1 ***Enterocytozoon hepatopenaei* (EHP) is a risk factor for acute hepatopancreatic**
2 **necrosis disease (AHPND) and septic hepatopancreatic necrosis (SHPN) in the**
3 **Pacific white shrimp *Penaeus vannamei***

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6 Luis Fernando Aranguren*, Jee Eun Han and Kathy F.J. Tang

7 School of Animal and Comparative Biomedical Sciences, University of Arizona, 1117 E.

8 Lowell Street. Tucson, Arizona, USA, 85721

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10 *Correspondence: Luis F. Aranguren, School of Animal and Comparative Biomedical
11 Sciences, University of Arizona, Tucson, Arizona 85721, USA. *1(520)621-4438
12 (lfarangu@email.arizona.edu).

ABSTRACT

The hepatopancreatic microsporidian *Enterocytozoon hepatopenaei* (EHP) is an emerging pathogen that affects cultured shrimp *Penaeus vannamei* in several SE Asian countries including China, Vietnam, Thailand, Indonesia, India and Malaysia. EHP infections are often accompanied by opportunistic infections of *Vibrio* spp. Laboratory challenges and a case control study were used to determine the effects of EHP infection on two *Vibrio* diseases: acute hepatopancreatic necrosis disease (AHPND) and septic hepatopancreatic necrosis (SHPN). To determine the effect of EHP on AHPND, two independent experimental infections were carried out. EHP-infected shrimp (EHP-AHPND group) and healthy shrimp (AHPND group) were challenged with 2.4×10^5 CFU/ml of AHPND-causing *Vibrio parahaemolyticus*. The results of the experimental infections showed that EHP-AHPND group exhibited higher mortalities (60 and 44%) than the AHPND group (0 and 18%). The pathological effects of AHPND vs. EHP-AHPND groups were compared during the first 12 hours post infection, 57% of the EHP-AHPND group displayed severe hepatopancreas necrosis and sloughing, features characteristic of AHPND infection while only 11% of AHPND group showed these features. This indicated that EHP-infected shrimp have a higher susceptibility to AHPND infection. To determine the effect of EHP on SHPN, we reviewed the histopathology of samples collected where EHP is endemic; and a case control study was carried out to determine the association between SHPN and EHP. We compared individual shrimp displaying histological signs of SHPN with the shrimp from the same ponds without these signs. A strong association was found between SHPN and EHP, indicating that shrimp with EHP have an increased susceptibility to SHPN. These findings suggested that EHP infection is a risk factor for both AHPND and SHPN.

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37 Key words: *Enterocytozoon hepatopenaei* (EHP), acute hepatopancreatic necrosis disease

38 (AHPND), septic hepatopancreatic necrosis (SHPN), *Penaeus vannamei*

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1. Introduction

Since 2003, hepatopancreas microsporidian (HPM) has been reported in *Penaeus monodon* cultured in Thailand. These shrimp displayed monodon slow growth syndrome (MSGs) and were co-infected with opportunistic pathogens such as monodon baculovirus (MBV), hepatopancreatic parvovirus (HPV), and *Vibrio* spp. (Chayaburakul et al., 2004). Years later, this microsporidian was described as *Enterocytozoon hepatopenaei* (EHP) (Tourtip et al., 2009) and it has been reported in *P. monodon* (Ha et al., 2010) and *P. vannamei* culture (Tangprasittipap et al., 2013) in SE Asia countries such as China, Indonesia, Malaysia, Vietnam, Thailand and India (Ha et al., 2010; Tang et al., 2015, 2016; Rajendran et al., 2016).

The main clinical sign of the hepatopancreatic microsporidiosis, a disease caused by EHP, is growth retardation (Sritunyalucksana et al., 2014; Newman 2015), which leads to an increased variability in size. In a more advanced stage, EHP-infected shrimp typically display soft shells, lethargy, reduced feed intake, and empty midgut. EHP is currently diagnosed through histological examination, *In situ* hybridization and PCR (Tangprasittipap et al., 2013; Tang et al., 2015). EHP is an intracellular microsporidian that replicates within the cytoplasm of the affected tubule epithelial cells in the hepatopancreas. Histology of infected tissues reveals several developmental stages, including basophilic inclusion bodies within the cytoplasm of the hepatopancreas epithelial cells, which appears to be the sporogonial plasmodium stage. Also, mild sloughing of the tubular epithelial cells and spores released into lumen are also observed (Tourtip et al., 2009).

64 During 2009-2012, a new, emerging disease called acute hepatopancreas necrosis disease
65 (AHPND) also known as “Early Mortality Syndrome (EMS)” started causing significant
66 production losses in most of the countries where EHP has been reported such as China,
67 Vietnam, Malaysia, Thailand, Mexico (Lightner et al., 2012; Tran et al., 2013; Flegel
68 2012; Leaña and Mohan 2012) and later on Philippines (Leobert et al., 2015). In some
69 cases, AHPND has been reported in co-infection with EHP (Ha et al., 2010; FAO 2015,
70 Chang 2016).

71 The causative agents of AHPND were determined to be *Vibriobacteria*
72 including *Vibriopara haemolyticus* (Han et al., 2015; Lee et al., 2015), *V campbellii* (Han
73 et al., 2016) and *V. harveyi* (Kondo et al., 2015) that contain a binary toxin, PirABvp, that
74 has shown to be the virulence factor of this disease. These *pirABvp* genes are located in a
75 large plasmid (69-70 kb) in these strains (Han et al., 2015; Lee et al., 2015; Sirikharin et
76 al., 2015).

77 AHPND appears during the first 20 to 30 days of culture, causing up to 100% mortality in
78 severe cases. In the acute phase, the infected hepatopancreas shows tubule epithelial
79 degeneration, round up and detachment of tubule epithelial cells from the basement
80 membrane, and sloughing of these cells into the tubule lumen. In the terminal stage, the
81 hepatopancreas shows extensive intertubular, hemocytic aggregations, which
82 causes hepatopancreas dysfunction and severe secondary *Vibrio* infections (Lightner et al.,
83 2012; Tran et al., 2013).

84 On the other hand, there are some SE countries, especially India and Indonesia, that have
85 only been affected by EHP (Tang et al., 2016; Rajendran et al., 2016), and so far, no cases
86 of AHPND have been reported. In these countries, there has been an increase of bacterial

problems contributing to hepatopancreas Vibriosis, which have been called with different names including the running mortality syndrome and white feces syndrome. Shrimp affected by these conditions display septic hepatopancreatic necrosis (SHPN) (Mastan and Begun, 2016; Ramsden 2015; Tang et al., 2016).

Based on the fact that EHP has been affecting the shrimp aquaculture industry before the occurrence of AHPND outbreaks, it is possible that EHP favors the establishment of AHPND and other bacterial diseases, such as SHPN. To determine the relationship of EHP to AHPND and SHPN, we evaluated the risk factor of EHP with these diseases, AHPND through experimental challenge tests and SHPN by a case-control analysis

2. Material and methods

2.1 Shrimp

Specific-pathogen-free (SPF) *Penaeus vannamei* (Shrimp Improvement Systems, Islamorada, FL) were used. All of the experimental infections were carried out in the Aquaculture Pathology Laboratory (APL) at the University of Arizona.

2.2 EHP infection

EHP-infected shrimp from Thailand were used to propagate EHP infection in SPF shrimp at the APL (Tang et al., 2015). Minced hepatopancreas tissue from infected shrimp was used to feed, at 10% of their body weight, to the SPF population; after three weeks the EHP infection was confirmed by PCR and histopathology.

2.3 AHPND bacterial strains and culture

A mutant of *Vibrio parahaemolyticus* 13-028/A3 (AHPND strain), was used for this study. For purpose of this paper it will be called VP_{AHPND}. This mutant carries a plasmid

encoding a chloramphenicol acetyl transferase (CAT) and a red fluorescent protein. Tryptic soy broth (TSB) with 2% NaCl (TSB+) and chloramphenicol (15 µg/ml) was used for culturing this strain at 28°C overnight with gentle shaking (100 rpm) to reach 1.5×10^9 CFU mL⁻¹.

2.4 AHPND pathogenicity bioassays and histopathology

Two AHPND experimental infections were conducted to compare the pathogenicity of VP_{AHPND} on shrimp juveniles pre-infected with EHP. The group “AHPND” was free of EHP prior of being infected with VP_{AHPND}, while the group “EHP-AHPND” was pre-infected with EHP for three weeks prior the AHPND infection. The “EHP negative control group” was a negative control group only exposed to EHP and not exposed to AHPND. The “SPF” was a negative control group that was not exposed to either EHP or VP_{AHPND}. For the first experimental infection, five treatments with five 90-L tanks and, for the second experimental infection, five treatments with ten 90-L tanks (2 replicates) were filled with artificial seawater (salinity of 25 ppt and temperature at 28°C), and 5±1 and 10±1 SPFP. *vannamei* (weights: 2.0-3.0 g) were stocked in each tank for the experimental infection #1 and #2 respectively. AHPND and EHP-AHPND groups were challenged by immersion with VP_{AHPND} by adding an overnight culture broth into the aquaria to obtain a final concentration of 2.4×10^5 VP_{AHPND} CFU mL⁻¹ (low dose). The positive control was challenged with VP_{AHPND} to obtain a final concentration of 2.4×10^6 VP_{AHPND} CFU mL⁻¹ (high dose). The details of the infection are presented in the Table 1. Mortality was recorded daily from the start of the experiment. Five EHP-infected shrimp were analyzed prior the AHPND experimental infection. Moribund shrimp were fixed in Davidson's AFA fixative (Bell and Lightner, 1988). Samples of dead shrimp

were collected to determine the presence of EHP and AHPND by PCR. The duration of the challenges were 10 and 14 days for the experimental infection #1 and #2, respectively.

2.5 Bacterial count and histopathology in a 12h time infection course

A 12h time infection course study was carried out to determine the histopathological changes and the colony forming units (CFU) in the two experimental groups (EHP-AHPND and AHPND). Twenty shrimp from each group were stocked in tanks and challenged by immersion to a high dose of VP_{AHPND} (2.4×10^6 CFU/ml water). Samples of 2-4 shrimp were taken out at 3h, 6h, 9h and 12h post AHPND-infection (h.p.i) for histopathology. In addition, at times 6 and 12 h.p.i, 5 samples were analyzed to determine the CFU g^{-1} of hepatopancreas. From each shrimp, the gastrointestinal tract (GI-tract) and posterior stomach was removed from the hepatopancreas, then, the hepatopancreas was longitudinally bisected just lateral of the mid-line. One half was preserved in Davidson's fixative; another half of the hepatopancreas was used for bacterial counting.

2.6 Relationship between hepatopancreatic microsporidiosis (HPM) and septic hepatopancreatic necrosis (SHPN) at farm level.

To determine the possible association between HPM and SHPN, a case-control study was carried out. For this, records of 19 grow-out ponds from farms sampled during 2015-2016 with history of EHP were analyzed. In total, 100 juvenile *Penaeus vannamei* samples were analyzed by histopathology. Proportion of cases: control were 1:4. As cases, juvenile *P. vannamei* with diagnosis of the SHPN were chosen. As controls, juveniles from the same farms with no presence of SHPN by histopathology were selected. Due to the fact that one of the main clinical signs for EHP is size variability, the variable "average

body weight (ABW)” was not included in this analysis. The diagnosis of SHPN by histopathology was characterized by generalized atrophy of the hepatopancreastubules with multifocal necrosis and hemocytic nodules, some melanized (Lightner 1996). The diagnosis of EHP was conducted by histopathology and confirmed by either *In situ* hybridization or PCR.

2.7 Histopathology and *In situ* hybridization (ISH) for EHP

The Davidson's alcohol-formalin-acetic acid (AFA)-fixed samples were processed, embedded in paraffin, and sectioned (4 µm thick) in accordance with standard methods (Lightner 1996). After staining with hematoxylin and eosin (H&E), the sections were analyzed by light microscopy. Severity grade of infection/lesion arranged from G0-G4 according Lightner (1996) being G0 absence of the disease and G4 indicate presence of severe lesions and advanced tissue destruction. For ISH, histological sections of 4 to 5 µm thickness were prepared from Davidson's fixed and paraffin-embedded shrimp tissues. Digoxigenin-labeled probes and colorimetric detection of the anti-DIG Fab fragment (alkaline phosphatase conjugate) with nitroblue tetrazolium and X-phosphate was used. The use of EHP probe is described in Tang et al.(2015).

2.8. EHP and AHPND PCR assay

For EHP detection by PCR, oligonucleotide primers EHP-510F (5'-GCC TGA GAG ATG GCT CCC ACG T) and EHP-510R (5'-GCG TAC TAT CCC CAG AGC CCG A), which generated a 510 bp amplicon, were used in the conventional PCR analysis (Tang et al., 2015). For AHPND detection by PCR, oligonucleotide primers PirB-F (5'-TGA TGA AGT GAT GGG TGC TC) and AHPND PirB-R (5'-TGT AAG CGC CGT TTA ACT CA), which generated a 392 bp amplicon, were used in conventional PCR analysis (Han

et al., 2015). PuReTaq Ready-to-Go PCR beads (GE Healthcare) were used. Amplifications for EHP and AHPND were performed with the following cycling parameters: initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 30s, and a final extension at 72°C for 5 min. Following amplifications, the PCR products were electrophoresed in 1.5% agarose gels containing 0.5 µg/ml ethidium bromide and visualized under ultraviolet light and digitally photographed by the AlphaImager (Alpha Innotech).

2.9 Statistical analysis

Comparison of mean bacterial load between the two groups was determined by twosided tailed t-test ($\alpha = 0.05$) using GraphPad. The difference in the survival curve was analyzed by Kaplan-Meier survival analysis using the software Med Calc 16.8.4. The association between SHPN and EHP was determined by calculating the Odds Ratio (OR) and 95% confidence intervals (CI) using the software OpenEpi (Dean et al., 2013).

3. RESULTS

3.1 EHP infection

Before AHPND infection, EHP was confirmed by histopathology in samples of EHP pre-infected shrimp and in the EHP-negative control group. Histological examination of samples of EHP-infected *P. vannamei* show basophilic inclusion bodies within the cytoplasm of the hepatopancreas tubule epithelial cells which appears to be a plasmodium stage (Tourtip et al., 2009). Mild sloughing of the tubular epithelial cells was noticed in shrimp-affected hepatopancreas and spores released in lumen were also observed. The severity of the affected shrimp ranged between G1-G2 according to a semi-quantitative

scale (Lightner,1996). The infection was more prominent in the medial and proximal region than in distal region. No lesions of AHPND were observed in any shrimp prior to the experimental infection.

3.2 AHPND Experimental infection

The results of the experimental VP_{AHPND} infection are shown in the Table 1. No mortality was observed in the negative control tanks in the two experiments. The VP_{AHPND} used in this experiment caused AHPND in the infected animals as its presence was confirmed by PCR and histopathology. High mortalities (83% and 64%) occurred in the groups exposed to a high dosage of *V. parahaemolyticus*. Juvenile from the EHP-AHPND group challenged with a low dose displayed higher mortalities than those from AHPND group: 60% vs. 0% and 44% vs. 18% in the experiment #1 and #2, respectively. A comparison of the survival curves between the AHPND vs. EHP-AHPND groups was carried out and a significant difference was found ($P < 0.05$) (Fig. 1).

By histology, moribund animals showed lesions typical of AHPND, including a massive sloughing of hepatopancreatic tubule epithelial cells in the medial and proximal regions of the hepatopancreas and progressing outward to the distal region. Affected tubule epithelial R and F cells displayed prominent nuclear karyomegaly. In some shrimp, the terminal phase characterized by a typical SHPN was observed. In EHP-infected shrimp, the plasmodium and spores stages were observed.

Dead animals were analyzed by PCR for AHPND and EHP. In the group AHPND and the positive control tanks, only positive results of AHPND were found. In the group EHP-AHPND, shrimp were positive for both: AHPND and EHP. At the end of the experiment, survivors shrimp did not show any histopathological lesions of AHPND.

3.3 Histopathology and bacterial count in a 12 h time infection course

During the first 6 hours post infection (h.p.i), no histological lesions of AHPND were observed in the AHPND group. Only a slight decrease of the hepatopancreas lipid droplets levels. At 12 h.p.i, two shrimp displayed an early phase of AHPND lesions with a severity grade G1 (Table 2); histological changes included sloughing of tubules cells and a focal necrosis of the medial proximal region (Fig. 2a, b, c). In the EHP-AHPND group, at 0 h.p.i only mild lesions of EHP were found and confirmed by ISH (Fig. 2g), however the hepatopancreas lipid droplets level was in the normal range (G3) (Fig. 2d). Mild histopathological lesions of AHPND were observed during the first 6 h.p.i including a decrease of the lipid droplets, karyomegaly, hepatopancreas epithelial cell blebbing and moderate sloughing of the tubule cells, (Fig. 2e). In comparison with the ISH, not all the sloughed cells had a positive reaction for EHP, which suggested that the sloughing was present in EHP-infected cells and EHP-uninfected cells. At 12 h.p.i, there was an absence of B cells, the necrosis progressed towards the distal region and a massive sloughing occurred (Fig. 2f). Interestingly, most of the sloughed cells were EHP-infected cells as it was confirmed by the ISH (Fig. 2i). At 12 h.p.i, 57% of the shrimp in the EHP-AHPND group displayed AHPND versus the 11% from the AHPND group. No histological signs of AHPND were found in the negative control shrimp (Table 2).

The CFU data is shown in the Fig. 3. VP_{AHPND} CFU g^{-1} of hepatopancreas at 0 h.p.i was not detected. It indicated that no other chloramphenicol resistant bacteria were present in the sample. There was not any significant difference between the CFU between the AHPND and EHP-AHPND groups at 6 h.p.i (1.1×10^8 vs. 3.4×10^7 CFU g^{-1} hepatopancreas) and 12 h.p.i (5.9×10^6 vs. 2.0×10^6 CFU g^{-1} hepatopancreas) respectively ($P > 0.05$).

However, combining the CFU of the two groups at 6 and 12 h.p.i, there was a significant difference in the bacterial loads being higher at 6 h.p.i with a mean of the 7.2×10^7 vs. 4.0×10^6 CFU g⁻¹ hepatopancreas at 12 h.p.i. ($P < 0.05$) (Fig.3).

3.4 Case control study: EHP and SHPN at farm level.

Records of 100 juvenile shrimp *P. vannamei* from 19 grow-out ponds from farms located in SE Asia with history of EHP were analyzed during 2015-2016. Based on our data (Table 3), there was a strong association between cases of SHPN and the EHP-infected shrimp: odds ratio (OR) = 4.2; 95% CI: 1.1-15.4. The frequency of SHPN was higher in shrimp with EHP than in those without EHP.

4. Discussion

Our study showed, through two independent methods, that EHP-infected *P. vannamei* have increased susceptibility to infection by *Vibrio* associated with AHPND and SHPN. In controlled laboratory trials, juveniles infected with EHP prior to being exposed to VP_{AHPND} displayed more severe histopathological lesions than those not pre-infected (Fig. 2f). Sloughing of hepatopancreas tubule cells was most severe in the EHP group, and the influence of EHP increased as the *Vibrio* infection progressed. At 6 h.p.i sloughed cells included both EHP-infected and uninfected cells, but, by 12 h.p.i almost all of the sloughed cells were EHP-infected (Fig. 2I). Interestingly, the VP_{AHPND} CFU was approximately 18 times higher at 6 h.p.i. than at 12 h.p.i., although mortalities were not seen until 12 h.p.i. The reasons underlying the declining CFU over the course of the trial are unclear; but obviously, in spite of this, damage to the hepatopancreas was severe enough to eventually cause mortality. Similar observations have been made by others

(Lee et al., 2015; Lai et al., 2015).

The dramatic effects of the co-infection are most likely explained by the synergistic actions of the microsporidian and bacterial pathogens on the tissue of the hepatopancreas.

As a part of the necrosis process, both EHP and AHPND cause sloughing by detachment of epithelial cells from the basement membrane (Lightner et al., 2012; Tran et al., 2013).

However, these two pathogens cause sloughing in different ways. EHP-infected cells contain plasmodia or spores in the cytoplasm and the microsporidian uses the cell nutrients to replicate. This depletes the cell nutrients resulting eventually in the death and rupture of the cell. In contrast, the sloughing caused by VP_{AHPND} is due to the effect of the toxin on the cytoskeletal proteins (such as microtubules, actin filaments) involved in the cell's attachment to the basement membrane. In this case, the whole cell, with an apparently intact nucleus, is sloughed off. Through their combined actions, co-infection of VP_{AHPND} and EHP dramatically increases the damage to the hepatopancreas, to the point of causing total tissue failure and morbidity.

Through use of a different method, a case-control study, a similar effect of EHP on shrimp susceptibility to SHPN was found. This is another disease resulting primarily from *Vibrio* spp. infection (Lightner 1996). The strong association between cases of SHPN and EHP that we found, suggests that EHP increases shrimp susceptibility to infection by *Vibrio* bacteria, which are known to be opportunistic pathogens that cause disease when shrimp health is compromised (Lightner 1996). EHP infection disrupts the cells of the hepatopancreatic tubules and allows *Vibrio* spp. already present to colonize the sloughed cells and the exposed basement membrane.

These findings may shed light on the patterns of emergence of disease outbreaks in

shrimp aquaculture worldwide. For example, countries in SE-Asia such as China, Vietnam, Thailand, and Malaysia that have faced EHP outbreaks during the last ten years, eventually were hit by AHPND years later. Other countries such as Indonesia and India have recently reported the presence of EHP but have not yet experienced outbreaks of AHPND. However, in Indonesia an emerging disease known as white feces appears to be due to the co-infection of EHP with other opportunistic *Vibrio* spp. that cause SHPN (Tang et al., 2016; Briggs 2012). Similarly, in India, the running mortality syndrome (RMS) (Ramsden 2015) has been recently reported with shrimp displaying severe histopathological lesions of the hepatopancreas, including sloughing of tubule epithelial cells and hemocytic inflammation (Mastan and Begun, 2016).

The development of measures to prevent EHP or to treat affected ponds could greatly reduce production losses in shrimp aquaculture. Research directed toward this end should be encouraged.

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Table 1 Comparative mortality of shrimp pre-infected by EHP (EHP-AHPND) vs. no pre-infected (AHPND), infected with VP_{AHPND}.

Expt	Experimental group	Treatment	Dosage (CFU/mL of water)	No. of dead shrimp/ no. total	Mortality (%)
1	AHPND	Low dose	2.4×10^5	0/5	0%
	EHP-AHPND	Low dose	2.4×10^5	3/5	60%
	Positive control	High dose	2.4×10^6	5/6	83%
	Tank Control	EHP Negative control	0	0/5	0.0%
	Tank Control	SPF Negative control	0	0/5	0.0%
2	AHPND	Low dose	2.4×10^5	2/11	18%
	EHP-AHPND	Low dose	2.4×10^5	4/9	44%
	Positive control	High dose	2.4×10^6	7/11	64%
	Tank Control	EHP Negative control	0	0/5	0.0%
	Tank Control	SPF Negative control	0	0/5	0.0%

Table 2 Histopathology results of AHPND and EHP-AHPND group exposed to VP_{AHPND} at a final dosage of 2.4×10^6 CFU mL⁻¹ of water

Time post infection (h)	Group	N° shrimp tested	AHPND lesions	AHPND cumulative lesions
0	AHPND	2	0	2/18 (11.1%)
3	AHPND	4	0	
6	AHPND	4	0	
9	AHPND	4	1 (G1)	
12	AHPND	4	1 (G1)	
0	EHP-AHPND	2	0	8/14 (57.1%)
3	EHP-AHPND	3	1 (G1)	
6	EHP-AHPND	3	2(G1)	
9	EHP-AHPND	3	2(G1)	
12	EHP-AHPND	3	2(G2)	

Table 3. Cases of SHPN and controls and association with EHP in juvenile *P. vannamei* shrimp farming in SE Asia on 2015-2016

		Cases SHPN	Controls	Total
EHP	Yes	17	46	63
	No	3	34	37
Total		20	80	100

Fig 1 Comparison of the cumulative survival curve between AHPND vs. EHP AHPND groups infected by AHPND. Experiment# 2

Fig 2. H&E (Mayer–Bennet haematoxylin and eosin-phloxine) histology and in situ hybridization of AHPND and Enterocytozoon hepatopenaei (EHP) in the infected shrimp. (A-F) H&E staining of hepatopancreas tissue of the *Penaeus vannamei*. (G-I) In situ hybridization with a digoxigenin-labeled EHP probe. The presence of dark blue precipitates indicates the presence of EHP. (A-C) H&E staining of *P. vannamei* challenge with VP_{AHPND} at 0 (A), 6 (B) & 12 (C) h.p.i ; (D-F) H&E staining of *P. vannamei* pre-infected with EHP & challenge with VP_{AHPND} at 0 (D), 6 (E) & 12 (F) h.p.i . Scale bars=100 μm.

Fig 3 Comparison of the VP_{AHPND} CFU g⁻¹ HP in shrimp exposed at 6 and 12 h.p.i. Data represent the mean $\pm$ SE. Different letters indicate significant difference ($P < 0.05$)