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Effects of low temperature acclimation on antioxidant defenses and ATPase activities in the muscle of mud crab (*Scylla paramamosain*)

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ABSTRACT

This study aims to determine the effects of low temperature acclimation on the antioxidant defenses and ATPase activities in mud crab (*Scylla paramamosain*). The activities of ATPases and antioxidative enzymes, as well as malondialdehyde (MDA) content, were measured in the muscle of *S. paramamosain* acclimated at 5, 10, 15, and 27 °C (control group). The activities of superoxide dismutase, catalase, and glutathione peroxidase gradually declined with decreasing temperatures and then significantly lowered at 5 and 10 °C compared with those at 27 °C ($P<0.01$ or $P<0.05$). Significant accumulation of MDA, a lipid peroxidation indicator, was observed at 5 and 10 °C ($P<0.01$). Compared with the control, the activities of four different ATPases significantly increased at 10 °C ($P<0.01$ or $P<0.05$). However, Na^+/K^+-ATPase and Mg^{2+}-ATPase activities in the 5 °C group showed no significance compared with those in the control ($P>0.05$). Ca^{2+}-ATPase and Ca^{2+}/Mg^{2+}-ATPase activities at 5 °C were remarkably lower compared with those at 27 °C ($P<0.01$). At low temperatures, antioxidant defense ability decreases with decreasing antioxidant enzyme activities, and MDA was accumulated in cell. The higher levels of ATPase activities at 10 °C, compared with the control, suggest compensation to maintain the physiological homeostasis of cytosol ion levels. However, ability to compensate for ATPase activity decreases beyond the critical value of low temperature (i.e., at 5 °C). Therefore, juvenile mud crabs should be cultivated in water at temperatures above 5 °C. This is an important guideline for farmers culturing mud crab in cold water. In addition, antioxidant parameters and ATPase activities can be used as biomarkers to assess the effects of low temperature acclimation on *S. paramamosain*.

1. Introduction

Fluctuating environmental temperatures impose stress effects on aquatic poikilotherm animals. To cope with and attenuate the effects of temperature changes, aquatic animals initiate corresponding modulations in physiological and biochemical metabolism. Previous observations on temperature effects have addressed many aquatic animals, such as crabs (Novo et al., 2005), shrimps (Furriel et al., 2000; Wang et al., 2006), and fish (Packer and Garvin, 1998; Ronisz et al., 1999). Research on the effects of temperature fluctuation and its mechanism is becoming increasingly important in the current aquaculture, especially for commercial species often transferred and reared at different temperatures. Mud crab (*Scylla paramamosain*, Estampador, 1949) is widely distributed along the coast of southeast China (Wang et al., 2005). *S. paramamosain* is an important inshore aquaculture species in the Indo-Pacific region. Aquaculture farmers usually transfer mud crabs situated in temperate waters to northern cold waters. One of the imminent issues in farming is the death of juvenile *S. paramamosain* caused by water temperatures beyond their adaptive capacity.

In both normal and fluctuating temperatures, managing the level of reactive oxygen species (ROS) produced through cellular metabolism is important to aquatic animals. A certain ROS level is necessary to meet the physiological demands of some chemical reactions (Kong et al., 2008). However, excessive ROS leads to oxidative stress (Di Guilio et al., 1989; Fang and Zheng, 2002; Finkel and Holbrook, 2000), which damages macromolecules, such as proteins, carbohydrates, nucleic acids, and lipids. Therefore, the removal of excess ROS and the maintenance of a dynamic balance between the production and removal of ROS are very important to protect organisms from oxidative stress and maintain normal physiological functions (Livingstone et al., 1992; Winston, 1991). Aerobic organisms, including aquatic animals, have an evolutionary-conserved antioxidant defense system for the removal of excess ROS. This defense system is composed of antioxidative enzymes and non-enzymatic antioxidants. The former includes superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and glutathione peroxidase (GPX, EC 1.11.1.9). The latter includes fat-soluble vitamins (e.g., α-tocopherol and β-carotene) and water-soluble small molecules [e.g., glutathione (GSH) and ascorbic acid]. When the capability to

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remove ROS is weakened, excess ROS initiates lipid peroxidation and generates malondialdehyde (MDA) as a final product, which has been used as a biomarker for oxidative stress (Doyotte et al., 1997; Viarengo et al., 1990, 1991a). Numerous studies on antioxidant defense systems have been carried out in aquatic animals, such as fish (e.g., Ronisz et al., 1999), crustaceans (e.g., Dandapat et al., 2000; Kong et al., 2004a, 2005, 2007a,b; Niyogi et al., 2001), cephalopods (e.g., Zielinski and Portner, 2000), and bivalve mollusks (e.g., Sheehan and Power, 1999). The effects of antioxidant defenses are closely correlated with temperature variations (Power and Sheehan, 1996; Ronisz et al., 1999; Viarengo et al., 1991a; Wilhelm et al., 2001), reproduction and food availability (Cancio et al., 1999), ontogenetic development (Livingstone et al., 1992; Rudneva, 1999; Viarengo et al., 1989, 1991b;), and xenobiotics (Di Guilio et al., 1989; Livingstone et al., 1989, 1992; Sheehan and Power, 1999; Viarengo et al., 1990; Winston, 1991). However, a majority of these studies have focused on bivalve mollusks, particularly in mussels *Mytilus edulis* and *Mytilus galloprovincialis* (e.g., Di Guilio et al., 1989; Livingstone et al., 1989, 1992; Sheehan and Power, 1999; Viarengo et al., 1990; Winston, 1991). Some studies on antioxidant defenses have been reported in crabs (e.g., Gamble et al., 1995; Kong et al., 2004a, 2005, 2007a,b; Orbea et al., 2002). However, few studies have analyzed the responses of antioxidant defenses to low temperature acclimation in the muscle of crabs.

Another set of enzymes important for aquatic animals in managing temperature change is adenosine triphosphatase (ATPase, EC 3.6.1.3). Transmembrane ATPase maintains ion equilibrium across cell membranes, and the disruption of this equilibrium often results in physiological dysfunction (Kong et al., 2007b). Na^+/K^+-ATPase is responsible for the osmoregulation of Na^+ and K^+ by transferring ions at the expense of energy supplied by ATP decomposition. Mg^{2+}-ATPase regulates the levels of Mg^{2+} ion, and Ca^{2+}-ATPase modulates the concentration of Ca^{2+} ions in the cytosol. Ca^{2+}/Mg^{2+}-ATPase is a mitochondrial membrane protein that transfers free Ca^{2+} from the cytosol into the mitochondria only in the presence of Mg^{2+} ions (Kong et al., 2004b; Zylinska and Legutko, 1998). The activities of these ATPases are regulated by environmental factors. In the gills of crabs, fluctuating ambient salinity regulates the activity of Na^+/K^+-ATPase, which then maintains stable physiological levels of Na^+ and K^+ (Ahearn et al., 1999; López Mañanes et al., 2002; Onken and Riestenpatt, 1998; Towle, 1997). The increased Na^+/K^+-ATPase levels in the gills at low salinity are mainly used to enhance the intake of more ions against the loss of ions in body fluid. The activity of Na^+/K^+-ATPase can also be regulated by temperature. Cold-acclimatized species such as crabs and fish have increased enzyme activity (Gabbianelli et al., 1996; Kong et al., 2007a; Staurnes et al., 1994; Stuenkel and Hillyard, 1980). Previous studies have addressed that ATPase activities depend on enzyme concentration (Schwarzbaum et al., 1992), isozyme expression (Lin and Somero, 1995; Somero, 1995), and membrane lipid composition (Gabbianelli et al., 1996; Hazel, 1995). The activities of Mg^{2+}-ATPase, Ca^{2+}-ATPase, and Ca^{2+}/Mg^{2+}-ATPase increase in the gills of mud crab *Scylla serrata* acclimated in cold water (Kong et al., 2005, 2007a). Gills are often used in the studies on ATPase activity because they are directly exposed to water and are greatly affected by environmental temperature. By contrast, the level of ATPase activity in the muscle of crabs is less studied. At the beginning of this study, in the muscle of crabs acclimated at low temperature, we speculate that these four ATPase activities will increase to compensate for weakened catalytic performance at low temperatures.

In this study, the activities of four ATPases and antioxidant enzymes, as well as MDA content, were measured in the muscle of *S. paramamosain* acclimated at low temperatures. The main objective of this study is to assess the effects of low temperatures on the ATPase activities and antioxidant defenses of *S. paramamosain*, as well as to understand the biochemical responses to low temperatures in mud crabs. The results of this study may provide practical guidelines for transferring mud crabs from temperate to cold waters.

2. Materials and methods

2.1. Experimental animals

Juvenile *S. paramamosain* from one brood (one mother), fed and managed under the same conditions, were sampled from a farm in Zhaoan, Fujian province. The average annual surface water temperature and water temperature during the sampling period were 21.4 and 28 °C, respectively. After the crabs were brought to the laboratory, 60 healthy male crabs without visible damage were selected for one week of adaptation, 40 crabs of which were grouped randomly into four after adaptation (Table 1).

2.2. Temperature acclimation

For each group of ten replicates, temperature acclimation was conducted in 2 L plastic tanks (one crab per tank with 1.5 L of water) with a 12 hour alternating light/dark cycle following the methods described by Cheng et al. (2005). Filtered and aerated seawater with a salinity of approximately 25 PSU was used in the tanks. A commercial crab diet supplemented with short-necked clam (*Ruditapes philippinarum*) was used to feed the crabs. After one week of adaptation, the water temperature was decreased in the acclimation groups at a rate of 1 °C/12 h until the assigned experimental temperatures of 5 °C ± 1.5 °C, 10 °C ± 1.5 °C, and 15 °C ± 1.5 °C were reached. Another group of crabs was kept at room temperature (27 °C ± 2.5 °C) as a control. The acclimation process started as soon as the water temperatures reached the desired temperatures. Water was changed every three days using prepared water with the same temperature for each group. During initial adaptation, temperature decrement, and temperature acclimation, the crabs were managed following the procedures described by Wang et al. (2007).

2.3. Sample preparation

After cold acclimation of three weeks, the muscles of the second appendage from the crabs in each group were carefully removed and stored immediately at −80 °C. To prepare samples for enzyme assay, approximately 0.2 g muscle tissue of each crab was homogenized in 1.8 mL of 0.9% sodium chloride solution using a hand-driven glass-Teflon homogenizer on ice. The homogenates were centrifuged at 3824 *g* for 15 min at 4 °C. The supernatant was carefully pipetted in aliquots and stored at −20 °C. All assays were completed within two weeks after preparation.

2.4. Enzyme assays and MDA measurements

The enzymatic activities of four ATPase were measured based on a method described by Kong et al. (2008). The standard incubation mixture (500 μL) for determining ATPase activity at 37 °C consisted of 30 mM imidazole buffer (pH 7.0), 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.1 mM ouabain, 0.1 mM EGTA, 3 mM ATP, and crude extract (approximately 0.2 mg/mL protein). This incubation mixture was modified slightly to measure each ATPase. Na^+/K^+- ATPase activity was measured in the incubation mixture without ouabain and

Table 1
Grouping status of juvenile *Scylla paramamosain* in experiment.

Items	groups			
	A (5 °C)	B (10 °C)	C (15 °C)	D (27 °C)
Carapace width/cm	3.06 ± 0.27	2.88 ± 0.24	2.95 ± 0.24	2.86 ± 0.18
Carapace length/cm	2.16 ± 0.21	2.09 ± 0.18	2.13 ± 0.18	2.08 ± 0.17
Body weight/g	5.04 ± 1.18	4.60 ± 1.13	4.69 ± 1.07	4.53 ± 0.49

Ca^{2+}. Ca^{2+} was omitted from the standard mixture to measure Mg^{2+}-ATPase activity. Mg^{2+} and EGTA were omitted to determine Ca^{2+}-ATPase activity. EGTA was omitted to measure Ca^{2+}/Mg^{2+}-ATPase activity. The activities of SOD and GPX were measured according to the methods reported by Orbea et al. (2002). SOD activity was determined by measuring the degree of inhibition of the reduction of cytochrome *c* by superoxide anion radicals generated by the xanthine:xanthine oxidase system. The rate of reduction was obtained spectrophotometrically at 550 nm (Spectrophotometer 752N, Shanghai Cany Precision instrument Co., Ltd, China). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 50 μM hypoxanthine, 1.87 mU/mL xanthine oxidase, and 10 μM cytochrome *c*. Using a spectrophotometer at 340 nm, GPX activity was measured by determining the decrease in NADPH during the formation of reduced glutathione via glutathione reductase. In this procedure, 0.2 mM H_2O_2 in 100 mM potassium phosphate buffer (2 mM glutathione, 0.5 mM sodium azide, 2 U/mL glutathione reductase, and NADPH 120 μM, pH 7.0) was used as the substrate. CAT activity was measured using the molybdate colorimetric method described by Goth (1991). All enzyme assays were performed at 37 °C in triplicates. MDA content was determined via the protocol provided by Draper and Hadley (1990). One unit of ATPase activity was defined as micromoles of inorganic phosphate (Pi) produced by ATP decomposition per milligram protein per hour. One unit of SOD activity is defined as the inhibition of 50% SOD activity in 1 mg protein. One unit of GPX activity is defined as the decrease of 1 μmol/L GSH (deducted non-enzyme action) in 1 mg protein per minute. One unit of CAT activity is defined as the decomposition of 1 μmol H_2O_2 in 1 mg protein in 1 min.

Protein concentration was determined according to the method described by Bradford (1976). Bovine serum albumin (AMRESCO Inc., USA) was used as the standard protein for the standard curve of protein concentration. The samples were diluted so that OD values are less than 0.5.

2.5. Statistical analysis

Statistical analysis was performed using the statistical analysis tools of EXCEL 2007. Homogeneity of variance was measured using one-way ANOVA. Significant differences between the acclimated group and the control were determined using Student's *t*-test. The significance levels were assigned at $P = 0.01$ and 0.05.

3. Results

3.1. Antioxidant defense in the muscle of *S. paramamosain* under low temperature acclimation

In the muscle of *S. paramamosain*, the changes in the activities of antioxidant enzymes SOD, CAT, and GPX at low temperatures are similar. In general, when measured at 37 °C, enzymatic activities in crab muscles collected at lower temperatures are lower than those in tissues collected at higher temperatures. The activity of SOD was 62.8 U/mg at 27 °C, a temperature similar to the native habitat of *S. paramamosain*. At 15 °C, SOD activity was 56.5 U/mg, slightly lower but not statistically significant. The SOD activities measured at 10 and 5 °C were 50.6 and 45.9 U/mg, respectively. Both values were significantly lower than those obtained at 27 °C ($P<0.05$ and $P<0.01$, respectively). The patterns of CAT and GPX activities are similar (Fig. 1). This result is consistent with what was previously observed in the gills (Kong et al., 2007a). The measurement of MDA content confirmed the build-up of oxidative stress. At 27 °C, approximately 0.8 nmol/mg of MDA was present in the muscle homogenate of *S. paramamosain*. A slightly higher MDA concentration (i.e., above 1 nmol/mg) was detected in the muscle tissue of *S. paramamosain* acclimated at 15 °C. The contents of MDA in the muscle of the 5 and 10 °C groups were 1.6 and 2.6 nmol/mg, respectively. Both values are significantly higher than that of the 27 °C group ($P<0.01$).

3.2. Four ATPase activities in *S. paramamosain* muscle under low temperature acclimation

Changes in ATPase activity are shown in Fig. 2. The Na^+/K^+-ATPase activity of the tissue samples at 10 and 15 °C was significantly higher ($P<0.05$) than that of the control at 27 °C. The Na^+/K^+-ATPase activity of the sample at 5 °C was similar to that of the control ($P>0.05$). For

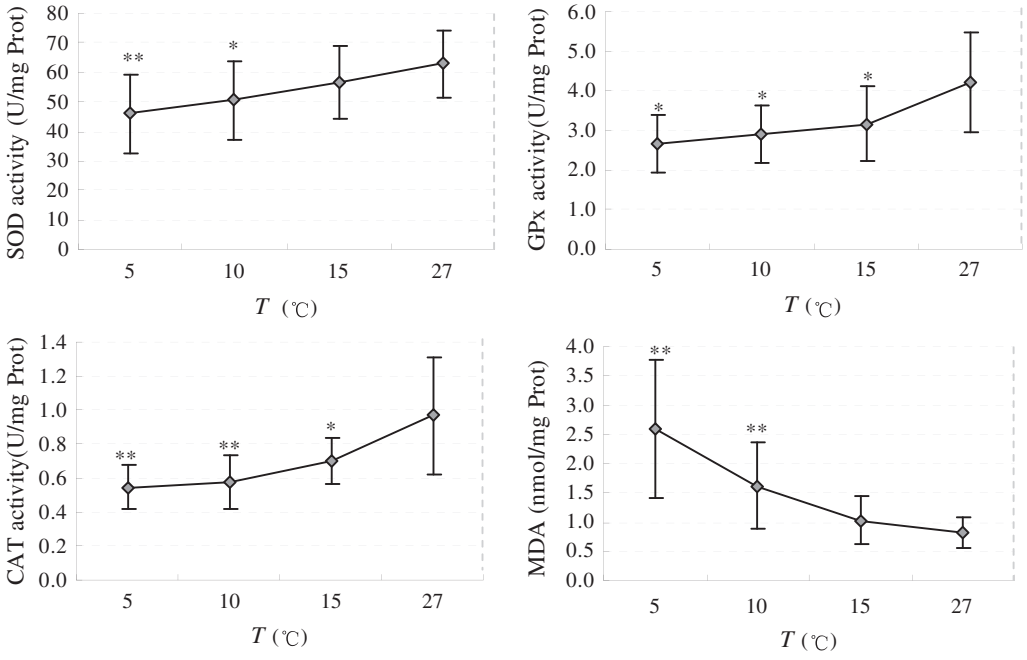

Fig. 1. Changes in SOD, CAT, and GPX activities, as well as MDA content in the muscle of *Scylla paramamosain* under low temperature acclimation. Note: All values in the figures are presented as means ± standard deviation (n = 10). '*' represents the significant difference between the acclimation and the 27 °C group ($P<0.05$); '**' represents highly significant difference ($P<0.01$).

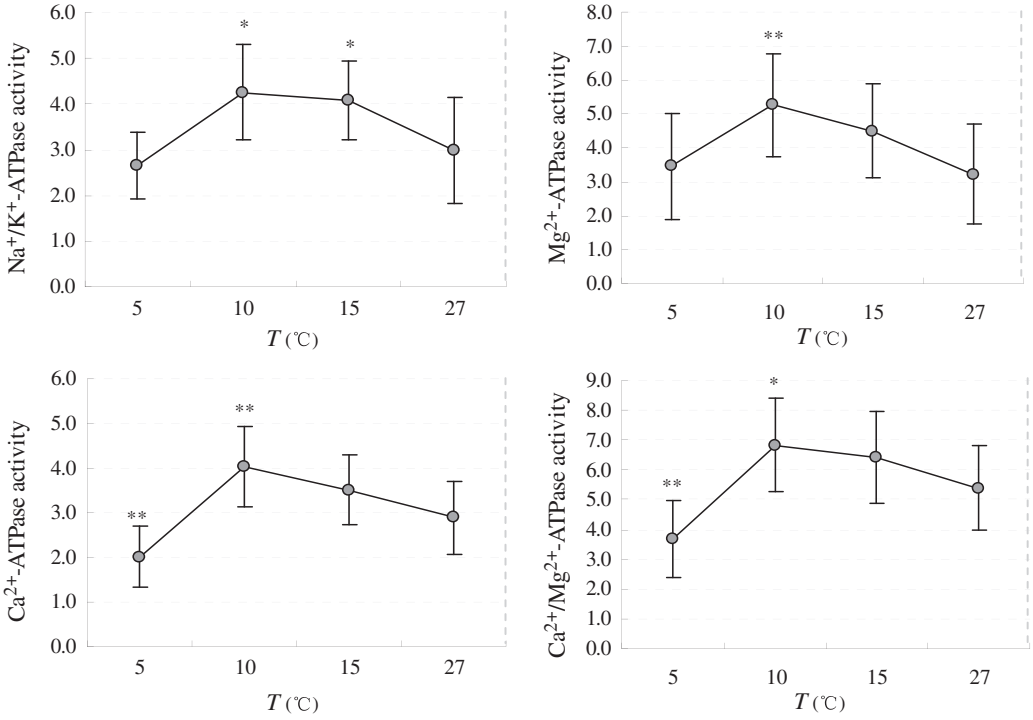

Fig. 2. Changes of four ATPase activities in the muscle of *Scylla paramamosain* under low temperature acclimation. Note: All values in the figures are presented as means ± standard deviation (n = 10). Enzyme activity unit is μmol(Pi)/h/mg Prot. '*' represents significant difference between the acclimation and the 27 °C group ($P<0.05$); '**' represents highly significant difference ($P<0.01$).

Mg^{2+}-ATPase, significantly higher enzymatic activity was detected only from the sample taken from the crabs acclimated at 10 °C ($P<0.01$). Similarly, Ca^{2+}/Mg^{2+}-ATPase and Ca^{2+}-ATPase activities were significantly higher in the samples from the 10 °C group compared with those of the samples from the 27 °C group. However, the activities of these two enzymes in the tissue samples from crabs acclimated at 5 °C were both significantly lower compared with those in the control group ($P<0.01$).

4. Discussion

Almost all organisms can adapt to certain temperature fluctuations through physiological and metabolic modifications, a compensated adjustment mechanism (Hochachka and Somero, 1984). Other mechanisms, such as increased mitochondrial density at low temperatures (Wang et al., 2007), are also important for organisms to ensure survival at extremely low temperatures. However, physiological and metabolic adjustments, as well as other mechanisms, have a critical limit of adjustable temperature range. Organisms with more flexible compensated abilities have a better chance of survival in an environment with dramatic temperature fluctuations because of their wider adaptive range of habitat temperatures through adaptation and natural selection (Hoang et al., 2002).

Aerobic organisms continuously produce ROS, even when they simply maintain a basal level of metabolism at low temperatures. At higher temperatures, organisms maintain a higher metabolic rate. As a result, more ROS is produced, thereby inducing the expression of more antioxidative enzymes. With the same logic, the expression of antioxidative enzyme is reduced because less ROS is produced at low temperatures. Previous studies on the gills of crabs (Kong et al., 2008) and on bivalves (Cancio et al., 1999; Sole et al., 1995; Viarengo et al., 1991a) reported that a positive correlation exists between antioxidative enzyme activities and seasonal temperatures. The present study clearly confirms that antioxidative enzyme activities decrease under low temperature acclimation. Interestingly, higher levels of MDA at 5 and 10 °C were detected. The elevated MDA content is believed to be the result of lipid peroxidation under oxidative stress (Kong et al., 2005). This finding implies the presence of more lipid peroxidation at 5 and 10 °C than at 27 °C, although less ROS was produced in metabolism at lower temperatures. When temperature is decreased, less ROS is produced. Simultaneously, the ability of scavenging ROS also reduces in crabs. When the antioxidant defense system cannot effectively remove the surplus of ROS produced at lower temperatures, MDA will accumulate in the cell. Therefore, in the present study, a higher level of MDA was observed at 5 and 10 °C. However, the mechanism by which antioxidative enzyme activity decreases at low temperatures remains unclear. As described by Kong (2004), the declined ability to synthesize antioxidative enzymes at low temperatures might present one possible reason.

The main function of transmembrane ATPase is to provide energy for ion transportation across membranes and thus maintain ion homeostasis in the cytoplasm. Previous studies suggested that ATPase activity is weakened in poikilothermal animals at low temperatures and that new ATPases are synthesized to compensate for this weakened activity (Hochachka and Somero, 1984; Kong et al., 2005). Therefore, higher ATPase concentration appears in the cells of poikilothermal animals at low temperatures, showing higher activity at the same measured temperature. The increased activities of all ATPases at 10 °C are believed to be the result of ATPase compensation for low temperatures. However, at 5 °C, the compensated ability of ATPase dramatically weakened. Therefore, the temperature 5 °C was beyond the critical value of adaptive temperature.

In addition, the observations in the current study regarding Ca^{2+}-ATPase and Ca^{2+}/Mg^{2+}-ATPase activities under low temperature acclimation in mud crab muscles are different from those of our previous study (Kong et al., 2005). As described by Kong et al. (2005), ATPase activities in the hepatopancreas of mud crab increase under low temperature acclimation. However, ATPase activities in the muscle increase at 10 and 15 °C but decrease at 5 °C. The minor difference between these ATPase activity changes is related to their functions and the ions they transport. Ca^{2+} is known to play a role in apoptosis. Previous

studies suggest that Ca^{2+} accumulation in the cytoplasm disturbs Ca^{2+} ion homeostasis, subsequently causing the dysfunction of mitochondria and endoplasmic reticulum and then finally leading to the apoptosis of muscle cells (Kong, 2004). At 5 °C, muscle cells cannot synthesize more Ca^{2+}-ATPase and Ca^{2+}/Mg^{2+}-ATPase to maintain a stable physiological level of Ca^{2+}. However, in the hepatopancreas, a more essential organ for the survival of crabs, an additional mechanism may be present to regulate Ca^{2+} level and thus ensure the integrity of physiological functions.

For mud crabs, the muscle plays an important role in activities, such as preying, mating, and fighting. The movement of crabs weakens at low temperatures because of insufficient energy. In the current study, mud crabs acclimated at 15 °C could move under the disturbance. At 10 °C, they could only move slightly when touched. However, at 5 °C, they became motionless even after being touched. After acclimation, the crabs kept at 5 °C were transferred back to room temperature. The crabs still could not move, except for the slight trembling of appendages after being kept at room temperature for more than 2 h. Behavioral observation indicated that the mud crabs at 5 °C were in the state of anesthesia. Therefore, 5 °C was suggested beyond a critical limit of low temperature adaptation for mud crabs. Results from biochemistry assays also support this suggestion. At 5 °C, the declined antioxidative enzymes result in remarkable MDA accumulation. Furthermore, Ca^{2+}-ATPase and Ca^{2+}/Mg^{2+}-ATPase activities at 5 °C also significantly decreased. These results, taken together, suggest that a temperature of 5 °C is very low for mud crabs and that it is beyond the critical value of adaptive low temperatures. At this temperature, mud crabs would not survive because of physiological function disturbance (Kong, 2004). The biochemistry analysis of a set of enzymes, which play vital roles in cellular function, not only explains the behavioral observation, but also functions as a set of valid biomarkers to monitor the acclimation of mud crabs.

The results imply that juvenile crabs at 5 °C could not survive with the extension of acclimation. Therefore, juvenile mud crabs should be cultured at temperatures above 5 °C. These findings provide guidelines for farmers to culture mud crabs during the winter or to produce acclimated crabs in cold water.

5. Conclusions

In summary, at the adjustable level of low temperature, the responses of antioxidative enzymes and four ATPases in the muscle of mud crabs were used to cope with cold stress and attenuate the effect of low temperatures. However, the ability to scavenge ROS decreases when temperature is decreased beyond the critical value (i.e., 5 °C). Lipid peroxidation was incurred at lower temperatures based on the gradually accumulated MDA. The reduced compensation ability of ATPase at 5 °C implies that the ability to maintain the ion homeostasis level is weakened. Therefore, juvenile mud crabs should be cultured at temperatures above 5 °C. These findings guide farmers in the cultivation of mud crabs during winter or in transferring the temperate species to culture in cold water.

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