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Effects of pH on survival, phosphorus concentration, adenylate energy charge and Na^+-K^+ ATPase activities of *Penaeus chinensis* Osbeck juveniles

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Abstract

Penaeus chinensis (Osbeck) juveniles were maintained for 14 days at pH 6.0, 7.0, 7.6, 8.0 and 8.5, respectively. The effects of pH on survival, phosphorus concentration, adenylate energy charge (AEC) and Na^+-K^+ adenosine triphosphatase (ATPase) activities of prawns were investigated. The results showed that survival of *P. chinensis* was impaired at low and high pH levels. The total phosphate level and AEC in abdominal muscle increased with pH level in range of 6.0–7.6 reaching the maximum values at pH 7.6. Thereafter, the levels declined with increasing pH level in range of 7.6–8.5. The change of Na^+-K^+ ATPase activity in gill of prawn was similar to that of total phosphorus content and AEC in muscle of prawn at different pH. The effect of pH on Na^+-K^+ ATPase activity in the muscle was lower than on that in gill.

Keywords: *Penaeus chinensis*; Phosphorus; Adenylate levels; pH; Na^+-K^+ ATPase

1. Introduction

Penaeus chinensis Osbeck (also known as *P. orientalis* Kishinouye) is distributed in Bohai and the Yellow Sea between the west of Korea and east of the Yellow Sea and extensively cultured on the China and Korea mainland.

The pH is an important factor affecting the life of euryhaline penaeids. In acidic waters, crustaceans and fish have suffered retarded growth and skeletal deformity (Haines, 1981) and impaired ionic regulation (Morgan and McMahon, 1982). The effect of ambient pH on urea-N excretion of *P. chinensis* was more pronounced at high pH than at low pH (Chen and Lin, 1995). In aquaculture ponds, pH reductions can be exacerbated during periods of heavy rain, as acidic soils in pond dykes erode into ponds (Boyd, 1989). However, rise in concentrations of soluble organic substances may result in formation of red tide in aquaculture ponds, thus, pH in aquaculture ponds can increase to 9.0 during periods of red tide (Wang and Wang, 1995). It also may be subject to temporary upward pH surges caused by the photosynthetic processes of algae (Murray and Zei-

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bell, 1984). The physiological response of prawn at different pH levels is of primary concern in prawn farming.

The adenylate energy charge (AEC) and the adenosine triphosphatase (ATPase) activity have been suggested as potentially useful indicators of an organism's physiological status. AEC and ATPase are related biochemically (Dehn et al., 1985). AEC is a measure of the metabolic energy available to an organism (Atkinson, 1968), and may provide a rapid and precise measure for the effect of environmental perturbations on specific organisms. The AEC of crustaceans, as well as other animals and plants (Ivanovici, 1980) has been observed to vary in response to stressors such as cadmium (Thébault et al., 1996).

Na^+-K^+ ATPase plays a significant role in both, whole body ion regulation and cellular water balance in marine animals (Towle, 1981). Pollutants have been shown to inhibit Na^+-K^+ ATPase activity in a variety of marine organisms (Haya and Waiwood, 1983) by disrupting energy producing metabolic pathways (Watson and Beamish, 1981; Verma et al., 1978). Therefore, examination of ATPase activity could be an indicator of the stresses caused by ambient water quality.

Until now, no papers on AEC and ATPase activity of *P. chinensis* in response to pH stress have been published. The aims of this study were to investigate effects of pH on survival, phosphorus levels, AEC and ATPase activity of prawn, *P. chinensis*.

2. Materials and methods

The postlarvae of the prawn, *P. chinensis*, were collected from Shan Dong Province, China, in May 2000. Postlarvae were reared together in the laboratory for 30 days until they reached an average size of 2–3 cm length. The water salinity at 27 ± 1 °C was 30 ± 1‰. They were fed on newly hatched *Artemia* sp. nauplii at a density of 10 individuals per ml.

Juveniles with mean lengths of 2.5 ± 0.2 cm in the intermolt stage were selected for the experiments. The intermolt stage was determined by examining the uropods, in which partial retraction of the epidermis can be distinguished prior to molt (Lim and Chao, 1990; Du and Jiang, 1997).

In the laboratory, duplicate treatments with five different levels of pH (6.0, 7.0, 7.6, 8.0 and 8.5) were established to assess the effects of ambient pH on *P. chinensis*. Ten glass aquaria ($40 \times 20 \times 15$ cm) were used and each aquarium pH was adjusted with 1 M HCl or 1 M NaOH. The pH levels were measured in each aquarium every 12 h during the experiment, using a model 720A, pH/ISE meter (Orion Laboratory Products Division). Thirty prawns were used in each aquarium. Each glass aquarium had recirculated water by means of an airlift. Each day, 25% of the water was replaced by a sea water supply of the same pH. The prawns were fed with commercial prawn pellets. Mortality of each aquarium was observed daily and dead animals and uneaten feed were removed daily. Acclimatization lasted 14 days and the prawns of each treatment in intermoult stage were selected for analysis.

To quantify total and inorganic phosphorus, the abdominal muscle was removed rapidly from six prawns per treatment and homogenized immediately at temperature between 0 and 4 °C. Samples for total phosphorus were digested in nitric acid and perchloric acid (Association of Official Analytical Chemists, 1990). Total and inorganic phosphorus were measured as described by Spaargaren et al. (1982).

2.1. Adenylate extraction

The abdominal muscle was rapidly removed from six prawns per treatment and freeze-clamped in liquid nitrogen. The nucleotides in muscles were extracted using a modified procedure of Zaroogian et al. (1982). Muscle sample was homogenized with nine volumes 5% cold perchloric acid. After centrifugation at 12 000 rpm at 4 °C, the supernatant was collected. The extract was stored at -80 °C until analysis.

2.2. Adenylate determination

The concentrations of ATP, ADP and AMP were determined using a TH-2 model high perfor-

mance capillary electrophoresis HPCE instrument (Tianhui Separation Science Institute, Baoding, China) equipped with a recorder (Dahua Meter Factory, Shanghai, China) to collect the electropherograms. The ribonucleotides were detected by on-column UV absorbance at 254 nm. In the instrument, the inlet was held at negative high voltage with respect to the ground outlet and sample introduction was performed by pressure. Electrophoresis was performed in a 48 cm × 75 μm I.D. fused silica capillary (Yongnian Optical Fiber Factory, Hebei Province, China), of which 37.5 cm was the effective length. Before the first use, capillary was sequentially pretreated with 0.1 M NaOH for 10 min, distilled water for 5 min, running buffer for 5 min, and finally equilibrated with the buffer. All micellar electrokinetic capillary chromatography (MECC) separations were operated at room temperature. The optimized conditions were as following: 50 mM Na_2HPO_4 + 20 mM CTAB + 1 mM EDTA (pH 8.0), −15 kV, 5 kPa for 6 s load and detection at 254 nm. The three adenosine phosphates were completely resolved on uncoated column within 10 min, as shown in Figs. 1 and 2. Peaks on electropherogram of both standard ribonucleotides (Sigma, St.Louis, MO) and extract were identified by spiking with pure single nucleotide added to the sample. Concentrations of the nucleotides were identified by using the peak areas of known nucleotide concentrations. Linear relationships between peak area and concentration are found for all three adenylates within the concentration range studied. Correlation coefficients were above 0.99715. The amount of each nucleotide in the muscle extract was determined by reading the concentration from its standard curve. The AEC, defined as (ATP + 1/2ADP)/(ATP + ADP + AMP) reflected the energy balance at a given time for an organism or a part of it (Atkinson and Walton, 1967).

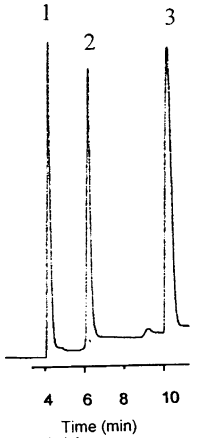

Fig. 1. Electropherogram of three standard adenylates. CE conditions, 50 mmol l^{-1} Na_2HPO_4, 20 mmol l^{-1} CTAB-1 mmol l^{-1} EDTA buffer (pH 8.0); −15 kV applied voltage (injection at cathode end); pressure inject at 5 kPa × 6 s. Absorbance was monitored at 254 nm. (1) AMP, (2) ADP, (3) ATP.

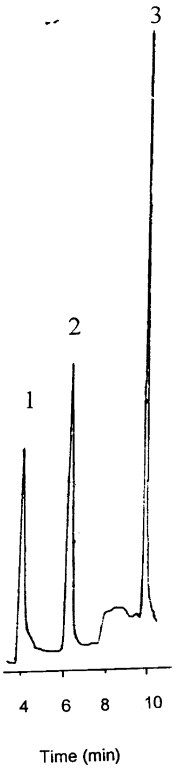

Fig. 2. Electropherogram of an extract of adenylates from prawn muscle. CE conditions, 50 mmol l^{-1} Na_2HPO_4, 20 mmol l^{-1} CTAB-1 mmol l^{-1} EDTA buffer (pH 8.0); −15 kV applied voltage (injection at cathode end); pressure inject at 5 kPa × 6 s. Absorbance was monitored at 254 nm. (1) AMP, (2) ADP, (3) ATP.

2.3. ATPase assay

The gills and muscles were excised rapidly from prawn, freeze-clamped in liquid nitrogen and were homogenized in 250 mM sucrose–5 mM EDTA. Final concentrations of the assay medium were: 50 mM NaCl, 10 mM KCl, 4 mM $MgCl_2$, 6 mM disodium ATP and 90 mM Tris–buffer (pH 7.6). Na^+–K^+ ATPase activity was measured according to a procedure already described by Dehn et al. (1985). The amount of inorganic phosphate (Pi) liberated in the reaction of $ATP \rightarrow ADP + Pi$ was expressed as μmol Pi per mg protein per hour. The protein content of the homogenate was determined by the Folin phenol method (Lowry et al., 1951), with bovine serum albumen as standard.

2.4. Statistics

Results were analyzed by one way analysis of variance (ANOVA) at the $P < 0.05$ confidence interval to detect differences among treatments.

3. Results

Survival of prawns acclimated 14 days to pH 7.0, 7.6 and 8.0 was 100%. It was 65.5 ± 0.5 and $71.5 \pm 0.5\%$ for prawns acclimated to pH 6.0 and 8.5, respectively (Fig. 3), and was significantly lower ($P < 0.05$) than at pH 7.0, 7.6 and 8.0.

The changes in adenylate levels and AEC in the muscle of prawn at different ambient pH are shown in Table 1 and Fig. 4. Fig. 4 also shows the changes in the phosphorus content of muscle at different pH. The level of inorganic phosphate in muscle at pH 7.0, 7.6 and 8.0 did not show significant ($P > 0.05$) variation, but they were significantly lower at pH 6 and 8.5 ($P < 0.05$). The total phosphate level in the muscle increased with pH level in the range of 6.0–7.6, reaching the maximum values at pH 7.6. Thereafter, the levels declined with increasing pH level in the range of 7.6–8.5. Total adenylate levels, ATP levels, and AEC values in the muscle at pH 6 and 8.5 were significantly lower ($P < 0.05$) compared with that at pH 7.6 (Table 1 and Fig. 4).

The gill Na^+–K^+ ATPase of prawn showed a very high activity at pH ranging from 7.0 to 8.0 (see Fig. 5). ATPase activity was very low at pH 6.0 and 8.5 and the maximal activity was measured at pH 7.6. Responses of muscle Na^+–K^+ ATPase activity of prawn to changes in pH were less pronounced than those of gill Na^+–K^+ ATPase activity. The maximum muscle Na^+–K^+ ATPase activity was found at pH 7.6 (see Fig. 5). The adverse effects of pH on survival, gill Na^+–K^+ ATPase activity and AEC in muscle of prawn were more pronounced at low pH (6.0) than at high pH (8.5).

4. Discussion

In an intensive culture system of *P. chinensis*, the shrimp experienced a fluctuation in pH level from pH 7.79 to 8.4 during the culture period (Wang et al., 1992). It is known that the pH levels in a pond may fall to 6.0 in the early morning or after heavy rain, and increase to 9.0 or more in the afternoon (Boyd, 1990) or during periods of red tide in aquaculture ponds (Wang and Wang, 1995) due to photosynthesis of aquatic algae. Mortality (Apud et al., 1985) and poor growth (Webber and Webber, 1978) of penaeids occurred

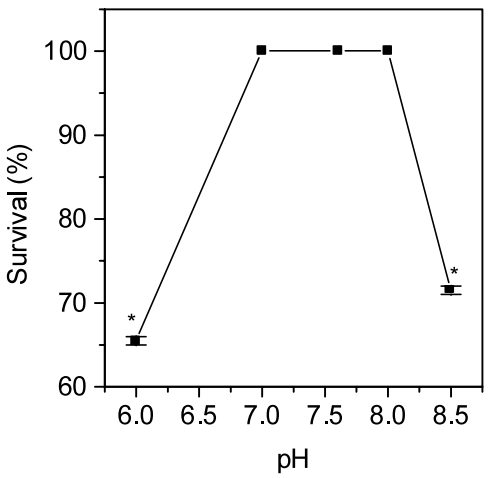

Fig. 3. The change in survival of prawn, *P. chinensis* at different pH levels. *, Significantly different from the corresponding values at pH 7.6, $P < 0.05$.

Table 1
The content of adenine nucleotides and energy charge (AEC) in the muscle of prawns, *P. chinensis* at different pH (mean (S.D.), $n = 12$)

pH	AMP (μmol g^{-1} wet weight muscle)	ADP (μmol g^{-1} wet weight muscle)	ATP (μmol g^{-1} wet weight muscle)	ΣAdN[a]	AEC
6	1.092*	1.330*	1.569*	3.991*	0.56*
	(0.054)	(0.025)	(0.058)	(0.087)	(0.001)
7	1.159	2.004	4.740*	7.902*	0.73
	(0.073)	(0.035)	(0.077)	(0.015)	(0.001)
7.6	1.295	2.1987	6.305	9.798	0.76
	(0.050)	(0.087)	(0.028)	(0.097)	(0.005)
8	0.775*	0.686*	2.368*	3.829*	0.71
	(0.021)	(0.108)	(0.035)	(0.109)	(0.006)
8.5	2.136*	1.146*	4.367*	7.648*	0.65*
	(0.260)	(0.042)	(0.096)	(0.137)	(0.001)

AEC = (ATP + 0.5ADP)/ΣAdN; *, significantly different from the corresponding values at pH 7.6; $P < 0.05$.
[a] ΣAdN = ATP + ADP + ATP.

when acid sulfate soils acidify pond waters. Allan and Magurire (1992) observed that the survival of *Penaeus monodon* was unaffected in acidified seawater with a pH 6.1 and mortality occurred at pH ≤ 5.1. However, in the present study, mortality of *P. chinensis* occurred at pH 6.0, indicating a higher degree of sensitivity to change in ambient pH. Juvenile prawn (in the present study) are more sensitive to low pH than the larger prawn, *P. monodon* (Allan and Magurire, 1992). In previous studies, ammonia-N excretion of *Macrobrachium rosenbergii* and *P. chinensis* decreased with increased pH in range 7.0–8.5 (Chen and Kou, 1996; Chen and Lin, 1995). Increasing pH by a unit caused a 10-fold increase in the concentration percentage of unionized ammonia (Bower and Bidwell, 1978; Boyd, 1982). Thus, the toxicity of ammonia at any particular concentration increases with increasing pH levels (Noor-Hamid et al., 1994). Accumulated ammonia-N in the hemolymph might also destroy the protein biosynthesis function of the hepatopancreas (Senkbeil and Wriston, 1981). Ammonia plays a role in the regulation of a number of metabolic pathways and could contribute to the altered metabolic status of these animals. In addition, ammonium ions are known to cause electrophysiological disruptions, particularly the displacement of K^+ in ion exchange mechanisms that could lead to the observed loss of swimming performance (Beaumont et al., 2000). This may also be a reason for the mortality of *P. chinensis* observed at pH 8.5. Therefore, a small increase or decrease in pH level may profoundly affect physiological function of cultured prawns.

In agreement with the pattern of enzyme change in trout gill (Nieminen et al., 1982), the gill Na^+–K^+ ATPase of prawn showed a very high activity at pH ranging from 7.0 to 8.0, whereas, it was very low at pH 6 and 8.5. Harris and Bayliss (1988) reported that potential Na^+

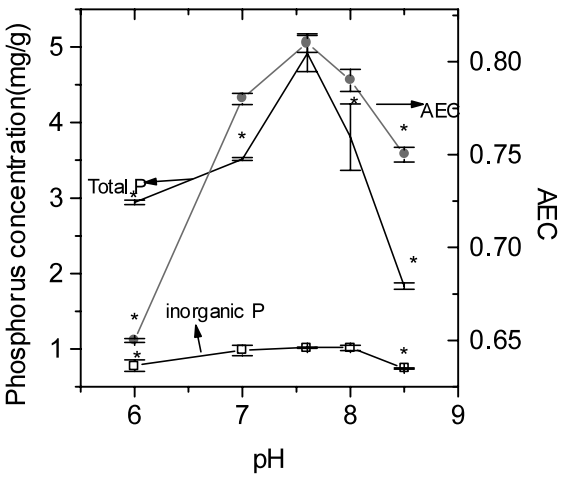

Fig. 4. The change in phosphorus levels and AEC of prawn, *P. chinensis* at different pH levels. *, Significantly different from the corresponding values at pH 7.6, $P < 0.05$.

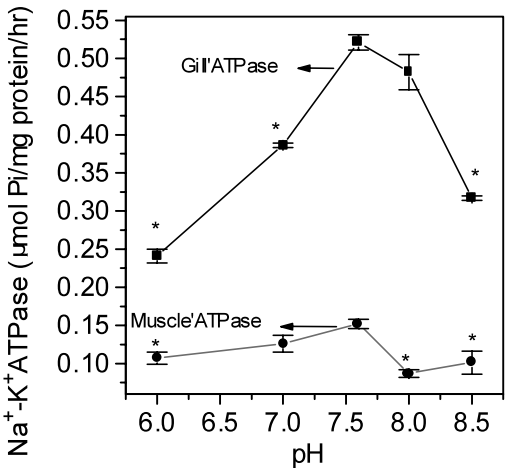

Fig. 5. The change in Na^+-K^+ ATPase activity in the gill and muscle of prawn, *P. chinensis* at different pH levels. *, Significantly different from the corresponding values at pH 7.6, $P < 0.05$.

pumping capacity of the gills was highly correlated with gill $(Na^+ + K^+)$-ATPase specific activity and more closely correlated with enzyme activity than Na^+ gradient. Reduced osmolarity, or a loss of haemolymph ions (in particular Na^-), has been recorded following exposure to acid water for several species of crustaceans (Morgan and McMahon, 1982). According to Heinzz (1967), in a variety of biological systems, the active transport of sodium and potassium was inhibited below pH 4.6–5.0, while the passive permeability for sodium of the mucosal barrier increases. Wilkie et al. (1999) showed that alkaline water caused transient decreases in the capacity of the ion transport system by directly acting on the gill's respective Cl^- and Na^+ transport sites. Thus the decrease of gill Na^+-K^+ ATPase activities at low and high pH in the present study likely accounted for most of the initial reductions in Na^+ influx at low and high pH. It seems probable, therefore, that impairment of the active transport mechanism for sodium ions through the gill epithelium was the primary cause of the deaths of prawn in acid and alkaline water. In the present study, the Na^+-K^+ ATPase activity in muscle of prawn at pH 7.6 was higher than that at other pH. These results showed the change of ambient pH not only affected gill Na^+ influx but also made changes in Na^+ transport capacity of cell membrane in muscle, although, the effect of pH on the Na^+ pump of cell membranes in muscle was less than that on gill. In addition, an increase in pH caused an increase in NH_3-N in media, a decrease in the ammonia-N excretion, and an increase in ammonia-N uptake by prawns (Chen and Lin, 1994). Chen and Nan (1992) reported that Na^+-K^+ ATPase activity of *P. chinensis* exposed to 5 and 10 mg l^{-1} ammonia-N for 4 h increased and decreased, respectively, as compared with those exposed to control solution. The fact that a decrease in Na^+-K^+ ATPase activity resulted from high pH in the present study indicated that there might be a malfunction of Na^+/NH_4^+ exchange.

AEC is a measure of the metabolic energy available to an organism (Atkinson, 1968), and might provide a rapid and precise measure for the effect of environmental perturbations on specific organisms. Previously, biochemical responses to various forms of stress (e.g. reduced salinity or exposure to pollutants) were coherent with the health state of organisms (Rainer et al., 1979; Thébault et al., 1996). A perturbation in optimal environment usually corresponds with a signal of energy consumption (low level of adenylates, low AEC). Since, AEC represents available biochemical energy, it is a sensitive biomarker for the effects of any stressor that has the potential to cause a recognizable shift of this ratio due to increased energy utilization or decreased energy production associated with physiological defense mechanisms or disruption of metabolism (Giesy, 1988). In the present study, AEC in the muscle of prawn varied with pH level indicating that there was an increased energy utilization or decreased energy production at low and high pH levels. Chen and Lin (1995) observed that oxygen consumption of *P. chinensis* juveniles decreased with increased pH level in the range of 7.0–8.5. The rate of oxygen consumption is associated with aerobic energy metabolism (Scholnick, 1995). In other invertebrates, a reduction in pH is considered to be the main factor in causing a decrease in metabolic rate (Hand and Gnaiger, 1988). These data indicated that part of the aerobic energy metabolism is arrested by low and high pH level.

The decreasing energy charge associated with metabolic depression has two implications. Firstly, it can reflect a temporary disconnection between the normally tightly coupled processes of energy production and consumption, resulting in a new equilibrium level of the adenylates. Secondly, a metabolically depressed system with a low energy charge has obviously become insensitive to its energy status. There may be two types of metabolically depressing systems: one that requires a stable energy charge, and thus a concerted decrease in energy production and utilization, and one that effects metabolic depression by limiting energy production, and in which energy producing processes must become insensitive to changes in energy status (Guppy et al., 1994). The change in AEC with changing pH also demonstrated that AEC ratios might provide a useful tool in monitoring the response of this prawn, to stressful environmental changes. It was noted that the changes of total and inorganic P levels in the muscle were similar to those of AEC and Na^+-K^+ ATPase activity in the muscle. This suggested that phosphorus levels in the muscle might be closely correlated with energy requirement and the transport capacity of cell membrane for prawn at different ambient pH. Phosphorus has an integral role in cellular function as it is a key component of nucleic acids, phospholipids, phosphoproteins, ATP and several key enzymes, and a deficiency of P can be produced in most species (Goote et al., 1996). In addition, phosphate serves as a buffer to maintain optimal pH in body fluids (Lovell, 1989). Blood phosphorus concentrations have been shown to fluctuate widely in response to factors such as temperature, salinity (Spaargaren et al., 1982), and disease status (Hille, 1982). In the present study, low and high ambient pH resulted in decrease of phosphorus concentrations in muscle of prawn. There are two possible explanations (1) the energy metabolic depression under low and high pH results in a decrease in P compounds; or (2) the dietary P intake and utilization were influenced by change in ambient pH thus decreasing P concentration in muscle of prawn. In addition, low pH can also influence the impact of potential toxins, e.g. heavy metals (Boyd, 1989). Their toxicity might be due to their interference with phosphate binding site of ATPase thereby arresting the release of energy.

We conclude that the pH optima for the survival of prawns, *P. chinensis* was in the range of 7.0–8.0.The low and high pH rearing water resulted in metabolic depression of *P. chinensis*, which involved inhibition of Na^+-K^+ ATPase activity and decreased AEC in muscle, which then impaired the survival of prawns. The effect of pH on survival, gill Na^+-K^+ ATPase activity and AEC in muscle of prawn was more pronounced at low pH (6.0) than at high pH (8.5). It was shown that AEC ratios provide a useful tool in monitoring the response of this crustacean to stressful environmental changes.

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