Immunological and histopathological changes in *Penaeus semisulcatus* challenged with *Vibrio harveyi*

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Abstract

Two-hundred and sixty five green tiger shrimp juveniles (*Penaeus semisulcatus*) with the average weight of 7-12g were collected from Helleh farms in Bushehr province and transported to Iran Shrimp Research Center of Bushehr in October, 2009. The juveniles were acclimated for two weeks. The experiment was designed in three treatments (named 3, 4 and 5) and two controls (named 1 and 2) in triplicate with 15 shrimp in each repetition prepared of glass aquarium. All the treatments and repetitions were exposed to *Vibrio harveyi* (NCBI: GU974342.1). The concentrations of the treatments were 10^8,10^6 and 10^4 CFU ml^{-1} in individual containers dedicated for each mentioned treatment (3, 4 and 5, respectively). The controls prepared with no any bacteria and fully filled with chlorinated and UV treated sea water were named 1 and 2 respectively. The hemolymph were withdrawn from abdominal segments of samples for measuring THC and TPC evaluation at designed hours (2, 6, 12, 24, 48, 96, 144, 192 and 240). The shrimp samples were also fixed in Davidson fixative for histopathological studies. The results showed that the difference of THC value between controls and group 3 during 12 till 96 hours of experiment was significant ($P< 0.05$). The differences between groups 4 and control of THC value during 24 to 144 hours of experiment were also significant ($P< 0.05$). There was no significant difference ($P> 0.05$) between group 5 and control groups of THC. The data showed that differences of TPC value between control groups during 24 to 96 hours were significant ($P< 0.05$), whereas the differences between controls with groups 4 and 5 during 48 to 144 hours and 192 hours, were significant ($P< 0.05$) respectively. TPC and THC were observed with an increase in the concentration of bacteria and passing the time as inverse bell shape procedure. In histopathology, gills showed melanization and color changed to brown and black. The hepatopancreas cells revealed necrosis and vacuolization of B, E, R and F cells. The bolitas ball and bacterial colonization was observed in the intestine. Our results showed that *Vibrio harveyi* with 10^8 and 10^6 cell/ml decreased immunity factors such as THC and TPC. The histopathological changes increased with increasing the concentration of bacterial level. This finding can be used for assessing the health of shrimp culture and prevention of vibriosis.

Keywords: *Vibrio harveyi*, *Penaeus semisulcatus*, Total hemocyte count, Total protein concentration, Histopathology

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Introduction

Iranian shrimp culture started from 1992 in the south of Iran. At the first time *P. semisulcatus* was chosen for culture as main species. The commercial production encountered by mass mortality, due to some virulent pathogens such as bacteria and viruses. *Vibrio harveyi* is the major pathogen infecting the cultured shrimp (Afsharnasab et al., 2008). In shrimp vibriosis is often related to injury, stress or other pathogens diseases. The pathogenic mechanisms and defense reactions during vibriosis are not yet clearly understood (Van de Braak et al., 2002). However, serious outbreaks of infectious diseases dealing with the shrimp culture industry, particularly by viruses and bacteria have been reported (Hsieh et al., 2008). Since 1998, shrimp farmers have experienced disease problems causing production losses due to a few serious outbreaks by Taura Syndrome Virus (TSV), and *Vibrio spp.* such as *Vibrio alginolyticus* and *Vibrio harveyi*. Disease outbreaks are often results of unfavorable environmental condition, e.g. stress associated with intensive shrimp culture (high stocking density) and increases in the proportion of potentially pathogenic species of cultured pond water (Hsu and Chen, 2007).

Because of interesting in the shrimp production and therefore intensive rearing systems, there are many reports on epizootic luminescent bacterial diseases, especially in the shrimp farms of Asian countries. The dominant species of Luminescent vibriosis are *V. harveyi, V. campbellii* and occasionally *V. splendidus* infecting larval, juveniles and adult stage of penaeid shrimp. Mass mortality with high percent in larvae occurred in some hatcheries of *penaeus monodon* in Philippines due to acute *vibrio harveyi* strains with density of 10^2 cell ml^{-1}. Other mass mortalities of shrimp larvae were also reported from Australia, South America and Mexico (Soto-Rodriguez et al., 2006). The studies of *vibrio .spp* prevalence have been done during 2004 to 2009 in Iran and the main species were identified as *V. parahemolyticus, V. vulnificus, V. harveyi* and *V. alginolyticus* (Afsharnasab, 2009). The evaluation of the immune system could be applied for assessing shrimp health, which is confirmed by aquaculture experts (Rodrıguez and Le Moullac, 2000). Haemocytes play a major role in crustacean immune system. First, they eliminate foreign particles in the hemocoel through phagocytosis, encapsulation and nodular aggregation. Secondly, haemocytes participate in wound healing by cellular clumping and begin coagulation processes through the release of factors required for plasma gelation (Kakoolaki et al., 2010). There is some information over the importance of THC in pathogen resistance. Evidence has been given regarding the physiological role of the plasma protein concentration and its susceptibility to environmental or physiological changes in the animal (Rodrıguez and Le Moullac, 2000). Histopathology is a suitable tool for monitoring and diagnosis health, where the changes at the cells and tissues due to the pathogen are interpreted to arrive at diagnosis. (Ambipillai et al, 2003).

The aim of the study was to assess the THC and TPC in the shrimp *P.*

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semisulcatus exposed to *V. harveyi* and histopathology study of some tissues.

Materials and methods

Two-hundred and sixty five green tiger shrimp juveniles (*Penaeus semisulcatus*) with average weight of 7-12g were collected from Helleh farms in Bushehr province and transported to Iran Shrimp Research Center of Bushehr in October, 2009. The juveniles were acclimated in the laboratory for two weeks. Shrimps which were in the intermolt stage were used for the study. The retraction of epidermis could be applied as an index to distinguish the molt stage (Robsertson et al., 1987).

During the acclimation and study span, shrimp were fed four times daily on a commercial shrimp diet (Havvorash Company, Bushehr, Iran). During the study, water conditions were measured so the temperature was 25± 1ºC, pH 8.0 6–8.34 and a salinity of 34ppt.

V. harveyi

A known pathogen strain named *V. harveyi* (NCBI: GU974342.1), which had been isolated from diseased shrimp of Bushehr province farms, identified in Iran Scientific and Industrial Research Organization (PTCC: 1755) and documented in the American Gene Bank (NCBI: GU974342.1) was used in the study. The stocked specimen of the bacteria was cultured on tryptic soy agar (TSA supplemented with 2.5% NaCl, Difco) for 24 h at 25-30 º C. It was then transferred to 10 ml tryptic soy broth (TSB supplemented with 2.5% NaCl, Difco) for 24 h at 25±1 ºC for use as a stock bacterial broth. For challenge experiments, Stocked cultured media were centrifuged at 7155× *g* for 20 min at 4ºC. The supernatant fluid was send out and the bacterial clog was resuspended in saline solution (0.85% NaCl) at 10^4, 10^6 and 10^8 colony-forming units (cfu) ml^{-1} for the exposure test.

Cell densities were photometrically obtained at 590 nm wavelength (Saulnier et al., 2000), based on the MacFarland Standard (equal to 10^4, 10^6 and 10^8 cfu ml^{-1}) and serially diluted to reach the density of 10^4, 10^6 and 10^8 CFU ml^{-1} .

Treatments

The experiment was designed in three treatments (named 3, 4, and 5) and two controls (named 1 and 2) in triplicate with 15 shrimp in each repetition prepared from a glass aquarium. The controls were prepared with no bacteria and full filled with cholorinated and UV treated sea water named 1 and 2 respectively. According to Robertson, et al. (1998) the exposure span of *P. semisulcatus* to *Vibrio sp*. was designed for 2 h in which good sterile aeration and no water exchange during the immersion were accompanied. Shrimp samples were then washed with sterile sea water and transferred back.

Haemolymph sampling

Haemolymph was obtained from the ventral part of the haemocoel of the second abdominal segment, using a 25 gauge needle and an 1 ml syringe filled with 0.4 ml cold modified Alsever's solution (AS:19.3 mM Na citrate, 239.8 mM NaCl, 182.5 mM glucose, pH 7.2, 6.2 mM EDTA (ethylene diaminetetra-acetic acid)) as an anticoagulant solution. The AS prevented melanisation and kept haemocytes in a quiescent state (Rodriguez et al., 1995).

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The puncture procedure prevented to extract tissue particles during the haemolymph sampling (Van de Braak et al., 1996).

The remaining haemolymph was stored in 5 ml Eppendorf cups and kept on ice until analysis within 1 h after sampling. Haemolymph were obtained at 2, 6, 12, 24, 48, 96, 144, 192 and 240 hours after exposure to different concentration of bacteria according to the study method. Signed and diseased shrimp were collected as samples of our study.

Haemocytes

A drop of the anticoagulant-hemolymph mixture was placed on a heamocytometer to measure THC (total hemocyte count) using a light microscope (Jiang et al., 2004). The remainder of the mixture was used for protein assay tests.

Haemolymph plasma

Haemolymph was centrifuged at 1500×g at 4° C for 10 minutes. The cell-free haemolymph (plasma) was collected for plasma protein investigation. Total plasma protein (TPP) was measured according to a modified Lowry-method, using bovine serum albumin (BSA) as a standard (Van de Braak et al., 1996)

Histopathology

Tissues samples were also collected at 2, 6, 12, 24, 48, 96, 144, 192 and 240 hours after exposure to different concentrations of bacteria according to the study method. Shrimp tissues were fixed in Davidson's fixative for 48 hours and transferred to 70% ethanol. After processing and hydration of tissues, wax impregnation was done. The paraffin wax embedded samples were sectioned, mounted on slides and stained with Myer-bennet haematoxylin and pheloxin/eosin (Lightner 1996).

Statistical analysis

A one-way ANOVA test was used to compare the differences between the groups (treatments and controls) affecting THC and TPC values at the confidence interval of 95% ($p< 0.05$). Multiple comparisons along with the Benferroni statistic was conducted to show that at least one group has a significant difference with others while the *P* value represented the value lower than 0.05. All statistical tests were evaluated using the V. 16 SPSS computer software.

Results

THC

According to our study there were no significant differences in THC for any of the shrimp from beginning to the following 6 h exposure to *V. harveyi* ($P>0.05$). For the shrimp which had been transferred to 10^8 CFU ml^{-1}, the THC decreased significantly by 29%, 52% and 39% after 24, 48 and 96 h, respectively ($P<0.05$). However significant differences in THC between 10^8 CFU ml^{-1} with controls and 10^4 CFU ml^{-1} from 12 h to 96 h after exposure time were observed ($P<0.05$). For the shrimp which had been transferred to 10^6 CFU ml^{-1}, the THC decreased significantly by 19%, 41% and 20% after 24, 48 and 96 h, respectively ($P<0.05$). There were significant differences in THC between 10^6 CFU ml^{-1} with controls and 10^4 CFU ml^{-1} from 24 h to 144 h after exposure time ($P>0.05$). No significant difference was observed in THC among the shrimp exposed to 10^4 CFUml^{-1} and controls at different sampling

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times (P>0.05). A similar trend was observed for the end of experiment. After 144 h, in eighth and ninth sampling (192 and 240 h respectively) there were no significant differences in THC for any of the groups (controls and treatments) (P>0.05) (Table 1 and Figure 1).

Table 1: The mean and standard deviation of THC at hours after exposure time of *V. harveyi* in different groups of study.

THC

	Sampling times (h)								
	2	*6*	*12*	*24*	*48*	*96*	*144*	*192*	*240*
Groups	*Mean(×10^5)± S.D.*	*Mean(×10^5) ± S.D.*	*Mean(×10^5) ± S.D.*	*Mean(×10^5) ± S.D.*	*Mean(×10^5) ± S.D.*	*Mean(×10^5) ± S.D.*	*Mean(×10^5) ± S.D.*	*Mean(×10^5) ± S.D.*	*Mean(×10^5) ± S.D.*
Control cl	*123.33± 2.51*	*121± 3*	*124± 4.35^a*	*125.66± 8.62^a*	*125.33± 7.63^a*	*122.33± 5.13^a*	*123.33± 5.50^a*	*121.66± 2.08*	*124± 3.60*
Control UV	*125± 7.97*	*122± 5.19*	*123.33± 2.30^a*	*125.66± 4.04^a*	*121.33± 3.21^a*	*123.33± 3.51^a*	*124.33± 4.04^a*	*121± 3.46*	*123.33± 6.65*
10^8 CFU ml^{-1}	*121.33± 3.51*	*119.33± 4.04*	*110.33± 2.51^b*	*85.66± 3.21^b*	*58± 5.56^b*	*73.33± 5.85^b*	*118.33± 4.04*	*121.66± 3.78*	*124± 4*
10^6 CFU ml^{-1}	*122.66± 4.04*	*121.66± 3.05*	*117.33± 3.05*	*99.33± 10.06^b*	*72.33± 3.78^b*	*98.33± 1.52^c*	*100± 11.53^b*	*125.66± 4.16*	*127.33± 4.16*
10^4 CFU ml^{-1}	*123.33± 1.15*	*123.33± 4.93*	*122.33± 5.50^a*	*121± 3.60^a*	*119.33± 4.50^a*	*122± 3.60^a*	*123.66± 5.13^a*	*127± 4.58*	*124.66± 4.04*

*significant differences are shown by dissimilar superscripts in same columns.

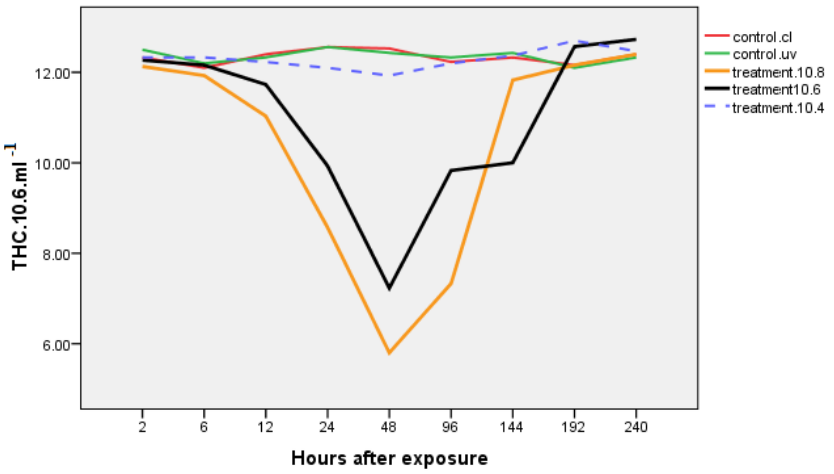


Figure 1: The effect of different bacteria on THC

TPC

Approximately a similar trend was observed for TPC parameter. There were no significant differences in THC for any of the shrimp from beginning to 12 h exposure to *V. harveyi* (*P*>0.05). For the shrimp which had been transferred to 10^8 CFU ml^{-1}, the TPC decreased significantly by 15%, 28%, 34%, 21% and 12% after 24, 48, 96, 144 and 192 h, respectively (P<0.05). However significant differences

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in TPC between 10^8 CFU ml^{-1} with controls and 10^4 CFU ml^{-1} from 24 h to 96 h after exposure time were observed ($P<0.05$), but after 96 h, from 144 to 192 h significant differences were shown in TPC between 10^8 CFU ml^{-1} with UV control and 10^4 CFU ml^{-1} ($P<0.05$). For the shrimp which had been transferred to 10^6 CFU ml^{-1}, the TPC decreased significantly by 15%, 29% and 20% after 48, 96 and 144 h, respectively ($P<0.05$). Our results showed significant differences in TPC between 10^6 CFU ml^{-1} with controls and 10^4 CFU ml^{-1} at 48 h and 144 h following exposure time ($P>0.05$), also significant difference was observed in TPC between 10^6 CFU ml^{-1} with 10^4 CFU ml^{-1} at 96 h following exposure time ($P>0.05$). There were no significant differences observed in TPC among the shrimps exposed to 10^4 CFUml^{-1} and controls at different sampling times except in 192 h that has a significant difference with chlorinated control ($P>0.05$). At the end of the experiment after 192 h, in the ninth sampling (240 h) there were no significant differences in TPC for any of the groups (controls and treatments except 10^8 CFU ml^{-1}) ($P>0.05$), that significant difference was observed between 10^8 CFU ml^{-1} and chlorinated control ($P>0.05$) (Table 2 and Figure 2).

Table 2: The mean and standard deviation of TPC at hours after exposure time of *V. harveyi* in different groups of study

TPC

	Sampling times (h)								
	2	*6*	*12*	*24*	*48*	*96*	*144*	*192*	*240*
Groups	*Mean± S.D.*	*Mean± S.D.*	*Mean± S.D.*	*Mean± S.D.*	*Mean± S.D.*	*Mean± S.D.*	*Mean± S.D.*	*Mean± S.D.*	*Mean± S.D.*
Control cl	*118.36± 7.58*	*109.76± 12.02*	*117.72± 5.71*	*125.01± 4.25^{a}*	*123.16± 3.01^{a}*	*108.14± 7.85^{ac}*	*113.82± 4.43*	*101.55± 7.54^{a}*	*126± 7.17^{a}*
Control UV	*115.66± 9.25*	*102.16± 6.27*	*123.36± 1.24*	*121.63± 4.96^{a}*	*113.33± 4.05^{ac}*	*110± 7.05^{ac}*	*125.18± 3.16^{ad}*	*126.66± 7.38^{b}*	*115.99± 8.83*
10^8 CFU ml^{-1}	*117.76± 7.72*	*105± 8.79*	*119.91± 5.38*	*99.85± 7.33^{b}*	*85± 8.63^{b}*	*77.16± 17.02^{b}*	*93.33± 12.38^{b}*	*103.33± 8.22^{a}*	*106.67± 6.74^{b}*
10^6 CFU ml^{-1}	*116± 7.55*	*105± 2.45*	*116.66± 5.24*	*115.10± 6.18*	*98.33± 7.19^{bc}*	*81.67± 11.61^{ab}*	*91.67± 10.39^{cb}*	*115± 5.00*	*113.33± 3.34*
10^4 CFU ml^{-1}	*101.67± 9.61*	*116.66± 9.26*	*113± 4.12*	*125.01± 3.68^{a}*	*118.33± 6.39^{a}*	*123.25± 2.08^{c}*	*126.67±5. 99^{d}*	*125± 4.99^{b}*	*110± 5.63*

*significant differences are shown by dissimilar superscripts in same columns.

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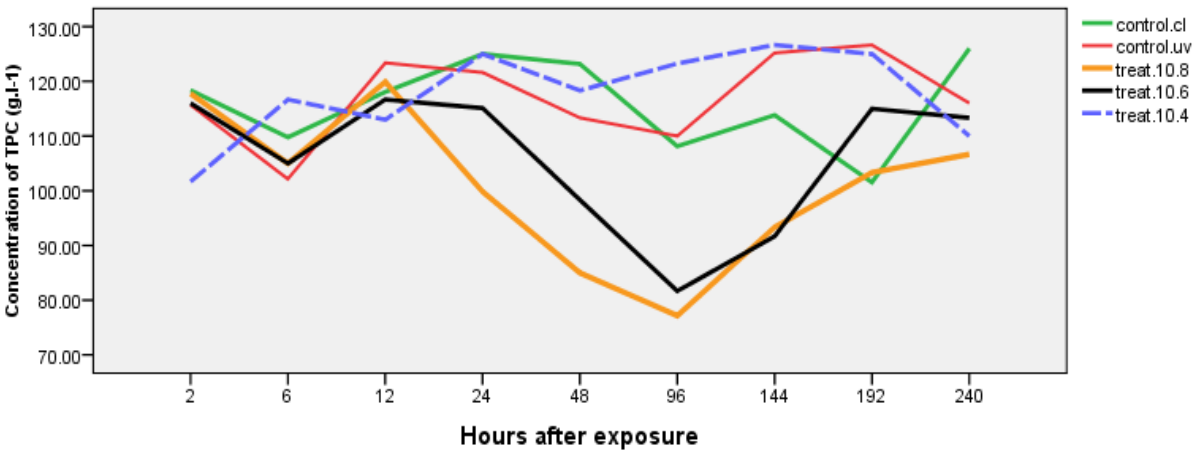


Figure 2: Effect of different bacteria on TPC

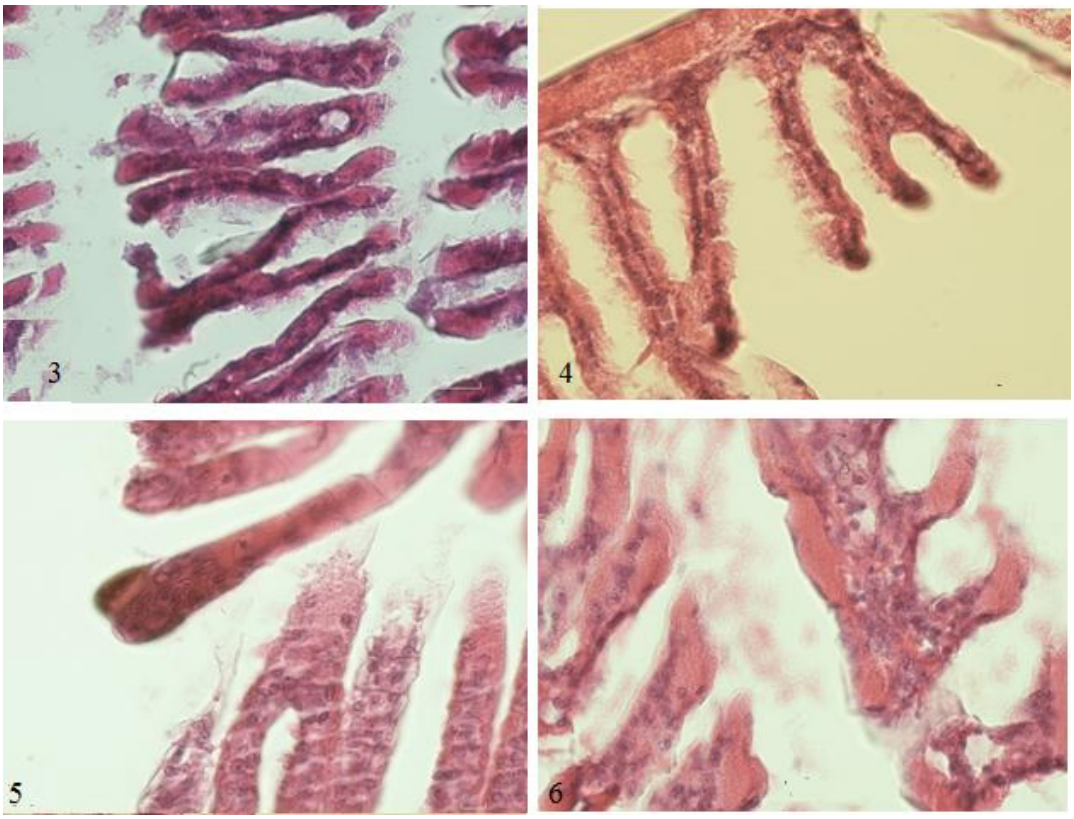


Figure 3: Infected gill lamellae of shrimps exposed to 10^8 CFU ml^{-1} *Vibrio harveyi* bacteria, 4 days after exposure time. All of lamellas are black and melanized. (H&E/Ph, Mag. 800).

Fig 4: Infected gill lamellas of shrimps exposed to 10^6 CFU ml^{-1} *Vibrio harveyi* bacteria, 4 days after exposure time. Nearly all lamellas are melanized. (H&E/Ph, Mag. 800).

Fig 5: Infected gill lamellas of shrimps exposed to 10^4 CFU ml^{-1} *Vibrio harveyi* bacteria, 4 days after exposure time. One lamella is melanized. (H&E/Ph, Mag.800).

Fig 6: Gill lamellas of control shrimps group (not exposed to *Vibrio harveyi* bacteria), 4 days after exposure time. Lamellae are intact and healthy. (H&E/Ph, Mag. 800)

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Histopathology findings

In histopathology sections, gills showed melanization and this appearance was higher with increase of bacterial dose. However shrimps that challenged with 10^8 CFU ml^{-1} bacteria almost of secondary lamellas were black and melanized. (Fig. 3) Shrimps that challenged with 10^6CFU ml^{-1} bacteria melanization of gills were less (Fig. 4). Also lesions in shrimps exposed with 10^4CFU ml^{-1} bacteria are lowest but this appearance wasn't observed in both control groups (Fig. 5 and Fig. 6).

Results of the histopathological examination showed lesions in haepatopancreas tissues were very extensive, necrosis and bolitas balls of haepatopancreas cells were observed, and increase in bacterial doses result higher lesions. Vacuolation in B, E, R and F cells of haepatopancreas show that all of the cells are involved in exposure to bacteria. Production of excretory enzymes due to vacuolation in cells results in the formation of balls in highest dose. Finally, necrosis was observed (Fig 7, 8, 9 and 10).

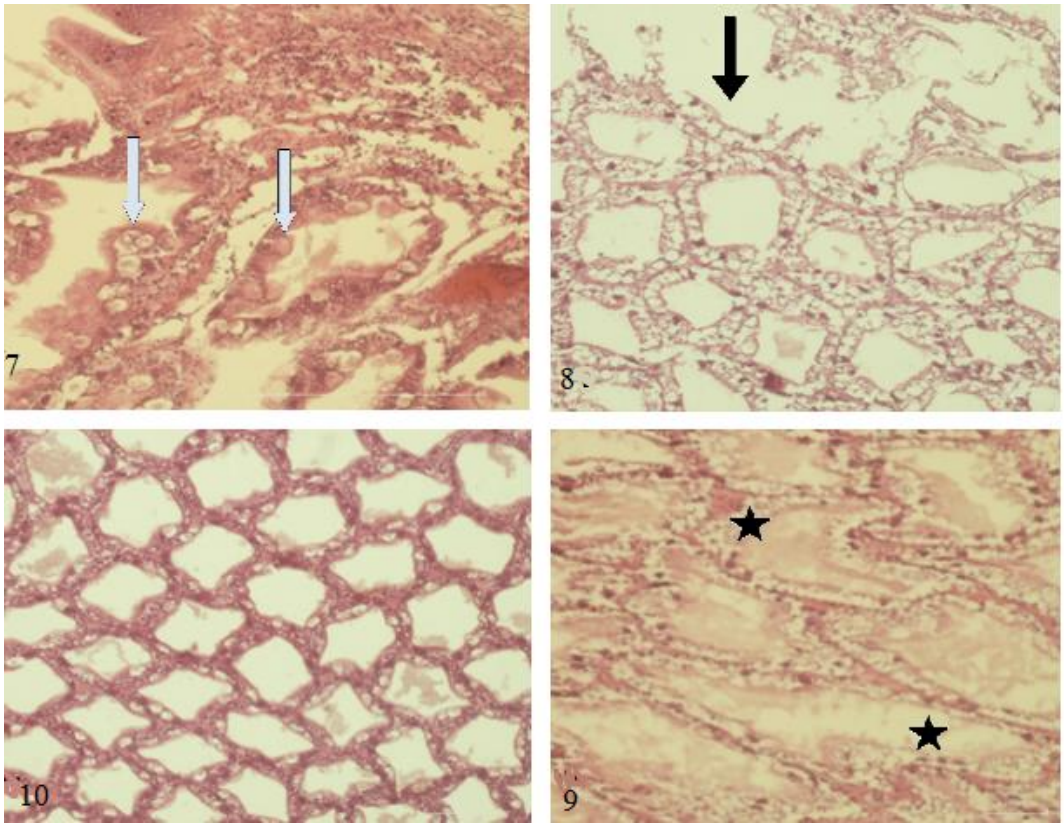


Fig 7: Infected haepatopancreas of shrimps exposed to 10^8 CFU ml^{-1} *Vibrio harveyi* bacteria, bolitas sign is observed (white arrows) destruction of haepatopancreas tissue and demolition of cells is shown. (H&E/Ph, Mag.800).

Fig 8: Infected haepatopancreas of shrimps exposed to 10^6 CFU ml^{-1} *Vibrio harveyi* bacteria. Severe vacuolation of cells (black arrow), cells are finally destroyed. (H&E/Ph, Mag.800).

Fig 9: Infected gill lamellas of shrimps exposed to 10^4 CFU ml^{-1} *Vibrio harveyi* bacteria. Lesions are low and some of tissues are healthy (stars) also vacuolation is observed. (H&E/Ph, Mag.800).

Fig 10: haepatopancreas of control shrimps group (not exposed to *Vibrio harveyi* bacteria). Cells are intact and healthy. (H&E/Ph, Mag.800).

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Discussion

According to the mean of THC and TPC in our study no significant differences were observed between groups (treatments and/or controls) in early time (2 and 6 hours) after the end of exposure time. It probably showed that the serological parameters could not be changed before 6 hours after encountering shrimp to bacterial pathogen such as *Vibrio harveyi*.

Of course the mean of TPC was stable until 12 hours after the exposure span. This result showed which TPP parameters could be changed as well as THC. Similar to another study as mentioned by Costa et al. 2009, low standard deviation in the mean of THC and decrease of it after exposure to the pathogen were observed in our study.

Chen et al. (1994) reported that total hemolymph proteins decreased at high levels of ammonia-N as a risk factor exposure, whereas free amino acids in hemolymph were increased concomitantly. These results have been explained as an occurrence of proteolysis under these situations, elevating the intracellular free amino acid pool for cell-volume maintenance as a result of the osmoregulatory disturbances caused by ammonia-N (Racotta & Herna´ndez-Herrera, 2000).

Decrease the THC in shrimp, *P. vannamei* challenged with TSV (Song et al., 2003) and in *P. indicus* and *P. monodon* with WSSV has been reported (Van de Braak et al., 2002).

Following exposure to WSSV In *P. monodon*, Hemolymph oxyhemocyanin, protein, ammonia and urea were 1.46 mM. Nitrite higher than that of the following exposure to 7.32 mM (cheng & chen, 2002). In our study, the variation of THC had clearly begun before TPC and the mean of TPC significantly decreased 12 hours after THC. Large variation was observed between the highest concentration of bacteria group (3) with controls and group 5. This result was constant from the time of 12 up to 96 hours after exposure span.The curve of THC in group 5 (the lowest concentration of bacteria) is relatively similar to the controls in all sampling times, after the exposure span. This showed that the THC of *P. semiculcatus* could not be affected by *V. harveyi* with the concentration of 10^4 CFU ml^{-1}. The mean of THC for group 3 was more affecting than the other two treatments, showing 1.3 fold greater than group 4 with the concentration of 10^6 CFU ml^{-1} of *V. harveyi.*According to our study, the intend curve of groups 3 and 4 showed an inverse bell shape in which the crown of the bell was located at the point of 48 hours after the exposure span. Based on our results the inversion bell shapes similar to THC of groups 3 and 4 (10^8 and 10^6 CFU ml^{-1} of *V. harveyi* respectively) were observed for the TPC in which the crown of the bell was located at the point of 96 hours after the exposure span. In invertebrates, the circulating hemocyte has a major role in the protection of the animal against aggressive microorganisms by participating in recognition, phagocytosis, melanization and cytotoxicity (Jiravanichpaisal *et al*., 2006). As mentioned in our study, after 6 hours of experiment the THC decreased and it means that the shrimp hemocyte is involved in vibrio protection by phagocytosis and melanization.

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The present study indicated that there were no significant differences observed in THC between the shrimps in the control groups and those infected by 10^4 cfu/ml vibrio. Because the vibrio is a bacterial flora of shrimp, this means the high concentration dosage of vibrio can attack the shrimp and induce the pathogencity, and so the shrimp at high concentration of vibrio could not resist in disease outbreaks. Therefore, differences in THC are due to the concentration of bacteria. Further research is needed to clarify whether the change in THC results from a consequence of lysis, diapedes, reduced recruitment or movement of the cells from circulation into the tissues. According to our findings gills showed melanization and this appearance was higher with increase of bacterial dose. Gills often appear brown (Anderson et al., 1988). Lesions of bacterial shell disease are brown or black and appear on the body cuticle, appendages or gills (Sinderman, 1990). We observed lesions in haepatopancreas tissues. There were very extensive, necrosis and bolitas balls of haepatopancreas cells observed and increase in bacterial doses result in higher lesions. B, E, R and F cells of haepatopancreas show vacuolation, all of the cells are infected in challenge to bacteria. Production of excretory enzymes results in vacuolation in cells that is due to formation of balls in the highest dose. Finally necrosis occurs. *Vibrio harveyi*, chronically infected *penaeus vannamei* larvae show grey haepatopancreas with balls of necrotic tissue that block the upper gut. (Robertson et al., 1998)

In Ecuador, Vibrio *harveyi* has been caused the Bolitas negricans disease, which results in degeneration of haepatopancreas and forms balls that move to the upper gut. (Karunasagar et al., 1994). Austin and Zhang (2006) reported Bolitas negricans cause the blocking the digestive gland with balls of tissue. Pathology of hepatopancreas in typical of oral/enteric vibriosis showing severe necrosis, loss of structure, atrophy of tubule epithelial cells, vacuolation and rounding and sloughing of cells into the lumen(Ambipillai et al., 2003) . These pathological changes in the haepatopancreasand gills are probably due to bacterial toxins. Extracellular products (ECP) have been considered as a virulent factor of *V. harveyi*.Furthermore liu, et al. (1996) studied the pathogenicity of strains in diseased *penaeus monodon*, and explained that virulence is determined with both live bacteria and ECP. Proteases, phospholipase, haemolysins and other toxins may have important roles in the pathogenecity of *V. harveyi*. (Austin and Zhang 2006)

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