies to colonize and proliferate in the shrimp organs and cause serious infection. In this study, *V. parahaemolyticus* in the rearing water adhered to the feeds added which facilitated the entry of the bacteria to the shrimp gut. This indicates that the oral cavity serves as an important portal of entry of potential pathogens from the shrimp rearing environment. *Artemia*, infected with pathogenic *V. harveyi* and *V. parahaemolyticus,* was able to successfully introduce the bacteria to apparently healthy *P. monodon* via the oral route. Both pathogens survive in the stomach and were able to proliferate and cause tissue damage particularly in the epithelial layer (Soonthornchai et al 2015). According to de la Peňa et al (1995), the stomach is the most probable establishment site of *Vibrio* spp. after entering kuruma shrimp *Penaeus japonicus* thru oral route. The stomach might also be where the bacteria multiply before released into the haemolymph and distributed to other organs. Higher counts of *V. parahaemolyticus* were recorded in the stomach of shrimp exposed to 35^{o}C. Aside from altering the shrimp immune functions, the result of the present study demonstrates that bacterial proliferation might be enhanced when the host was exposed to temperature stress. Several studies documented that high temperature can cause higher mortality rate of animals challenged with bacterial pathogens (Cheng & Chen 1998; Cheng et al 2003, 2005).

The effect of temperature on the colonization of *V. parahaemolyticus* on shrimp gut is most notable when shrimp was subjected to hyperthermal stress for 96 hours prior to bacterial exposure which indicate that prolonged temperature stress can cause shrimp to become more susceptible to pathogen invasion which may result in diseases. As unexpected fluctuations in environmental temperature is always possible, employing innovative shrimp culture practices such tilapia green water technique which can suppress growth of potential pathogens like *V. parahaemolyticus* (Cadiz et al 2016) as well as use of immunostimulants proven to improve shrimp survival when infected by the AHPND-causing microorganism (Solidum et al 2016) could help reduce losses due to such diseases.

Conclusions. The present study demonstrated that increasing temperature can cause alterations in the respiratory burst and phenoloxidase activities of juvenile *P. vannamei*. In addition to changes in the immune responses, prolonged exposure to hyperthermal stress can enhance the capacity of *V. parahaemolyticus* to colonize and proliferate in the shrimp gut which could compromise the animal's health.

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