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RESEARCH ARTICLE

Seasonal variations of ATPase activity and antioxidant defenses in gills of the mud crab *Scylla serrata* (Crustacea, Decapoda)

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Abstract The mud crab *Scylla serrata* is an important commercial crustacean being widely distributed along the southeastern coast of China. This crab and particularly its gills are subjected to seasonal fluctuations of environmental factors due to direct exposure to seawater. The investigation of seasonal variations of ATPase and antioxidant defenses in gills could be helpful for the understanding of physiological regulation mechanism of seasonal adaptations. In this study, mud crabs were collected from the subtropical waters near Xiamen island, Southeast China (24°26′46′′N, 118°04′04′′E), in August and November, 2002, and February and May, 2003, respectively, being considered as specimens from summer, autumn, winter and spring, respectively. Only healthy intermoult male crabs without carapace or appendage damage were used, having a carapace width of 7.05 ± 0.52 cm and a wet weight of 130 ± 20 g. The activities of four ATPases and three antioxidation enzymes (superoxide dismutase, catalase, glutathione peroxidase) and the content of malondialdehyde in gills were measured. The results showed that the activities of Na^+, K^+-ATPase, Mg^{2+}-ATPase, Ca^{2+}-ATPase and Ca^{2+}, Mg^{2+}-ATPase reached maximum levels similarly in summer and decreased in winter. The activities of superoxide dismutase and catalase were higher in summer and lower in winter, with a significant decrease in winter compared to the other seasons ($p < 0.01$). Glutathione peroxidase activity was higher in summer, and lower in autumn, being significantly higher in summer or winter than in spring or autumn ($p < 0.01$). Malondialdehyde content was higher in summer and lower in spring with significant differences among the different seasons ($p < 0.01$). In summary, the obvious seasonal activity variations of four ATPases and antioxidant enzymes and the content of malondialdehyde reflect a seasonal regulation of the physiological metabolising enzyme and the antioxidant capacity to cope with seasonal alterations of environment factors such as fluctuating salinities and temperatures.

Abbreviations

ATPase	Adenosine triphosphatase
ROS	Reactive oxygen species
SOD	Superoxide dismutase
CAT	Catalase
GPX	Glutathione peroxidase
GSH	Reduced glutathione
MDA	Malondialdehyde

Introduction

As seasonal temperature fluctuates, aquatic animals, such as crab (Novo et al. 2005), shrimp (Furriel et al. 2000; Wang et al. 2006) and fish (Ronisz et al. 1999; Packer and Garvin 1998) cope with these changes and attenuate the impact on physiological homeostasis. In the case of crabs, particularly the gills, due to their exposure to water are susceptible to environmental factors such as temperature (Kong et al. 2007a, b) and salinity (Novo et al. 2005).

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Hence, several studies focus on the physiological adaptation of gills as adaptations to fluctuating environmental scenarios (Goncalves et al. 2006; Torres et al. 2007; Kong et al. 2007a, b). Here the determination of physiological baseline values at different seasons will be important for future studies on physiological responses to environmental changes.

ATPase (adenosine triphosphatase, EC 3.6.1.3) plays an important role in osmoregulation, in the cytosol and humoral fluids, in maintaining several ions at equilibrium physiological levels. The Na^+, K^+-ATPase is responsible for the osmoregulation of Na^+ and K^+ ions by transferring ions at the expense of energy supplied by ATP decomposition. The Mg^{2+}-ATPase is used to modulate levels of the Mg^{2+} ion, the Ca^{2+}-ATPase adjusts the concentration of the Ca^{2+} ion in the cytosol, the Ca^{2+}, Mg^{2+}-ATPase being situated in mitochondrial membranes, transfers free Ca^{2+} from the cytosol into the mitochondria only in the presence of Mg^{2+} ions (Zylinska and Legutko 1998; Kong et al. 2004b). As ambient salinity is fluctuating, the activity of Na^+, K^+-ATPase in the gills of crabs will change to maintain Na^+ and K^+ at physiological levels (López Mańanes et al. 2002; Towle 1997; Onken and Riestenpatt 1998; Ahearn et al. 1999). At the same time, the activity of Na^+, K^+-ATPase was shown to increase in cold acclimatized specimens measured at the same conditions (Kong et al. 2007a). Similar results were also achieved in cold acclimated fish (Stuenkel and Hillyard 1980; Staurnes et al. 1994; Gabbianelli et al. 1996). For Mg^{2+}-ATPase, Ca^{2+}-ATPase and Ca^{2+}, Mg^{2+}-ATPase, they perform important regulatory functions to maintain Ca^{2+} and Mg^{2+} ions at physiological equilibria (Kong et al. 2004b, 2005, 2007a, b). The disruption of homeostasis of Ca^{2+} and Mg^{2+} will lead to a physiological dysfunction and diseases (Kong et al. 2007b). In our previous studies, Mg^{2+}-ATPase, Ca^{2+}-ATPase and Ca^{2+}, Mg^{2+}-ATPase activities were also shown to increase in mud crab *Scylla serrata* acclimated in cold water (Kong et al. 2005, 2007a). However, actually, the previous studies in aquatic animals demonstrated that seasonal changes of ATPase activities were complicated and involved in enzyme concentration (Schwarzbaum et al. 1992), isozyme expression (Lin and Somero 1995; Somero 1995) and membrane lipid composition (Hazel 1995; Gabbianelli et al. 1996). In the present study, we put forward a hypothesis that the activities of four ATPases increase in gills of cold acclimatized crabs in winter. However, seasonal changes of Mg^{2+}-ATPase, Ca^{2+}-ATPase and Ca^{2+}, Mg^{2+}-ATPase activities in crustaceans remain unclear.

Aerobic organisms continuously produce endogenous reactive oxygen species (ROS) in the process of metabolism, which could affect macromolecules, such as proteins, carbohydrates, nucleic acids and lipids, in a process of oxidative damage or oxidative stress (Di Guilio et al. 1989; Finkel and Holbrook 2000; Fang and Zheng 2002). Therefore, mechanisms are in place to remove excessive ROS by an antioxidant defense system comprising an enzymatic system, such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and glutathione peroxidase (GPX, EC 1.11.1.9) and non-enzymatic antioxidants, such as fat-soluble vitamins (e.g., β-carotene and α-tocopherol) and water-soluble low molecular [e.g., glutathione (GSH) and ascorbic acid]. An antioxidant defense system that scavenges ROS plays an important role in the maintenance of physiological homeostasis (Winston 1991; Livingstone et al. 1992). Imbalance in the producing and removal of ROS could result in lipid peroxidation initiated by a surplus of ROS, being used as an indicator to evaluate oxidative damages (Di Guilio et al. 1989; Viarengo et al. 1991a; Wilhelm et al. 2001). Malondialdehyde (MDA) is a final product of lipid peroxidation, being used as another biomarker for oxidative stress (Viarengo et al. 1990; Viarengo et al. 1991a; Doyotte et al. 1997).

A number of 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; Niyogi et al. 2001; Kong et al. 2004a, 2005, 2007a, b), cephalopods (e.g., Zielinski and Portner 2000) and bivalve mollusks (e.g., Sheehan and Power 1999). The results of these studies showed that physiological effects of antioxidant defenses were closely correlated with seasonal variations (Viarengo et al. 1991a; Power and Sheehan 1996; Ronisz et al. 1999; Wilhelm et al. 2001), reproduction and food availability (Cancio et al. 1999), ontogenetic development (Viarengo et al. 1989, 1991b; Livingstone et al. 1992; Rudneva 1999) and xenobiotics (Di Guilio et al. 1989; Livingstone et al. 1989, 1992; Viarengo et al. 1990; Winston 1991; Sheehan and Power 1999). However, the majority of these studies were focusing on bivalve mollusks, particularly using the mussels *Mytilus edulis* and *M. galloprovincialis* as experimental systems (e.g., Di Guilio et al. 1989; Livingstone et al. 1989; Viarengo et al. 1990; Winston 1991; Livingstone et al. 1992; Sheehan and Power 1999). Although there are some studies on antioxidant defense in crabs (e.g., Gamble et al. 1995; Orbea et al. 2002; Kong et al. 2004a, 2005, 2007a, b), no studies have been made on seasonal variations of antioxidant defense system.

The mud crab *S. serrata* is an important commercial crustacean widely distributed along the coast of southeast China (Wang et al. 2005), which inhabits temperate waters and becomes one of the important inshore fishery and aquaculture species in the Indo-Pacific region. In this study, the activities of four ATPase and antioxidant enzymes, and the content of malondialdehyde were measured in the gills of *S. serrata* sampled in different seasons. The main objective of this work was to investigate the seasonal variations of ATPases and antioxidation activities in gills of mud crab

S. serrata, and to evaluate the effects of environmental parameters on these variations.

Materials and methods

Crabs

Specimens of the mud crab *S. serrata* were collected from subtropical Xiamen waters, southeast China (24°26′46′′N, 118°04′04′′E), which were sampled in August and November in 2002, and February and May in 2003, respectively, representing specimens of summer, autumn, winter and spring. The collected crabs, immediately immersed in tanks in ambient water, were transferred to the laboratory within half an hour. Only healthy, intermoult male crabs, without carapace or appendage damage, were used to analyze physiological parameters. The average carapace width and wet weight of crabs used were 7.05 ± 0.52 cm and 130 ± 20 g, respectively. Time of collecting crab and temperature of ambient air and surface water are summarized in Table 1.

Sample preparation

After the carapaces of 12 male crabs were opened, the gills were carefully removed and stored at −80°C. Tissue portions of about 0.2 g were homogenized in a 1:9 (w/v, tissue wet weight: buffer solution of 0.9% sodium chloride) using a hand-driven glass–teflon homogenizer. The homogenates were centrifuged at 3,824*g* for 15 min. All operations mentioned above were carried out at 4°C. The supernatant was stored at −80°C until analysis.

Enzyme assays and MDA measurements

The various ATPase activities were based on the methods described by McConnell et al. (1999), Chauhan et al. (2002) and Cheng et al. (2005) and modified as the following details. The standard incubation mixture (500 μL) for determining the various ATPase activities 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 enzyme crude extract (about 0.2 mg/mL protein). To assess Na^+, K^+-ATPase activity, ouabain and Ca^{2+} were omitted from the incubation mixture; to measure Mg^{2+}-ATPase activity, Ca^{2+} was omitted from the reaction mixture; Mg^{2+} and EGTA were omitted to determine Ca^{2+}-ATPase activity; EGTA was omitted to measure Ca^{2+}, Mg^{2+}-ATPase activity. The reaction was started by the addition of 3 mM ATP. After 10 min, the reaction was stopped by the addition of 50 μL of ice-cold 15% TCA. A control for nonenzymatic decomposition of ATP was performed in parallel (enzyme crude extract was added after the end of reaction). The reaction solution was followed by centrifugation at 1,000*g* for 15 min, then, the 200 μL supernatant was added to 2 ml of a mixture (1% lubrol, 1% ammonium molybdate and 0.9 M H_2SO_4). The amount of inorganic phosphate (P_i) released from the substrate ATP was colorimetrically determined at 660 nm. The standard tube contained 200 μL of 0.5 mM standard inorganic phosphorus was also determined in parallel. The content of inorganic phosphate (P_i) was calculated by the difference of OD values between each ATPase reaction and nonenzyme control, multiplied by the ratio of P_i content to OD value in standard tube.

The activity of SOD and GPX were measured according to Orbea et al. (2002). SOD activity was conducted by measuring the degree of inhibition of the reduction of cytochrome *c* by superoxide radicals generated by the xanthine:xanthine oxidase system at 550 nm. The reaction mixture contains 50 mM potassium phosphate buffer (pH 7.8), 50 μM hypoxanthine, 1.87 mU/mL xanthine oxidase and 10 μM cytochrome *c*. GPX activity was measured by the NADPH consumption monitored at 340 nm during the formation of reduced glutathione by glutathione reductase using 0.2 mM H_2O_2 as substrate in 100 mM potassium phosphate buffer (pH 7.0) (containing 2 mM glutathione, 0.5 mM sodium azide, 2 U/mL glutathione reductase and NADPH 120 μM). CAT activity was measured using the molybdate colorimetric method described by Goth (1991). All enzyme assays were preformed at 37°C in triplicates. MDA content was determined by a protocol provided by Draper and Hadley (1990).

One unit of ATPase activity was expressed as micromoles inorganic phosphate (P_i) produced by ATP decomposition per milligram protein per hour. One unit of SOD activity is defined as the inhibition of 50% of 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)

Table 1 Average temperature of air and surface water in different months of Xiamen waters (°C)

Date	02–6	02–7	02–8[a]	02–9	02–10	02–11[a]	02–12	03–1	03–2[a]	03–3	03–4	03–5[a]
Air temp	27.35	30.85	32.65[a]	28.95	26.05	21.90[a]	17.65	14.70	16.65[a]	16.75	20.90	23.50[a]
Water temp	24.55	27.15	28.15[a]	26.05	20.85	19.15[a]	18.60	14.55	15.30[a]	16.20	18.95	22.80[a]

[a] Mud crabs were collected in 2002–8, 2002–11, 2003–2 and 2003–5, which correspondingly represent specimens from summer, autumn, winter and spring, respectively. Data were supplied partially by X. Jiang

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 is determined according to the method described by Bradford (Bradford 1976). Bovine serum albumin (BSA) (from AMRESCO) provided a standard curve of enzyme activity. The protein concentration of sample was optimally diluted to an OD level <0.5.

Statistical analysis

Statistical analysis was performed using one-way ANOVA (checked for homogeneity of variance) and Student's *t*-test by statistical analysis tool supplied by EXCEL 2003. The significance level were assigned at $p = 0.01$ and 0.05.

Results

Seasonal variations of four ATPase activities in gills of *S. serrata*

Seasonal variations of four ATPase activities in the gill tissues of *S. serrata* were similar in different seasons (Fig. 1). The activity of Na^+, K^+-ATPase was 4.97 ± 0.95 μmol P_i/h/mg Prot in summer, without significant difference compared with spring, and decreased significantly in winter to its lowest level (1.73 ± 0.79 μmol P_i/h/mg Prot) ($p < 0.01$ or <0.05). The activity of Mg^{2+}-ATPase was shown to be the highest in summer (3.27 ± 0.58 μmol P_i/h/mg Prot) and lower in autumn and winter. Statistical analysis indicated that Mg^{2+}-ATPase activity was significantly higher in spring or

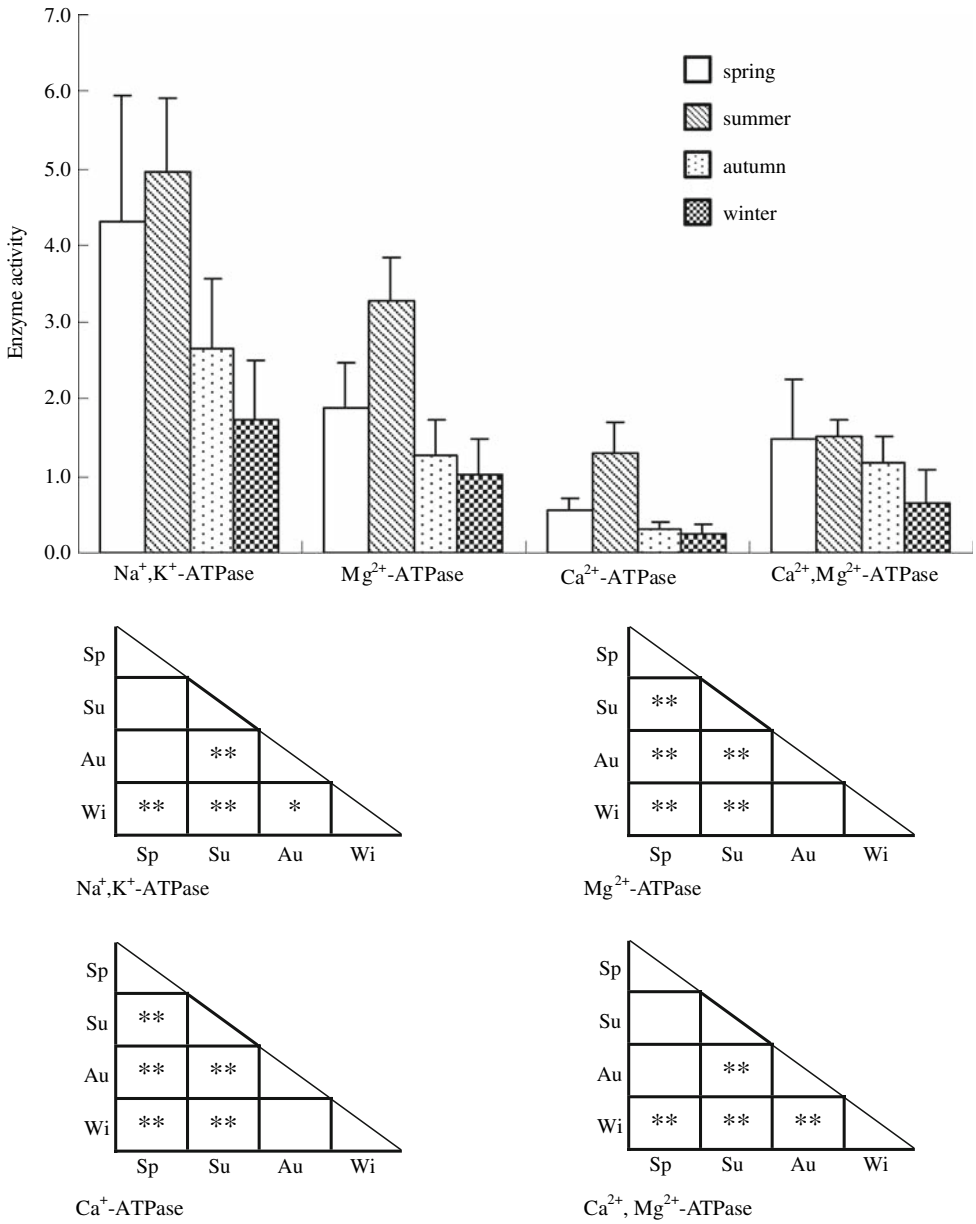

Fig. 1 Seasonal variations of Na^+, K^+-ATPase, Mg^{2+}-ATPase, Ca^{2+}-ATPase and Ca^{2+}, Mg^{2+}-ATPase activity in gill of *S. serrata.* All values present in figure are in mean + standard deviation (M + SD), the unit of enzyme activity refers to μmol P_i/h/mg Prot. The results of statistical analysis are shown in four half-tables, respectively. *Indicates significant differences ($p < 0.05$). **Indicates highly significant differences ($p < 0.01$). Sp, Su, Au and Wi represent spring, summer, autumn and winter, respectively

summer than in autumn or winter ($p < 0.01$). Ca^{2+}-ATPase activity indicated the similar seasonal changes with Mg^{2+}-ATPase, also being higher significantly in summer (1.29 ± 0.42 μmol P_i/h/mg Prot) than in other seasons ($p < 0.01$); and reached minimal values in winter (0.23 ± 0.12 μmol P_i/h/mg Prot), being significantly lower than in spring or summer ($p < 0.01$). The activity of Ca^{2+}, Mg^{2+}-ATPase was also higher in summer (1.52 ± 0.21 μmol P_i/h/mg Prot) than in winter (0.65 ± 0.42 μmol P_i/h/mg Prot) ($p < 0.01$). In general, the ranking of seasons, with regard to decreasing enzyme activity, was summer, spring, autumn and winter.

Seasonal variations of antioxidant enzyme activity in gills of *S. serrata*

Seasonal variations of antioxidant enzyme activity and MDA content in gills of *S. serrata* are shown in Fig. 2. SOD activity appeared to be higher in summer (27.08 ± 4.79 U/mg Prot) than in winter (15.05 ± 6.51 U/mg Prot). CAT activity also reached its maximum in summer (1.35 ± 0.42 U/mg Prot) and lowest values in winter (0.37 ± 0.12 U/mg Prot). The activities of SOD and CAT were significantly lower in winter than in the other seasons ($p < 0.01$). GPX activity was shown to be higher in summer (23.54 ± 4.75 U/mg Prot) and lower in autumn (2.92 ± 0.38 U/mg Prot). The activity of GPX was significantly higher in summer or winter than in spring or autumn ($p < 0.01$). The content of MDA was maximal in summer (3.03 ± 0.48 nmol/mg Prot) and lowest in spring (0.32 ± 0.07 nmol/mg Prot) with significant differences ($p < 0.01$) among all four seasons. Similar seasonal changes of SOD and CAT activity were higher in summer and lower in winter. The activity of GPX was higher in summer and winter than in spring and autumn. Interestingly, seasonal variations of GPX activity were in accordance with corresponding changes in MDA content.

Discussion

Seasonal variations of four ATPase activities in gills of *S. serrata*

The four ATPases studied here are essential for osmoregulation in the supply of energy for physiological processes

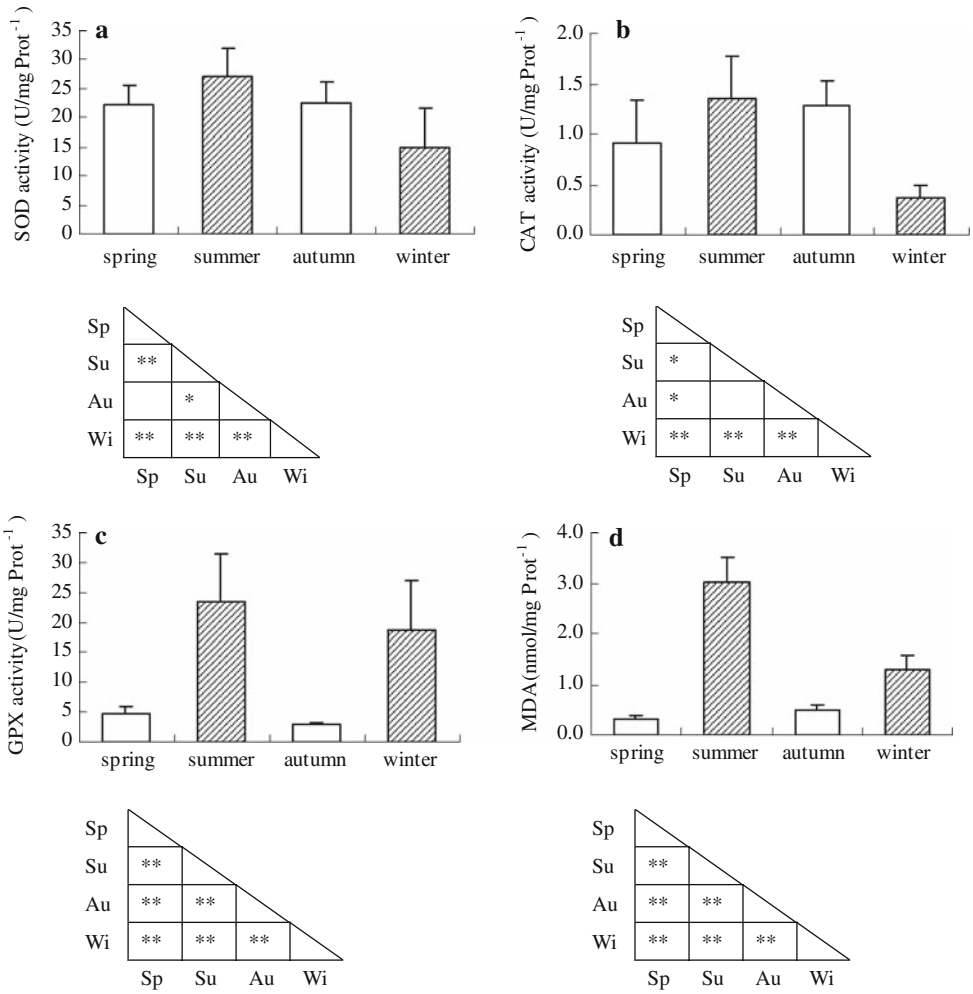

Fig. 2 Seasonal variations of SOD, CAT, GPX activity and MDA content. All values present in figure are in mean + standard deviation (M + SD). Statistics analyses are shown in four tables respectively. *Indicates significant differences ($p < 0.05$). **Indicates highly significant differences ($p < 0.01$). Sp, Su, Au and Wi represent spring, summer, autumn and winter, respectively

(Novo et al. 2005). To maintain ions such as Na^+, K^+, Mg^{2+} and Ca^{2+} in the cytosol at physiological equilibria is important for cell to perform biological functions (Kong et al. 2007a, b). When particularly Ca^{2+}-ATPase and Ca^{2+}, Mg^{2+}-ATPase would decrease, excessive Ca^{2+} would be accumulated in the cytosol and result in metabolic malfunction and cellular abnormalities (Kong et al. 2007b).

In this study, the activities of Na^+, K^+-ATPase, Mg^{2+}-ATPase, Ca^{2+}-ATPase and Ca^{2+}, Mg^{2+}-ATPase in the gills of *S. serrata* appeared to be higher in summer and lower in winter. Gills, being exposed to ambient waters, are key ion exchange organs for osmoregulation and ionoregulation, particularly during salinity fluctuations (Novo et al. 2005). As euryhaline crustacean were subjected to lower salinities, the activities of Na^+, K^+-ATPase increase in gills (Pequeux 1995; Lima et al. 1997; Towle 1997; López Mańanes et al. 2002). In summer, the salinity of surface waters in Xiamen generally decrease from 31 to 20 PSU due to influx from the Jiulong River with rising levels due to summer rains. In this study, the increase of Na^+, K^+-ATPase activity in summer might therefore be due to lower salinities. In winter, the decrease of it might result from the elevated salinity levels. The results of our study are in agreement with proposals from previous studies (Pequeux 1995; Lima et al. 1997; Towle 1997; López Mańanes et al. 2002). Temperature has been shown to have a crucial influence on metabolic activities in eurythermal aquatic animals (Hochachka and Somero 1984). In general, the concentration of metabolic enzyme increases compensatively at low temperatures, to maintain a basal level of metabolic activity because of the weakened ability of enzyme catalysis. At the same salinities, the activity of Na^+, K^+-ATPase increases in crabs as water temperature drops (Kong et al. 2007a, b). Such is the case in fish (Staurnes et al. 1994; Sun et al. 1994; Gabbianelli et al. 1996). The present increase of Na^+, K^+-ATPase activity in summer is hence interpreted to be mainly caused by seasonal salinity fluctuations. Of course, temperature and other environmental factors also have influence on enzyme activity but only of minor level.

Mg^{2+}-ATPase, Ca^{2+}-ATPase and Ca^{2+}, Mg^{2+}-ATPase in crab gills are responsible for the ionoregulation of Ca^{2+} and Mg^{2+} (Kong et al. 2004b, 2007a, b). In previous studies, the activities of these three ATPases in mud crabs increased at low-temperature acclimation (Kong et al. 2005, 2007a), which also compensated for the weakening of enzyme catalysis at lower temperatures. In the present study, in summer, the increases of these three ATPase activities might be most probably due to salinity stress. Until now, few studies have been reported for crustaceans on seasonal changes of these three ATPases. This makes it difficult to compare the present results with those of other investigations. In present study, three ATPases were demonstrated to show similar seasonal variations with Na^+, K^+-ATPase activity. Therefore, seasonal variations of four ATPase, mentioned above, showed that the regulation of Na^+ and K^+ performed by Na^+, K^+-ATPase was coincident with the modulation of Ca^{2+} and Mg^{2+} mediated by Mg^{2+}-ATPase, Ca^{2+}-ATPase and Ca^{2+}, Mg^{2+}-ATPase in the process of seasonal adaptation.

Seasonal variation of antioxidant defense in gills of *S. serrata*

Reactive oxidative species are continuously produced in physiologically active aerobic organisms. However, there activity is considered to be closely related to environmental factor (Di Guilio et al. 1989). At higher temperatures, excessive ROS is produced at accelerated metabolic rates (Finkel and Holbrook 2000), leading to an accumulation, without being removed with time. The accumulated ROS might induce antioxidant enzymes (SOD, CAT and GPX) to be removed (Fang and Zheng 2002). Gills, of *S. serrata*, with direct exposure to water, are shown to be susceptible to environmental changes (Kong et al. 2007a, b). In the summer results of the present study, the activities of SOD, CAT and GPX were induced to remove surplus ROS. These results were consistent with previous studies on bivalves by Viarengo et al. (1991a), Solé et al. (1995) and Cancio et al. (1999).

MDA as the final product of lipid peroxidation is considered to be a biomarker for oxidative stress (Doyotte et al. 1997). In the present study, the content of MDA were maximal due to oxidative damage resulted from excessive ROS produced in summer, which could induce antioxidant enzymes (SOD, CAT and GPX) to enhance the scavenging of ROS. However, as the increase of antioxidant enzyme activities is not sufficient to remove accumulated ROS, oxidative stress will occur. Therefore, in the present study, the content of MDA increases in summer. On the other hand, the content of MDA was the second highest in winter, which might be due to the weakening ability to remove ROS, since the activities of SOD and CAT slow down with decreasing temperature. As a result, ROS was accumulated to lead to an increase of MDA content in winter. These results were in accordance with studies on mollusks by Solé et al. (1995), Power and Sheehan (1996), Regoli (1998) and Niyogi et al. (2001). Interestingly, in winter, GPX activity increased significantly to become the second highest level, which could compensate for insufficient of CAT activity in removing ROS. The complexity of seasonal changes, however, warrants further studies on the seasonal variation of MDA content.

The harmoniousness of enzyme activity in seasonal regulation

The gills of *S. serrata* are susceptible to changes of environmental factors such as temperature and salinity. The increase

of antioxidant enzyme activities in summer was shown to be used to remove excessive ROS that resulted from enhanced metabolic activity. Here, increasing temperature is considered to be a major effect factor. In the present study, the increase of four ATPase activities was demonstrated to be used to perform osmoregulation and ionoregulation and to maintain ions at basal physiological level in summer. Here, salinity, decreasing in summer, might be thought as a mainly effective factor. Of course, temperature and other environmental factors also have impacts on enzyme activity but only of minor level. Therefore, in this study, seasonal change of enzyme activity is suggested to be an integrated effect of various seasonal factors, and the aim of these changes is speculated to maintain basic physiological function.

From the previous studies, seasonal variation of metabolic enzyme in aquatic animals is affected by both exogenous (e.g., temperature, salinity and xenobiotics) (Viarengo et al. 1989, 1990; Packer and Garvin 1998; Lucu and Devescovi 1999; Satyavathi and Prabhakara Rao 2000) and endogenous factors (e.g., reproduction, hormone and immunity) (Winston 1991; Holmblad and Soderhall 1999; Forlin and Haux 1990; Hylland et al. 1998), as well as ontogenesis and ageing (Viarengo et al. 1991b). Therefore, in order to obtain an accurate explanation for seasonal variations of these physiological parameters, multiple environmental factors should be taken into account, and further studies need to be carried out under the control experimental conditions in the future.

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