

## Effect of *Aeromonas hydrophila* infection with high and normal water temperature on total hemocyte count, total plasma protein and histopathological differences in freshwater crayfish *Astacus leptodactylus*

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### Abstract

In this study, the effect of water temperature on pathogenesis of *Aeromonas hydrophila* in freshwater Crayfish *Astacus leptodactylus* were investigated. In our study, The treatments Crayfish have exposed to *A. hydrophila* with concentration of  $3 \times 10^6$  CFUml<sup>-1</sup> in high water temperature (25°C) and normal water temperature (10-15°C). The untreated control groups in high water temperature (25°C) and normal water temperature (10-15°C) were disinfected by oxytetracyclin at concentration of 100 ppm for 24 hours. The haemolymph samples were withdrawn for measuring of THC and TPP. For histopathological study, the Crayfish samples were fixed in Davidson fixative. The results showed that the value of THC in control group1 in comparing with control group2 and two other treatments at 48h after experiment time were significant ( $p < 0.05$ ). Also the difference of THC value between control group 2 with treatment group2 at 144h after experiment time was significant ( $p < 0.05$ ). The finding of TPP value showed that there was significant difference between control group1 and treatment4 in last time after experiment time (336h) ( $p < 0.05$ ). In infected Crayfish to bacteria with high temperature, red and black spots and also wound were observed. The result of histopathology in hepatopancreas, gill, heart and digestive tract showed hemocyte aggregation, necrotized tissue, dead cells within pyknosis and karyorrhexis of nucleus and vaculation of hepatopancreas. In treatment group1 and control group1 no pathological changes of heart were observed. In digestive tract no changes were appeared in treatment1 and two others control group, but aggregation of hemocyte with pyknosis of nuclei was revealed in concentration of  $3 \times 10^6$  CFUml<sup>-1</sup> with high temperature.

**Keywords:** *Astacus leptodactylus*, *Aeromonas hydrophila*, Total hemocyte count, Total plasma protein, Histopathology.

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## Introduction

Bacterial infections have been reported in freshwater crayfish, often associated with the exoskeleton, enteric tract and hemolymph (Quaglio *et al.*, 2006). Studies showed that both gram-negative and gram-positive bacteria have been isolated from crayfish hemolymph. The most frequently reported Gram-negative genera are *Pseudomonas*, *Aeromonas*, *Acinetobacter*, *Flavobacterium* and *Vibrio micrococcus* and *Staphylococcus* are the most often reported Gram-positive genera (Scott and Thune, 1986; Wong *et al.*, 1995; Madetoja and Jussila, 1996). Among these species, *Aeromonas* spp are opportunistic pathogens causing a wide range of conditions including septicemia, meningitis and localized wound infections in susceptible hosts (Blair *et al.*, 1999). The members of the genus *Aeromonas* are chemoorganotrophic facultative anaerobes demonstrating both respiratory and fermentative metabolism. These bacteria are Gram-negative bacilli of 0.3-1  $\mu\text{m}$  diameter and 1-3.5  $\mu\text{m}$  length which are usually present singly, in pairs or in short chains (Blair *et al.*, 1999). *A. hydrophila* is a member of the family Aeromonadaceae and in only one of six species, *Aeromonas* species, that are known to be pathogenic in humans. It can cause both intestinal and nonintestinal infections in human and can often

be fatal (Carnahan and Altwegg, 1996; Adamski *et al.*, 2006). *A. hydrophila* is widespread in nature and is usually found in freshwater, brackishwater, moist soil, and nonfecal organic material (Ko and Chuang, 1995). Under condition of stress, it is even likely that some strains of motile aeromonads that are ordinarily part of the normal gut flora become pathogenic. These bacteria are found associated with black spot necrosis in juvenile and adult *Macrobrachium* spp. As well (Brady and Lassodelavega, 1992). The pathogenesis of *A. hydrophila* has been reported to involve a variety of biologically active extracellular products (Ecps) and enzymes including hemolysins, cytotoxins, enterotoxins and proteases. Which are believed to be associated with the virulence of *A. hydrophila* (Allan and Stevenson, 1981; Ljungh and Wadstron, 1981; Pansare *et al.*, 1986). Studies showed that severity of disease is influenced by a number of interrelated factors, including bacterial virulence, the kind and degree of stress exerted on a population of fish, the physiologic condition of the host, and the degree of genetic resistance inherent within specific population of fishes (Cipriano, 2001). The distribution of *A. hydrophila* in many aquatic systems globally indicates the successful adaptation of the bacterium to such environments (Daskalov, 2006). *A. hydrophila*

infection is the scourge of fresh and warm water fish farming worldwide and is considered as a significant economic problem (Rahman *et al.*, 2001; Hu *et al.*, 2005). The bacterium is of scientific and economic interest because of its pathogenicity to human and fishes (Austin *et al.*, 1998; Elwitigala *et al.*, 2005). Normally, crayfish hemolymph is free of bacteria (Alderman and Polglase, 1988), but under poor conditions, e.g. high density, lack food or poor water quality, bacterial infections in otherwise healthy crayfish may occur (Vey, 1977). The bacterial infections can be taken as indications of decreased immune resistance and thus poor condition of the crayfish (Alderman and Polglase, 1988). Studies showed that the freshwater crayfish mounts an immune system against pathogens through the use of both humoral and cellular responses to activate multiple cellular and humoral response including melanization, blood coagulation, the production of AMPs, and the phagocytosis/encapsulation of invading microorganisms by hemocytes (Liu, 2008). Crayfish hemocytes play important roles in the initiation of several immune response and production of antimicrobial peptides (Jiravanichpaisal *et al.*, 2007). Researches indicated that proteins are major contributors to hemolymph density (Lorenzon *et al.*, 2011).

Blood protein levels fluctuate with changes in environmental and physiological conditions and play fundamental roles in the physiology of crustaceans from O<sub>2</sub> transport to reproduction up to stress responses (Hagerman, 1983; Lorenzon *et al.*, 2008). In fact, moulting, reproduction, nutritional state, infection, hypoxia, and salinity variations are the major factors affecting the relative proportions and total quantities of the hemolymph proteins (Depledge and Bjerregaard, 1989; Arcos *et al.*, 2003). The level of total protein in the hemolymph is also depending on the species, with highest concentration observed in penaeid and lower ones in freshwater Crayfish (Shafir *et al.*, 1992; Palacios *et al.*, 2000; Da Silva-Castiglioni *et al.*, 2007; Buckup *et al.*, 2008). The reports indicated that Total Hemocyte Count (THC) and Total Plasma Protein (TPP) can fluctuate due to crustacean diseases (Rodrigues and Lemoullac, 2000). Histopathology studies on freshwater Crayfish *A. leptodactylus* and *P. leniusculus* with *A. hydrophila* infection demonstrated necrotic tissue with pyknotic nuclei and clump-infiltrated hemocyte in the gill, heart, hepatopancreas and circulatory system (Jiravanichpaisal *et al.*, 2009; Samcookiyaie *et al.*, 2011). The aim of this study was to determine the total hemocyte count (THC), total plasma protein (TPP) and histopathological differences in

the Crayfish *A. leptodactylus* due to *A. hydrophila* with high and normal water temperature.

### **Materials and methods**

#### *Collection, Mangment and Adaptation of samples:*

Two-hundred and forty freshwater Crayfish, *A. leptodactylus* with average weight of 25-40g were purchased from Aras dam reservoir in West Azerbaijan province and transported to Iranian Artemia Research Center of Urmia city in September 2010. Before experiment, the Crayfish were acclimated in the laboratory for ten days. During the adaptation and experiment time, crayfish were fed one time daily with commercial diet (trout, Biomar and Blood warm). Water of Crayfish aquarium was changed one time daily and the uneaten food was cleaned up regularly. During the study, water temperature, pH and dissolved oxygen were recorded.  $15\pm 1^{\circ}\text{C}$ , 7-8 and 5-5.5 ppm, respectively.

#### *Isolation of Aeromonas hydrophila*

Many of infected Crayfish from Aras dam reservoir were used for isolation of bacteria. Haemolymph sample had been gathered from infected Crayfish with cutting their antennules and transferred to TSA medium (tryptic soy agar). Then haemolymph specimens cultured on TSA for 24h at  $25^{\circ}\text{C}$ . After 24h of incubation The many of colonies of

bacteria had been grown in TSA. Eventually, *A. hydrophila* were determined in order to biochemical test. Also Rimler Shotts Agar, specific medium, was used for confirmation of the bacteria. Cell densities were photometrically obtained at 590nm wavelength (Saulnier *et al.*, 2000), based on the Mcfarland standard and serially diluted to reach the density of  $3\times 10^6$  CFU  $\text{ml}^{-1}$ .

#### *Orgnazation of groups*

This experiment was designed in two group treatments (Numbers 3 and 4) and two controls (Numbers 1 and 2). About 12 glass aquarium were designed for transportation of crayfish and Crayfish were transported in these glass aquarium in triplicate with 20 crayfish in each glass aquarium in order to temperature and concentration of bacteria. The control groups (1 and 2) were prepared without any bacteria and were disinfected by oxytetracyclin antibiotic at concentration of 100 ppm for 24 hours. Treatments and repetitions in individual containers have exposed for 2 h by *A. hydrophila* and no water changed during the exposure time. Two hours after challenge, the Crayfish samples finally washed with chlorinated and disinfected water and transferred to glass Aquarium.

### *Haemolymph sampling*

Haemolymph was obtained from the haemocoel second belly segment of Crayfish with using from 25 gauge needle and 1 ml syringe. For preventing coagulation, haemolymph sample were mixed 1 to 1 with an anticoagulant solution (Smith and Soderhall, 1983) (26 mm citric Acid, 30 mm trisodium citrate, 10 mm EDTA (Ethylene diamine tetra-acetic acid) (pH=5.4). Haemolymph specimen transported in differential gamma plastic tubes for study THC and TPP and maintained in cold box for time analysis. The haemolymph samples (One sample for each glass aquarium) were withdrawn from crayfish for measuring of THC and TPC within interval hours 2, 6, 12, 24, 48, 96, 144, 240 and 336 after exposure to concentration of bacteria. In this study, implementation of moribund and diseased Crayfish was mostly sampled.

### *Analysis of total hemocyte count*

Hemocytometer was used for study of hemocytes. A drop of the anticoagulant-haemolymph mixture was transferred on a hemocytometer and the rate of THC (total hemocyte count) measured by the light microscope (Jiang *et al.*, 2004).

### *Analysis of total Plasma Protein*

Haemolymph samples were centrifuged at  $2900 \times g$  at  $4^{\circ}C$  for 10 minutes. Then a drop of centrifuged samples was transferred in special

cups of analysis and total plasma protein (TPP) concentration was measured according to a Hitachi 902 auto analyzer by the Biuret-method at 520-560 nm wavelengths (Silverman and Christenson, 1996).

### *Histopathology*

Tissues samples (heart, gill, haepatopancreas and digestive tube) were also collected at 2, 6, 12, 24, 48, 96, 144, 240 and 336 hours after exposure time. The crayfish tissues were then fixed in Davidsons fixative for 48 hours and then transferred to 70% ethylic alcohol. After this time The tissue samples were processed for histopathological study by Bell and Lighthner (1988) and sections were prepared for H&E staining and viewed under light microscopy.

### *Statistical analysis*

A one-way ANOVA test was used to compare the differences of THC and TPP value among treatments and control groups at confidence interval of 95% ( $p < 0.05$ ). Multiple comparisons along with the Tukey HSD post hoc test was conducted to show that at least a group has a significant difference with other Groups while the P value indicated the value lower than 0.05. All Statistical tests were evaluated using the Version. 18 SPSS computer software.

## Results

### 1: THC changes in treatment and control groups after challenging time with *A. hydrophila*

Finding showed (Table 1 and Fig. 1) that the value of THC in Control group (1) (temperature = 10-15 °C) at 48h after experiment time in comparison with control group (2) (temperature=25 °C) and two others treatment ( $3 \times 10^6$  CFU ml<sup>-1</sup>, Temperature=10-15 °C (3) and  $3 \times 10^6$  CFU ml<sup>-1</sup>, temperature = 25 (4)) was significant and the value of THC in these groups in comparing with control group1 significantly decreased ( $p < 0.05$ ). The value of THC between control group (2) and treatment (4) ( $3 \times 10^6$  CFU ml<sup>-1</sup>,

Temperature=25 °C) was also significant at 144h after expouser time and the value of THC in treatment (4) ( $3 \times 10^6$  CFU ml<sup>-1</sup>, Temperature=25 °C) in compare to control group 2 (temperature=25 °C) significantly increased ( $p < 0.05$ ). In other times, no significant difference in THC was observed between treatments together and treatments with control groups ( $p > 0.05$ ). In our study, also the highest mean value of THC was  $119 \times 10^4$  CFU ml<sup>-1</sup> in control group 1 at 12h after experiment time and the lowest mean value of THC was  $9 \times 10^4$  CFU ml<sup>-1</sup> in control group 2 at 144h after experiment time.

**Table1: The mean and standard deviation of THC at difference hours after challenging to *Aeromonas hydrophila* in control and treatment groups THC result (Hemocyte  $\times 10^4$ ).**

Groups	Times (hour)								
	2	6	12	24	48	96	144	240	336
Control 1 Temperature =10-15°C	34±17.34	90.33±45.78	119±72.38	43.33±23.02	*72.33±15.04	39.67±19.50	28±7.21	80.33±24.94	42±33.18
Control 2 Temperature =25 °C	18±5.29	82.33±8.62	86.33±11.59	19.33±10.40	18±12.49	22±11.35	*9±6.24	61.67±4.16	25.33±11.01
$3 \times 10^6$ CFU ml <sup>-1</sup> Temperature= 10-15 °C	35.33±16.16	54.67±15.04	37.67±35.36	25.33±4.50	13.33±5.85	22.33±14.01	24±14.93	47±22.60	29±12.12
$3 \times 10^6$ CFU ml <sup>-1</sup> Temperature=25 °C	19.67± 15.17	52.67±27.53	39±16.09	18.67±12.85	13.67±14.94	74±41.58	*58± 28.85	48.67±26.57	78.67±12.58

Star sign (\*) have indicated significantly differences of THC in similar times

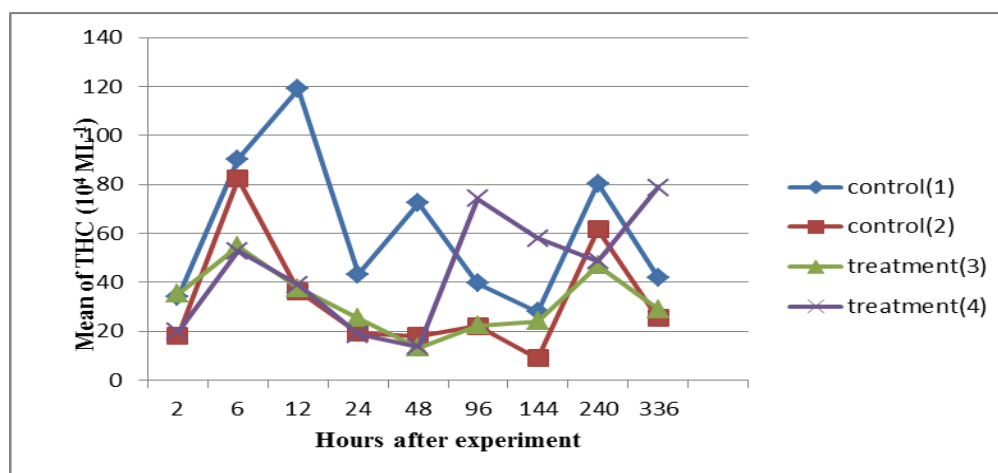


Figure 1: THC differences in treatment and control groups after experiment time.

2: TPP changes in treatment and control groups after challenging time with *A. hydrophila*

The result based on TPP demonstrated (Table 2 and Fig. 2) that the value of TPP between treatment (4) ( $3 \times 10^6$  CFU ml<sup>-1</sup>, temperature=25 °C) and control group (1) (temperature=10-15 °C) in last time after experiment time (336h) was significant and the value of TPP in treatment (4) in

comparing with control group (1) significantly increased ( $p < 0.05$ ). In other times, no significant difference in TPP was observed between treatments together and treatments with control groups ( $p > 0.05$ ). Also in order to our study the highest mean value of TPP was 2.36 Gr dl<sup>-1</sup> in treatment (4) and the lowest mean value of TPP was .9 Gr dl<sup>-1</sup> in control group (1).

Table 2: The mean and standard deviation of TPP at difference hours after challenging to *Aeromonas hydrophila* in control and treatment groups.

TPP Result (Gr dl <sup>-1</sup> )										
Times (hour)										
	2	6	12	24	48	96	144	240	336	
<b>Groups</b>	<b>Mean</b>	<b>S.D</b>	<b>Mean±S.D</b>	<b>Mean±S.D</b>	<b>Mean±S.D</b>	<b>Mean±S.D</b>	<b>Mean±S.D</b>	<b>Mean±S.D</b>	<b>Mean±S.D</b>	
Control 1 Temperature =10-15 °C	1.13±.45	1.96±.60	1.90±.00	1.46±.70	1.33±.58	1.23±.55	1.63±.75	.90±.10	*1.00±.50	
Control 2 Temperature =25 °C	1.40±.40	1.43±.30	1.90±.26	1.46±.40	1.53±.30	1.16±.15	1.63±.32	1.60±.17	2.06±.11	
3×10 <sup>6</sup> CFU ml <sup>-1</sup> Temperature= 10-15 °C	1.53±.05	1.43±.47	1.50±.60	1.53±.58	1.30±.26	1.40±.20	1.23±.55	1.46±.64	1.40±.52	
3×10 <sup>6</sup> CFU ml <sup>-1</sup> Temperature =25 °C	1.16±.72	1.20±.36	2.23±.89	1.73±.47	1.70±.20	1.03±.35	1.70±.26	1.46±.23	*2.36±.30	

Star sign (\*) have indicated significanty differences of TPP in similar times

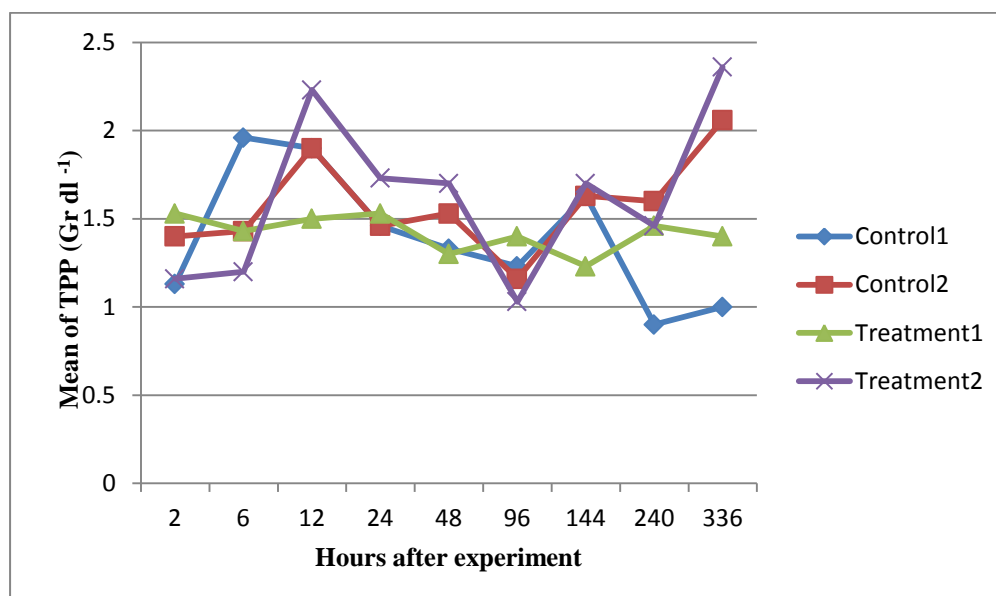


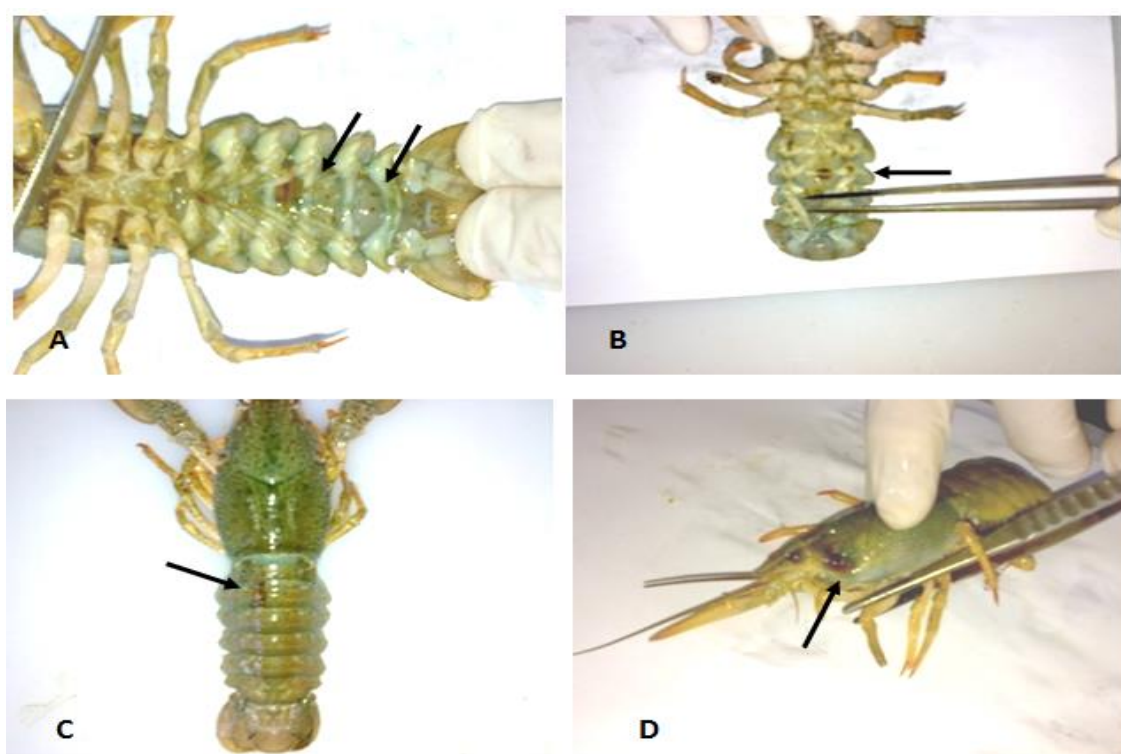
Figure 2: TPP differences in treatment and control groups after experiment time.

### 3: Clinical signs and mortality due to *A. hydrophila* in Crayfish *A. leptodactylus*

Our finding showed that there was no obvious signs observed in *A. leptodactylus* in early times after affecting to bacteria (2,6). But there were seen, weakness, lethargy, anorexia and accumulation in corner of aquarium in treatment (4) and control group (2) crayfish *A. leptodactylus* by passing time. Our result indicated that in crayfish challenging to *A. hydrophila* with high temperature (treatment 4), wound, redspots, and many of black spots on the abdominal segment, carapas and walking legs along with abdominal blakness were observed. And It was also observed that the

moribund animals lose their balance and fall on their dorsal surface (belly up) before dying. In order to our study, the highest levels of mortality (33 crayfish) was observed in Crayfish challenging to bacteria with high temperature (treatment 4), and the rate of mortality in this group in comparing with other groups was significant. The rate of mortality in control group (2) in comparing with treatment (3) and control group (1) were also significant (21 crayfish). But the rate of mortality wasn't significant between treatment (3) and control group (1).





**Figure 3:** Clinical sign due to *Aeromonas hydrophila* with high temperature in crayfish *Astacus leptodactylus*. Numerous red spots on internal surface of abdominal segments (A) , various black melanized spots on lateral and internal surface of abdominal segments (B), wound on dorsal surface of abdominal second segments (C) and lateral surface of carapas (D) (arrows) were occurred.

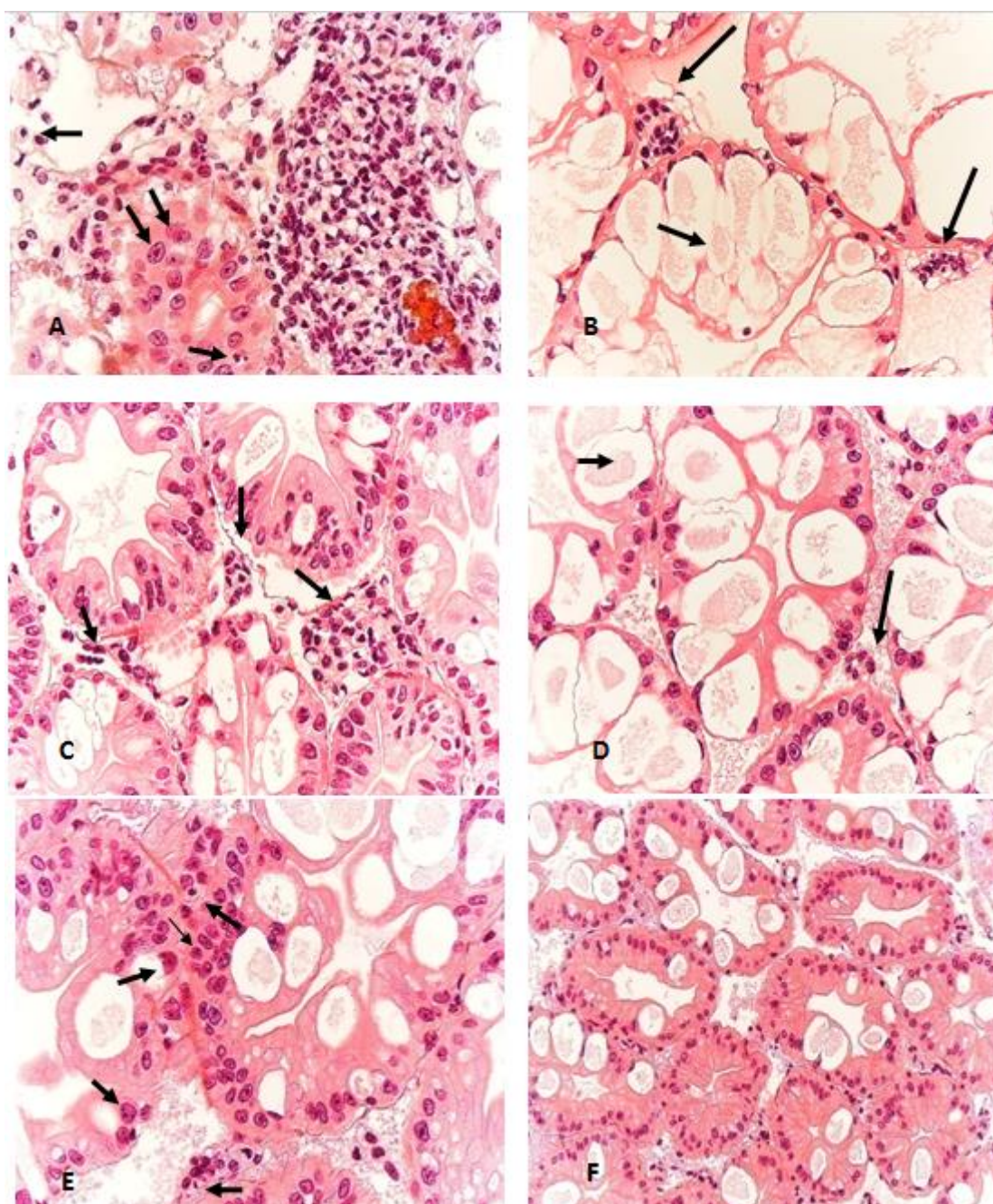
**Table 3:** Rate of mortality in treatment and control groups in freshwater crayfish *Astacus leptodactylus* after experiment times.

Time (hour)	Rate of mortality				Sum
	Control 1 Temperature= 10-15 °C	Control 2 Temperature=25 °C	$3 \times 10^6$ CFU ml <sup>-1</sup> Temperature= 10- 15 °C	$3 \times 10^6$ CFU ml <sup>-1</sup> Temperature=25 °C	
2	-	-	-	-	-
6	-	-	-	-	-
12	-	-	-	-	-
24	-	-	-	-	-
48	2	2	1	4	9
72	1	1	1	2	5
96	2	4	-	7	13
120	-	5	1	7	13
144	1	2	1	2	6
168	-	-	-	-	-
192	1	-	-	2	3
216	-	2	1	1	4
240	-	1	1	3	5
264	-	2	-	2	4
288	-	1	-	1	2
312	-	-	-	1	1
336	1	1	-	1	3

#### 4: *Histopathology Conclusion in treatment and control groups*

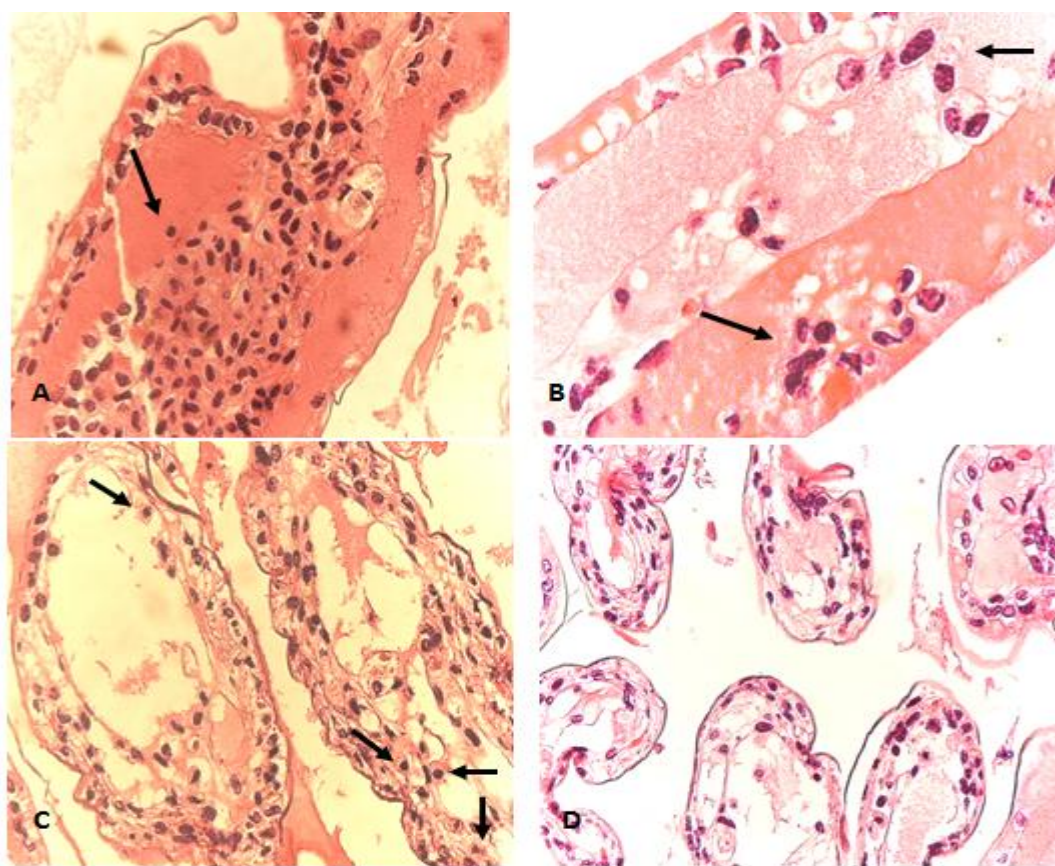
In histopathology studies, the massive hemocyte aggregated in hepatopancreas were observed, exclusively in crayfish challenging to  $3 \times 10^6$  CFU ml<sup>-1</sup> *A. hydrophila* with high temperature (Fig. A), as well as hemocyte aggregated were observed in crayfish infected to  $3 \times 10^6$  CFU ml<sup>-1</sup> *A. hydrophila* with normal temperature (temperature=10-15 °C) and control group (2), but in these groups, hemocyte aggregated in comparing with infectious crayfish to  $3 \times 10^6$  CFU ml<sup>-1</sup> with high temperature were lower. In crayfish affecting to bacteria with high temperature and control group (2) were also observed vaculation and decreasing of hemocyte accumulation by passing time (Fig. B). As seen in hepatopancreas, necrosis, and dead cells along with the many of pyknotic and karyorhexis nuclei (Figs. B, C). But this appearance wasn't observed in hepatopancreas tissue in control group (1) (Fig. D).

In histopathology results, Gills indicated necrosis, hemocyte aggregated, dead cells and pyknotic nuclei. In infected crayfish to  $3 \times 10^6$  CFU ml<sup>-1</sup> *A. hydrophila* with high temperature severe gill differences and high necrosis were noticed. Also hemocyte aggregated and cell death were observed (Fig. A). In crayfish affecting to  $3 \times 10^6$  CFU ml<sup>-1</sup> *A. hydrophila* with normal temperature, necrosis of gill and hemocyte aggregated were also observed, however these differences in comparing to  $3 \times 10^6$  CFU ml<sup>-1</sup> *A. hydrophila* with high temperature were lower (Figs B, C). In Crayfish affecting to high temperature (temperature=25 °C) hemocyte aggregated along with decreasing of gill lamella normal cells were seen. But this appearance wasn't observed in gill tissue in control group1(temperature=10-15 °C) (Fig. D).



**Figure 4:** Hepatopancreas tissue sections of crayfish *Astacus leptodactylus* in treatments due to *Aeromonas hydrophila* and control groups after experiment times. Massive hemocyte aggregated and clumped together in hemal sinus (long arrow) along with dead cells with pyknotic and karyorrhexis nuclei (short arrows) and necotized tissue were observed in haepatopancreas of crayfish challenged to *A. hydrophila* with high temperature (Fig. A), Vaculization of the cytoplasm (long arrows) and decreasing of hemocyte aggregated (short arrows) due to *A. hydrophila* with high temperature and Control group 2 crayfish (without of bacteria *A. hydrophila* with high temperature) after passing time (96h) were seen (Figs B and D), hemocyte aggregated (arrows) were observed in many sites of hemal sinus of hepatopancreas in control group 2 crayfish (without of bacteria *A. hydrophila*) (Fig. C), few hemocyte aggregated in hemal sinus of hepatopancreas along with many of pyknotic and karyorrhexis nuclei were noticed in crayfish infected to *A. hydrophila* with normal temperature (Fig. E), no pathological differences observed in control 1 crayfish (without of bacteria) and hepatopancreas was safe (Fig. F).





**Figure 5:** Gill lamella tissue sections of crayfish *Astacus leptodactylus* in treatments due to *Aeromonas hydrophila* and control groups after experiment times. High necrosis, cell death and hemocyte aggregated have been observed in gill lamella in crayfish infected to  $3 \times 10^6$  CFU ml<sup>-1</sup> *A. hydrophila* with high temperature (Fig. A). (H&E/Mag, 440), Distribution of many hemocyte (arrows) were seen in control group 2 crayfish (without any of bacteria with high temperature) (Fig. B) (H&E/Mag, 880), low necrosis, distribution of low hemocytes along with many of pyknotic nuclei (short arrows) in comparing to infected crayfish to  $3 \times 10^6$  CFU ml<sup>-1</sup> *A. hydrophila* with high temperature were observed in infected crayfish to  $3 \times 10^6$  CFU ml<sup>-1</sup> *A. hydrophila* with normal temperature (Fig. C) (H&E/Mag.440), Histopathological changes wasn't significant in control group 1(without any of bacteria) and lamellas of gill were proximately safe in this group (Fig. D) (H&E/Mag.440).

Histopathological finding of heart and digestive system showed that in crayfish infected to  $3 \times 10^6$  CFU ml<sup>-1</sup> *A. hydrophila* with high temperature, massive hemocyte aggregated and distributed in the myocardium along with many of pyknotic nuclei and distribution of many hemocytes in digestive system have been observed (Fig. A). Low hemocyte aggregated

were also observed in heart in control group 2 (Fig. B) but digestive system was safe in this group. According to our study heart and digestive system were Safe in Crayfish infected to  $3 \times 10^6$  CFU ml<sup>-1</sup> *A. hydrophila* with normal temperature and control group 1. (Figs. C, B).

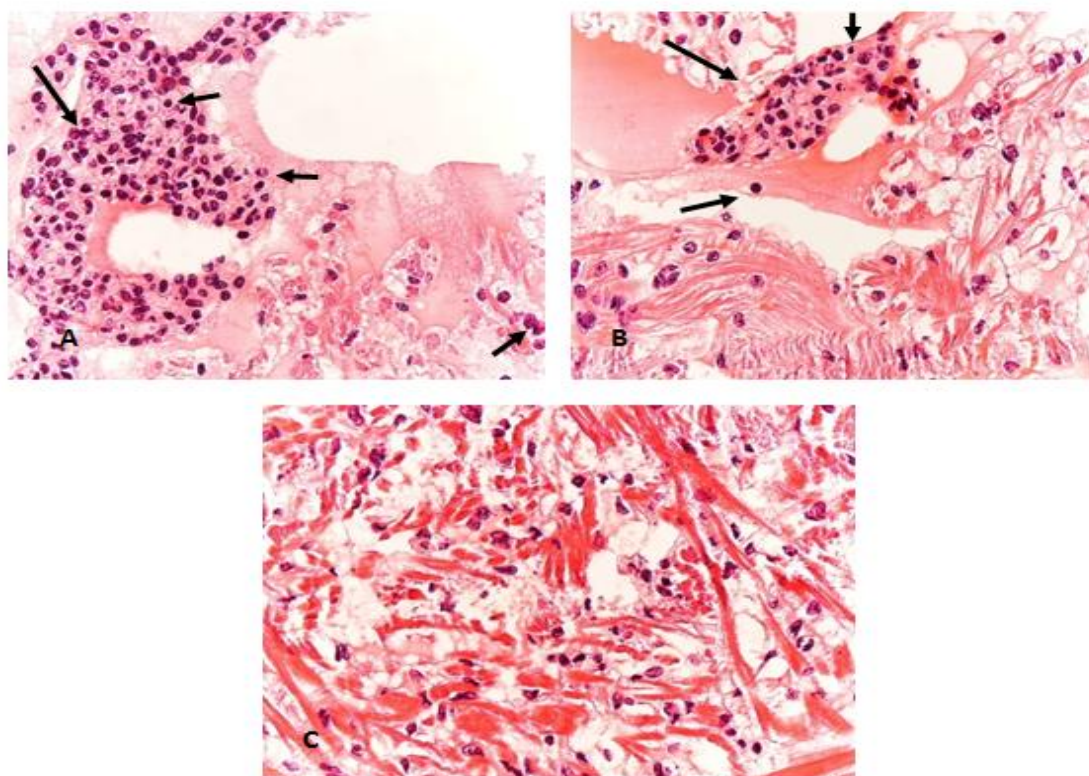


Figure 6: Heart tissue sections of crayfish *Astacus leptodactylus* in treatments due to *Aeromonas hydrophila* and control groups after experiment times. Massive hemocyte aggregated (long arrow), dead cells and many of pyknotic nuclei (short arrows) were observed in the myocardium of infected crayfish to  $3 \times 10^6$  CFU ml<sup>-1</sup> *A. hydrophila* with high temperature (Fig. A), this appearance were also observed in control group 2 crayfish but in this group, hemocyte aggregated in compare to crayfish expouser to  $3 \times 10^6$  CFU ml<sup>-1</sup> *A. hydrophila* with high temperature were lower (Fig. B), No pathological changes observed in heart in Crayfish challenged to  $3 \times 10^6$  CFU ml<sup>-1</sup> *A. hydrophila* with normal temperature and control group 1 (Fig. C), H&E, Mag, 440).

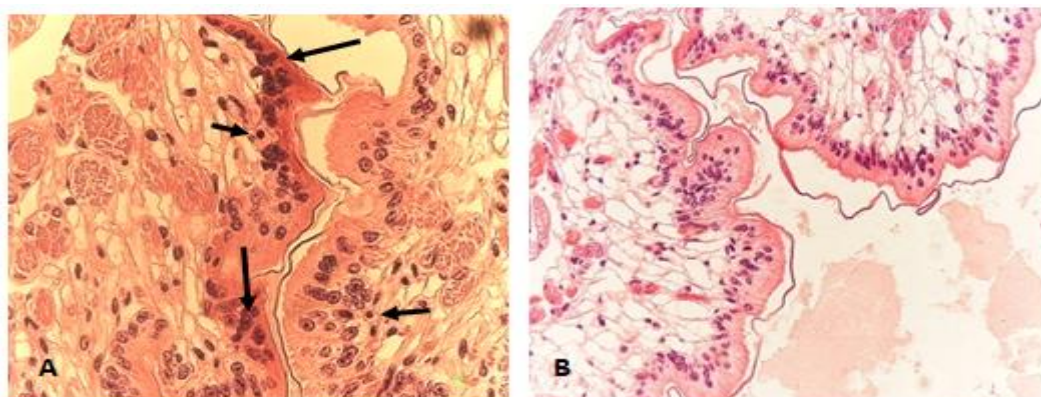


Figure 7: Digestive tube tissue sections of crayfish *Astacus leptodactylus* in treatments due to *Aeromonas hydrophila* and control groups after experiment times. Hemocyte aggregated (long arrow) with pyknotic nuclei (short arrows) were observed in crayfish challenged to  $3 \times 10^6$  CFU ml<sup>-1</sup> *A. hydrophila* with high temperature (Fig. A) (H&E/Mag.880), but no pathological changes observed in crayfish infected to  $3 \times 10^6$  CFU ml<sup>-1</sup> *A. hydrophila* with normal temperature and control group 1 and 2 (Fig. B) (H&E/Mag.440).

## Discussion

*A. hydrophila* is an opportunistic as well as primary pathogen of variety of aquatic and terrestrial animals including man (Dhasarathan *et al.*, 2010). One study indicated that the value of total hemocyte count (THC) could be changed in response to various concentration of *A. hydrophila* (Samcookiyaie *et al.*, 2011). In crustaceans and other invertebrates, circulating hemocytes are essential in immunity, performing functions such as phagocytosis, encapsulation, and lysis of foreign cells (Smith and Soderhall, 1983; Ratcliffe *et al.*, 1985; Soderhall and Smith, 1986; Johansson and Soderhall, 1989; Soderhall and Cerenius, 1992). Hemocyte counts maybe a valuable tool in monitoring the health status of crustacean species (Jussila *et al.*, 1997; Mix and Sparks, 1980). Many studies showed that total and differential hemocyte counts could be changed in result of the factors such as water temperature, sex, moulting cycle and starvation (Manjula *et al.*, 1997; Johansson *et al.*, 2000; Lemoullac and Haffner, 2000). According to our study, there was no significant differences on THC in early time after challenging to bacteria (2, 6, 12, 24h). It seems that the serological and physiological parameters could not affect in this time. Or probably indicated that the concentration of bacteria was lower, thereby the value of THC could not

change to bacteria. Samcookiyaie *et al.* (2011) showed that the value of THC in crayfish *A. leptodactylus* showed significant differences in first time (2h) after challenging to high concentration of *A. hydrophila* ( $3 \times 10^8$  CFU ml<sup>-1</sup>) in comparing with lower concentration of bacteria and control group (unchallenged with bacteria). They suggest that the stress of high concentration of bacteria or probably interference of Antimicrobial peptides (AMPs) can cause to it. Based on our study, there was significant differences on THC at 48h after experiment time, and the value of THC in treatments and control group 2 in comparing with control group 1 significantly decreased. It probably suggest that the value of THC could be changed cause to differences of physiological and serological parameters of Crayfish *A. leptodactylus* due to *A. hydrophila* and high temperature as a stressor factor with passing time. The differences of THC in infected crayfish to *A. hydrophila* in high water temperature in compare to control group 2 at 144h after experiment times were also significant, and the value of THC in this treatment in comparison with control group 2 significantly increased. This finding may be showed that the concentration of bacteria due to stressing of high water temperature was increased with passing time. Thereby the value of THC for response to high



concentration of bacteria in comparing with control group 2 significantly increased. Or may be similar to other studies (Jiravanichpaisal *et al.*, 2006; Jiravanichpaisal *et al.*, 2007; Liu *et al.*, 2008), the presence of Antimicrobial peptides such as PL crustin1 as an innate immune system in hematopoietic and hemolymph tissue of Crayfish, and also activated of phenol oxidase system by hemocyte in against to *A. hydrophila* cause to it. But in this time (144h), there were no significant differences between challenged crayfish with *A. hydrophila* in normal water temperature with unchallenged crayfish in control group 1, and also between this groups in comparing with two others treatment and control groups that can cause of adapted of crayfish to environment condition and decreased of stressor factors and bacterial concentration due to normal water temperature. Studies demonstrated that Alteration in total plasma or serum protein concentration relative to a reference interval have been used as a broad clinical indicator of health, stress, and wellbeing of terrestrial and aquatic organisms (Kiche, 2007). Consequently, measuring the blood protein concentration of a crustacean sample group can provide valuable information to identify its condition (Ozbay and Riley, 2002). Many studies indicated that moulting,

reproduction, nutritional state, infection, hypoxia, salinity variations and stocking density are some of the factors affecting the relative proportions and total quantities of the hemolymph proteins (Depledge and Bjerregard, 1989; Oliver and Macdiarmid, 2001; Arcos *et al.*, 2003; Ocampo *et al.*, 2003; Coeurdacier *et al.*, 2011). In order to previous study, the value of TPP didn't showed significant differences in infected Crayfish with various concentration of *A. leptodactylus* in comparison with control group (unchallenged crayfish). which probably indicated that the value of TPP could not be changed based on low concentration of bacteria or short period of study (Samcookiyaee *et al.*, 2011). Our study showed that the value of TPP can be affected due to concentration of bacteria in high water temperature. Based on our study, the value of TPP in challenged crayfish to *A. hydrophila* in high water temperature in comparison with control group 1 in last time of experiment (336h) significantly increased. It seems that the value of TPP could not be changed in short period time, but the value of TPP could be changed with changes in environment condition and physiological and serological parameters of crayfish due to pathogenesis of *A. hydrophila* in high water temperature with passing time. In our study, wound, red and black melanized

spots were observed in challenged crayfish with *A. hydrophila* in high water temperature. Also histopathological changes was higher in this treatment in comparing with other treatment and control groups. Because of high water temperature in challenged crayfish in this group, the pathogenesis of *A. hydrophila* was revealed, and following severity symptoms and histopathological differences were observed. Because of massive hemocyte aggregated, dead cells and high necrosis in hepatopancreas and gill tissues, it seems that these tissues are target organs for pathogenesis of *A. hydrophila* in crayfish *A. leptodactylus*. Functional studies of crayfish PO (Phenol oxidase) suggest that PO was required in crayfish defence against an infection by a highly virulent and pathogenic bacterium, *A. hydrophila*, to crayfish. Silencing of crayfish proPO led to increased bacterial growth, lower phagocytosis, lower PO activity, lower nodule formation, and higher mortality when infected with this bacterium (Liu, 2008). Studies showed that the transcript of PLcrustin 1 was increased in both hemocytes and hematopoietic tissue as a response to challenge by a highly pathogenic Gram-negative bacterium *A. hydrophila* (Jiravanichpaisal *et al.*, 2007). Secreted extracellular toxins and enzymes have been described which were suggested to be associated with

the virulence of *A. hydrophila* (Allan and Stevenson, 1981; Ljungh and wadstrom, 1981). In our research, the rate of mortality in challenged crayfish in high water temperature was high. It may be suggested that the increasing of bacterial concentration due to high temperature led to eradication of hemocyte cells and following decreased of phenol oxidase (PO) activity and antimicrobial peptides (specially PLcrustin 1) as important defence immune system against *A. hydrophila* and finally will lead to the secretion of toxins and enzymes (such as hemolysins, cytotoxins, enterotoxins) from this bacterium to Crayfish. Therefore the crayfish after challenging with *A. hydrophila* in high water temperature will die. We can conclude that the pathogenesis of *A. hydrophila* in high water temperature is too high. Therefore the presence of crayfish in this water body will lead to destruction of immune system due to secretion of toxins by *A. hydrophila* and following the crayfish will die. This finding is similar to another study as mentioned by Jiravanichpaisal *et al.*, (2009). The result of this study suggested that the value of THC due to *A. hydrophila* and high water temperature could be changed. Especially in challenged crayfish in high water temperature. But the value of TPP could only be changed in the crayfish challenge with *A. hydrophila* in high water temperature.



Our study also showed that the histopathological differences and the rate of mortality were highest in the crayfish challenge with *A. hydrophila* in high water temperature.

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