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Some effects of experimental acidification on phenoloxidase, trypsin and lysozyme activities in freshwater crayfish (*Pontastacus leptodactylus*)

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Abstract: Levels of phenoloxidase (Po), trypsin and lysozyme activities were determined following 48 hours exposing freshwater crayfish (*Pontastacus leptodactylus*) weighing 59.8±3.4g to different low pH values of 6.5, 5.5, 4 and 2.5 at 20-23˚C. Significant decrease were found ($P<0.01$) in levels of Po and trypsin activities in crayfish haemocyte lysate supernatant (HLS) at pHs 5.5 and 4 compared to the control group (pH 8). Also, plasma lysozyme activity showed a significant increase in water with pHs of 6.5, 5.5 and 4 ($P<0.01$). Furthermore, measurement of calcium and magnesium concentrations in sera samples showed a significant decrease for calcium ion at pHs 5.5 and 4, while magnesium concentration was significantly decreased only at pH 4 compared to control group ($P<0.05$). Crayfish exposed to pH 2.5 died within 24 hours post-exposure.

Keywords: Crayfish, *Pontastacus leptodactylus*, phenoloxidase, trypsin, lysozyme, serum calcium and magnesium, acidification

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Introduction

Many stressors have been identified that impact aquaculture operations among which are rapid changes in water temperature, pH, insufficient oxygen, elevated CO_2, heavy metals, salinity and nitrites (Bayne, 1975; Lignot *et al.*, 2000). Acidification of natural waters has become a global problem and can be due to acid rain, industrial effluent including oxidation of both sulphuric and nitric acid (McMahon & Stuart 1989), elevated levels of CO_2, ammonia, cyanides and irons (Paxton, 2003). Effects of acidification on the distribution of crayfish have been reported by some authors (e.g. Appelberg, 1989) indicating most crayfish species are negatively affected by acid water at a pH below 5.5. However, depending on the crayfish species, various levels of sensitivity have been reported (Berill *et al.*, 1985). Several factors are probably responsible for decrease in crayfish population in acidified waters including direct lethal effects, reproductive failure, decreased resistance to diseases and increased vulnerability to cannibalism and predation (France, 1983). In acidified waters, disturbed calcium metabolism leads to low exoskeleton rigidity resulting in a potential increase in the risk of cannibalism and predation (France, 1987). Also, low rigidity of exoskeleton can cause an enhancement in the animal susceptibility to pathogenic microorganisms because of failure in immunophysical barriers. In addition, low pH waters can internally cause several changes in animal immunophysiological functions resulting in increasing in the animal susceptibility to secondary pathogenic micro/macro-organisms such as bacteria, fungi and parasites (France, 1987).

Freshwater crayfish (*P. leptodactylus*) is mainly distributed in the north-west rivers of Iran and is a valuable species as a new candidate for commercial aquaculture (Nezami, 1997; Skurdal & Taugbol, 2002). The action of stressors on this species is varied and not widely studied. Monitoring of its immune status may be a very important tool for determining what relative degree of stress a population is under and thus how susceptible it might be to an infectious disease process. Minimum data are available concerning the side effects of acidified waters on immunophysiological parameters of this species. Therefore, the aim of this study was to assess the acidification effects on the phenoloxidase, trypsin and lysozyme

activities as indicators of *P. leptodactylus* immune system, defining what levels of low pH are problematic and lethal for the animal.

Materials and methods

Sixty adult males of freshwater crayfish (*P. leptodactylus*) with mean carapace length of 43.2±0.7mm and mean body weight of 59.8±3.4g were obtained from a commercial farm in the province of Tehran. Only intermoult and healthy animals were used in this experiment. Groups of control and test comprised 30 crayfish each were kept in 2000L glass aquaria provided in the Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran with suitable aeration, water temperature at 20-23˚C and pH 8 (Perazzolo & Barracco, 1997). They were fed with raw pieces of fish each night and acclimated to the laboratory conditions one week prior to the experiment.

The pH values of 6.5, 5.5, 4 and 2.5 were prepared by adding an appropriate volume of 10% sulphuric acid to 1 L of normal water with pH 8 at 20-23˚C. Test groups were then transferred to these low pH waters and were kept for 48 hours under the specified water quality conditions. Control group was kept in water with pH 8. Sample collection and processing of samples were undertaken at the end of 48 hours exposure and the following variables were measured.

A volume of 1 ml of haemolymph was taken from each animal using a 22 ¼ gauge needle inserted into the perioarthrodal membrane at the base of the walking legs. Sera samples were prepared as described by Perazzolo and Barracco (1997). Haemolymph samples were transferred to the glass tubes, allowed to coagulate and stored at 4˚C for 24 hours, and centrifuged at 2000g for 20 minutes at 4˚C to remove the sera. The separated sera samples were then used to measure calcium and magnesium concentrations (Perazzolo & Barracco, 1997).

Calcium and magnesium concentrations of sera samples were determined using a standard kit (Pars Azmoon Co., Tehran, Iran) and a modified sequential Auto Analyzer (EPPOS-506 Eppendorf) (Burtis & Ashwood, 1999).

Adequate haemolymph samples were obtained from each animal using syringes containing precooled anticoagulant solution (0.015M potassium dihydrogen

phosphate, 0.01 M sodium citrate, 0.14 M NaCl, 0.15M glucose, 0.02 M citric acid, pH 4.6, 2:1 (v/v) haemolymph:anticoagulant solution). The haemolymph was then centrifuged at 100g for 10 minutes at 4°C and the supernatant (plasma) was removed and stored at -20°C until used. The collected haemocyte pellet was first washed in 0.01 M sodium cacodylate (100mM $CaCl_2$ pH 7.0) and then homogenized in 0.01 M sodium cacodylate buffer (5mM $CaCl_2$, pH 7.0). After homogenisation the sample was centrifuged at 20000 g for 20 minutes at 4°C to remove the cell debris. The supernatant was collected, referred to as HLS and used immediately in the experiments as described by Perazzolo and Barracco (1997); Stritunyalucksana *et al.* (2001) Soderhall and Soderhall (2002).

Phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-Dopa) as described by Perazzolo and Barracco (1997) and Herandez-Lopez *et al.* (1996). A volume of 50µL of each HLS sample was preincubated with 50µL of 10% sodium dodecyl sulphate (SDS) as an elicitor at room temperature for 30 minutes. A volume of 50µL of L-Dope (3mg/ml) was then added followed by adding 850µL of distilled water to slow the enzymatic reaction. The optical density was measured at 490 nm after 5, 10 and 20 minutes spectrophotometrically.

The activity of trypsin was measured by method described by Perazzolo and Barracco (1997). A volume of 100µL of each HLS sample was preincubated with 100µL of *Aeromonas hydrophila* lypopolysaccharide (LPS) (1mg/ml) for 15 minutes at room temperature. Each sample then received 500µL of Tris-buffferd solution (TBS) (pH 8.0) and 100µl of chromogenicpeptide: Na-Benzoyl-DL-arginine-4-Nitroanilide-hydrochloride (BAPNA) (Sigma) ($0.05mol.L^{-1}$) and incubated at 30°C for 1 hour. The samples then received 200µL of 50% (v/v) acetic acid to stop the enzymatic reaction. The optical density was measured at 405nm. The control samples were used by replacing the HLS with TBS (Perazzolo & Barracco, 1997).

Lysozyme activity of plasma samples was measured using a turbidimetric method described by Ellis (1990) with slight modifications. Briefly, 175µl of *Micrococcus lysodeikticus* suspension (0.375mg/ml 0.05M phosphate buffer sodium (PBS), pH 6.2 was mixed with 25µl of *each* sample and the optical density

was measured after 180 seconds by spectrophotometer at 600nm wavelength. PBS was considered as the blank and known amounts of hen egg white lysozyme (Sigma) were used as standard. Lysozyme activity was defined as a reduction in light absorbance (Ellis, 1990).

The data were analysed using independent sample t-test and ANOVA subjected to SPSS software.

Results

Effects of low pH values on calcium and magnesium contents in serum of crayfish are shown in Table 1 and Figures 1 and 2. The levels of serum calcium were significantly reduced at pH 5.5 (29.3±2.6mg/dl) and pH 4 (27.3±2.4mg/dl) compared to control samples at pH 8 (39.3±2.7mg/dl) ($P<0.01$), while magnesium concentration of serum showed a significant decrease only at pH 4 (4.1±0.07 mg/dl) compared to control samples (4.5±0.09mg/dl) ($P<0.05$).

The results of Po activity in HLS of crayfish kept in waters with different pHs are shown in Table 2 and figures 3, 4 and 5. No significant changes at pH 6.5 was obtained in Po activity after 5, 10 and 20 minutes compared to control samples ($P<0.05$). Po activity at pH 5.5 showed significant decreases after 5, 10 and 20 minutes compared to control samples ($P<0.01$). The level of Po activity was also significantly reduced at pH 4 compared to control samples ($P<0.01$).

Trypsin activity in HLS of crayfish kept in waters of different pHs are shown in Figure 6. Trypsin activity was significantly reduced at pH 5.5 (340.8±6.4) and pH 4 (309.4±6.2), while no significant difference was found at pH 6.5 (386±17) compared to control samples (408.5±12.3) ($P<0.01$).

Lysozyme activities in plasma of crayfish kept in waters of different pHs are shown in Figure 7. Optical density at 600 nm for lysozyme activities were significantly decreased at pH 6.5 (857±3.3), pH 5.5 (828.8±3.6) and pH 4 (795.2±2.5) compared to control samples (867.0±3.1) ($P<0.01$), showing significant increases in the level of lysozyme activity at all three ranges of low pH. When crayfish were exposed to pH 2.5, all died within 24 hours post-exposure.

Table 1: Calcium and magnesium concentration in *P. leptodactylus* serum following short term exposure to low pH waters of 6.5, 5.5 and 4 (pH 8 as control group). Values are means ± SE (n=30).

pH values	Calcium concentration (mg dl^{-1})	Magnesium concentration (mg dl^{-1})
8	39.3 ± 2.7	4.5 ± 0.09
6.5	38.09±2.7^{NS}	4.4 ± 0.09 ^{NS}
5.5	29.3 ± 2.6**	4.3 ± 0.08 ^{NS}
4	27.3 ± 2.4**	4.1 ± 0.07*

NS = not significant
** = significantly different at 1% level
* = significantly different at 5% level

Table 2: Po activity in *P. leptodactylus* HLS following short term exposure to low pH of 6.5, 5.5 and 4 (pH 8 as control group) after 5, 10 and 20 minutes. Values are means ±SE of optical density ×1000 at 490 nm (n=30).

Time per minute	pH 8.0	pH 6.5	pH 5.5	pH 4.0
5	863.0±4.9	858.0±4.9^{NS}	747.6±11.9**	717.6±11.9**
10	940.2±4.7	932.2±4.7 ^{NS}	908.2±9.1**	848.2±9.1**
20	971.6±8.6	961.6±8.6 ^{NS}	943.4±10.6*	860.4±10.6**

NS = not significant
** = significantly different at 1% level
* = significantly different at 5% level

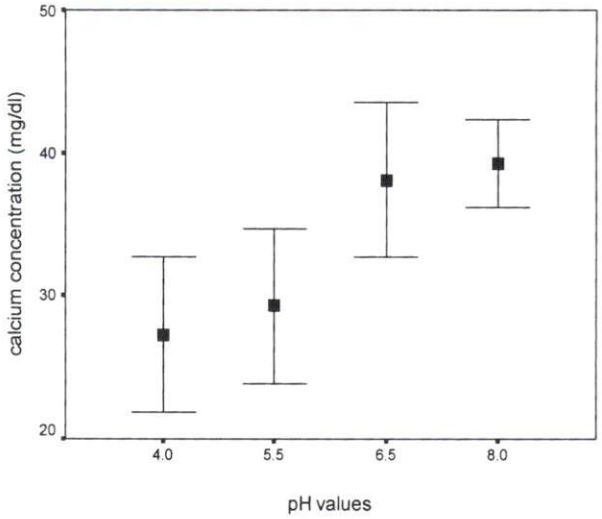

Figure 1: Effect of acidity on calcium concentration in *P. leptodactylus* serum at low pH of 6.5, 5.5 and 4 (pH 8 as control group). Means ± 2 SE are shown.

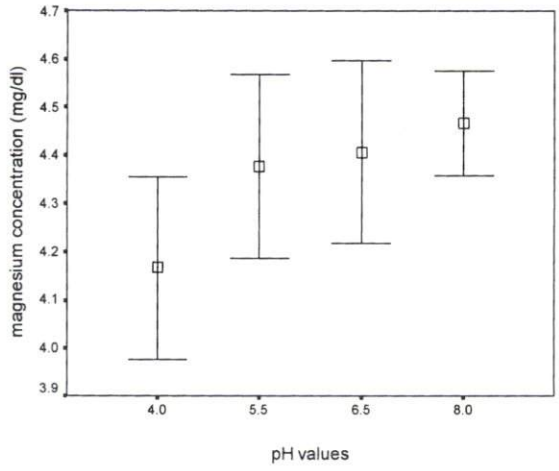

Figure 2: Effect of acidity on magnesium concentration in *P. leptodactylus* serum at low pH waters of 6.5, 5.5 and 4 (pH 8 as control group). Means ±2 SE are shown.

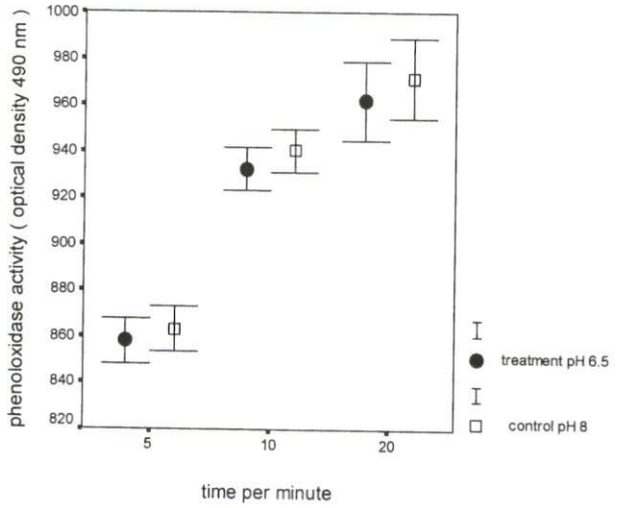

Figure 3: Effect of acidity on phenoloxidase activity in *P. leptodactylus* HLS at pH 6.5. Means ±2 SE of optical density × 1000 at 490 nm.

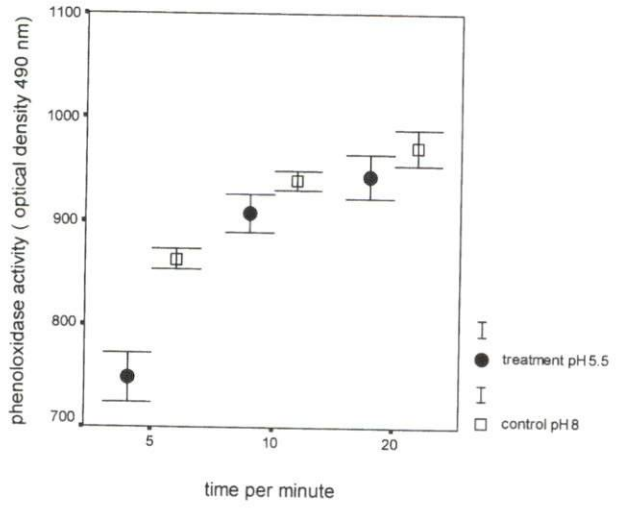

Figure 4: Effect of acidity on phenoloxidase activity in *P. leptodactylus* HLS at pH 5.5. Means ±2 SE of optical density × 1000 at 490nm.

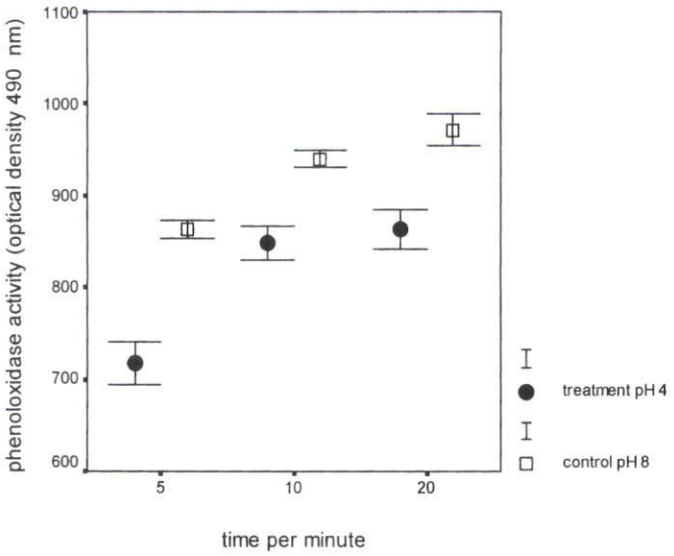

Figure 5: Effect of acidity on phenoloxidase activity *in P. leptodactylus* HLS at pH 4. Means ±2 SE of optical density ×1000 at 490nm.

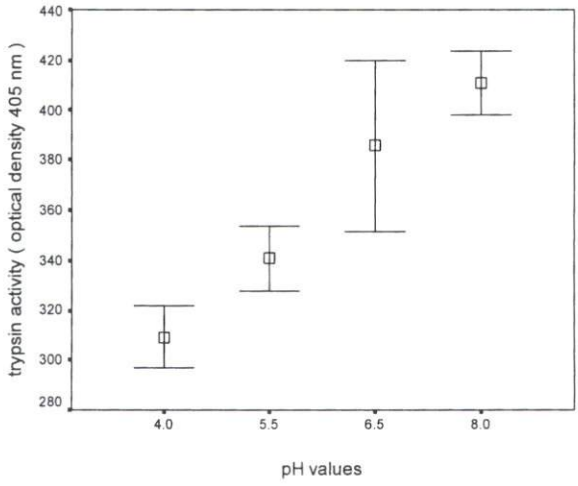

Figure 6: Effect of acidity on trypsin activity in *P. leptodactylus* HLS at low pH waters of 6.5, 5.5 and 4 (pH 8 as control group). Means ±2 SE of optical density ×1000 at 405nm.

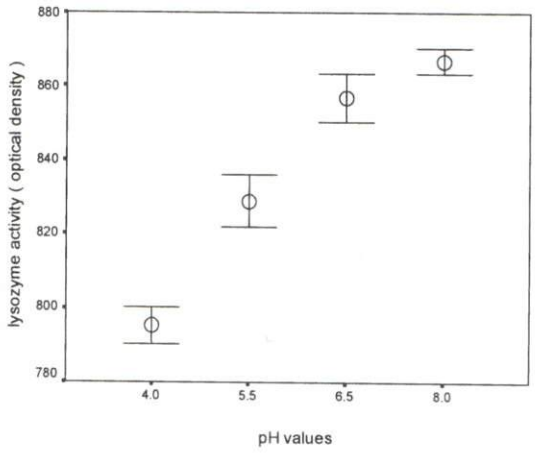

Figure 7: Effect of acidity on lysozyme activity in *P. leptodactylus* plasma at low pH waters of 6.5, 5.5 and 4 (pH 8 as control group). Means ±2 SE of optical density ×1000 at 600λ are shown.

Discussion

Significant reduction was observed in calcium concentration of *P. leptodactylus* serum after 48 h exposure to pH 5.5 and 4. Also, concentration of serum magnesium showed significant decrease at pH 4. Result of other studies shows that acid stress causes marked acidification of the haemolymph and marked ion loss in haemolymph and tissues of both crayfish and fish (Morgan & McMahon, 1982; McMahon & Morgan, 1983; Appelberg, 1985; Wood & Rogano, 1986; Mauro & Moore, 1987; McMahon & Stuart, 1989; Jensen & Malte, 1990). Also, McMahon and Stuart (1989) reported that sublethal acid (pH 4) exposure in *Procambarus clarkii* resulted in haemolymph ion loss, although after 60 days exposure both haemolymph pH and the concentrations of most ions returned to levels near control animals. Furthermore, exposure of *A. astacus* to low pH waters resulted in disturbance of osmoregulation and acid-base status of the crayfish (Malley, 1980; Appelberg, 1985; Wood & Rogano, 1986).

We previously investigated the cation dependence of Po activity in *P. leptodactylus* HLS (Khazraeenia *et al.*, 2005_a) as it has been reported by some authors in other decapod crustaceans (Durliat, 1985; Perazzolo & Barracco, 1997;

Gollas-Galvan *et al.*, 1997). Also, calcium dependence of trypsin like serine proteinase activity in crustaceans HLS has been reported by some authors (Gollas-Galvan *et al.*, 1997). In this study both Po and trypsin activities of *P. leptodactylus* showed significant decrease after 48 hours exposure to pH 5.5 and 4. This might be in part due to depletion in haemolymph ion as mentioned earlier. Changes in pH can also biochemically affect the enzyme structure and function (Malhotra, 1998). Cheng *et al.* (2002) express the circulating haemocytes of decapod crustaceans are affected by extrinsic factors such as pH, temperature, salinity and dissolved oxygen. Cheng and Chen (2000) reported that both Po activity and total haemocyte count (THC) of *Macrobrachium rosenbergii* were significantly higher at pH 7.5-7.7 and 30-31°C. However, they found significantly lower values after exposing the animal to pH 4.6-5.0 and 9-9.5 at 33-34°C. As *P. leptodactylus* haemocytes are the major source of Po activity (Khazraeenia *et al.*, 2005_b), a decrease in Po activity in *P. leptodactylus* under low pH condition can consequently reduce THC.

In this study, an increase in lysozyme activity in plasma was observed following 48 hrs exposure of *P. leptodactylus* to pH 6.5, 5.5 and 4 compared to control pH 8. This may be in part due to the animal immunological reaction to unsuitable environmental conditions. This is also supported by the point that the lysozyme activity is suppressed following a chronic stress while it is often elevated after exposing to an acute stress (Kubilay & Ulukoy, 2002). Therefore, it is suggested that elevated lysozyme activity in *P. leptodactylus* at low pH water may be more of an indication that the animal is stressed rather than more resistant to disease when the crayfish is exposed to an acute stress due to low pH condition. 100% mortality in *P. leptodactylus* occurred after 24 hour exposure to pH 2.5.

Other reports show that crayfish is more resistant to acidified water than fish, and in some species of crayfish very low pH (i.e. pH 2) can kill the animal in a short term exposure (i.e. 24 hrs) (Morgan & McMahon, 1982; Jensen & Malte, 1990; Ellis & Morris, 1995_a). As already mentioned, acid stress causes a marked acidification of the haemolymph and ion loss in haemolymph and tissues and, therefore, such mortality at low pH is probably more related to the ion loss than to the haemolymph acidification. Acidification may also affect haemolymph oxygenation

and, as a consequence, animals undergo ventilatory and circulatory disturbance as described by Patterson and DeFur (1988). For example, hypometabolism has been reported in *Cherax destructor* following 20 days acid exposure (Ellis & Morris, 1995_b). In conclusion, the present study shows that low pH waters affects *P. leptodactylus* immunophysiological functions including Po, trypsin and lysozyme activities, and short-term exposure to pH 2.5 will cause death of the crayfish. Therefore, evaluation of enzyme activities is a useful tool to monitor crayfish immunophysiological status, particularly when the animal is exposed to unsuitable environmental conditions such as acidified waters.

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