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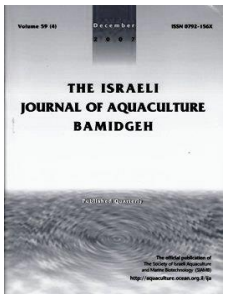

The Effects of High Temperatures on Survival Rates and Immunity of Saltwater Clam *Meretrix meretrix* from Different Geographical Populations

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Keywords: *Meretrix meretrix*; geographical populations; high temperature; immunity; Hsp

ABSTRACT

High temperature has significant effects on the survival and immunity of organisms. In order to conduct a detailed investigation into the response of the saltwater clam *Meretrix meretrix* from different geographical populations (GuangXi, JiangSu, ShanDong, LiaoNing) to high temperatures, we examined the survival rates and antioxidant enzyme activities of these clams that were exposed to different temperature treatments (25°C, 35°C, 37.5°C, 40°C). Results showed that the survival rate of clams from GuangXi was highest at 40°C, followed by JiangSu. SOD and CAT activities of all the clams increased with the increasing temperatures, and then gradually decreased at 40°C over time. The expression levels of Hsp70 of *M. meretrix* from four populations were analyzed by qPCR sampled from the hepatopancreas after 72 h under different temperatures. Higher Hsp mRNA expression of GuangXi at 40°C indicated that clams of GuangXi have stronger high temperature resistance than those of the other geographical populations. Our findings provide a basis for the choice of cultured clams with a high temperature tolerance.

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Introduction

Temperature is one of the most vital and relevant abiotic factors which affects the biochemistry, physiology, and behavior of aquatic animals (Beitinger et al., 2000; Gillooly et al., 2001). Many studies show that temperature stress can influence major physiological processes, metabolism, reproduction, and disrupt cellular homeostasis (Abele et al., 2002; Fearman and Moltschaniwskyj, 2010). Under high temperature conditions, antioxidant enzymes such as superoxide dismutase (SOD), and catalase (CAT) can respond to the thermal stress, accompanied with changes of enzyme activities to avoid oxidative damage. They also serve as biomarkers to study the impact of stressful conditions (Pérez-Casanova et al., 2008; Radovanović et al., 2010; Sellami et al., 2015).

Heat shock proteins (Hsps) are present in all organisms. They are responsible for maintaining cellular viability by performing essential biological functions under various stressful conditions such as high temperature, pathogenic bacteria, hypoxia stress, and other negative factors (Srivastava, 2002; Rungrassamee et al., 2010). Hsps have become useful models for studying transcriptional regulation, stress response, and molecular evolution (Xu et al., 2015). Hsps are involved in immune responses which can protect shellfish from both environmental stress and biological stress (Wang et al., 2009; Fu et al., 2011; Yue et al., 2011; Wu et al., 2014; Li et al., 2016).

The clam *Meretrix meretrix* is an important commercial mollusk in the coastal areas of Asia and is cultured widely in China (Jayabal and Kalyani, 1986; Ho and Zheng, 1994). In recent years, *M. meretrix* have shown high mortality rates in the summer, resulting in heavy economic losses in clam farming. In this study, we selected *M. meretrix* of different geographical populations from south to north China (GuangXi, JiangSu, ShanDong, LiaoNing). We examined survival rates, antioxidant enzyme activities, and expression of the heat shock protein 70 (Hsp70) in clams from different geographical populations under high temperature treatments. The aim of this study was to examine the heat tolerance (under high temperatures) of clams from different populations and provide basic information for new cultivars with high temperature tolerance.

Materials and methods

Experimental animals.

Healthy clams were collected from GuangXi, JiangSu, Shandong, LiaoNing geographical populations (Fig.1). All clams were kept at a constant temperature (25±1°C) with sifted sand and aerated filtered seawater, and were fed with *Isochrysis galbana* twice per day. After two weeks, individuals from each natural population were assigned to four treatments for the temperature tolerance experiment.

Fig. 1. The geographical location of clam collection.

Survival rate and sampling.

The clams of each population were maintained at a temperature of 25°C (control), 35°C, 37.5°C, and 40°C, with 20 clams per tank. Three replicates were employed for each temperature, totaling 48 tanks. Numbers of deaths were counted every 12 hours and survival rate was calculated using the following formula: Survival Rate =
1- (numbers of deaths/total number of individuals at the beginning of the experiment).

Hepatopancreas were dissected after 1 h, 6 h, 12 h, 24 h, 36 h, 48 h, and 72 h, from three randomly selected individuals per tank. All samples were washed with 1 × PBS (phosphate-buffered saline, 0.01 M), frozen immediately in liquid nitrogen and stored at -80 °C for qPCR.

Antioxidant capacity.

All samples were washed with 1 × PBS (phosphate-buffered saline, 0.01 M), frozen immediately in liquid nitrogen, and stored at -80°C for later examination of antioxidant enzymes.

Superoxide dismutase (SOD) and catalase (CAT) were determined spectrophotometrically using commercial assay kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions. All the assays were tested in triplicate. In the reaction, superoxide anion generated by the xanthine and xanthine oxidase reaction system was combined with SOD, forming nitrite that can react with chromogenic agent and generate a purple color. One unit of SOD activity is defined as the amount of enzyme necessary to inhibit 50% of the color formation measured at 550 nm. SOD activity was expressed as unit per milligram hepatic protein. One unit of CAT activity is defined as the amount of the enzyme that consumes 1 μmol of $H_2O_{2/}$min at 405 nm. CAT activity was expressed in international units per mg protein.

Total RNA isolation and cDNA synthesis.

Total RNA was extracted from tissues using RNAprep Pure Tissue Kit (TianGen, BeiJing, China). DNase I (TianGen, BeiJing, China) was used to eliminate genomic DNA of RNA samples to ensure the purity of RNA. Quantity and quality of the RNA were assessed by OD260/280 method and electrophoresis with 1.2% agarose gel. First strand cDNA was synthesized from 1 μg of total RNA using a QuantScript RT Kit (TianGen, BeiJing, China). The cDNA was kept at -20°C for further assays.

Tissue expression analysis by quantitative real-time PCR.

Hsp70 mRNA expression patterns were evaluated using a quantitative real-time PCR assay on an Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems, USA). qPCR amplification was carried out in a total volume of 20 μl that contained 1 μl of cDNA (100 ng), 10 μl of 2×SuperReal Premix Plus, 0.6 μl of 10 μM specific forward and reverse primers (Table 1), 2 μl of 50×ROX Reference Dye, and 5.8 μl of nuclear water. The qPCR program was set as: 1 cycle of initial denaturation at 95°C for 15 min, then 40 cycles of denaturation at 95°C for 10 s, and annealing at 60°C for 30 s, at the end of the qPCR reaction, the melting cure analysis was performed at 65−95°C (with increments of 0.5°C) for 10s. Three replicates were performed per sample and *β*-actin was used as a reference standard to normalize the expression levels between samples (Yue et al., 2011). Significant differences in expression were detected with values of $P<0.05$. The relative copy number of hsp70 mRNA was calculated according to the $2^{-\Delta\Delta CT}$ comparative CT method ($\Delta\Delta C_T = \Delta C_{Target} - \Delta C_{\beta\text{-actin}}$) (Livak and Schmittgen, 2001).

Table 1. Primers used for qPCR

Primer Name	*Sequence(5′→3′)*	*Description*
Hsp-F	CTTAGCAAGGAAGACATTGACAG	Primer for qRT- PCR
Hsp-R	AGTTCCTTCTGCTGGTGCTCAA	Primer for qRT- PCR
β-actinF	TTGTCTGGTGGTTCAACTATG	primer for qRT- PCR
β-actinR	TCCACATCTGCTGGAAGGTG	primer for qRT- PCR

Data analysis.

Quantitative data were expressed as means ± SD (N=3). Statistical analysis was performed using SPSS 20.0. Statistical differences were estimated by one-way ANOVA followed by Duncan's multiple range tests. Significance was set at $P<0.05$.

Results

Mortalities of the clams under different temperature conditions were recorded. These deaths occurred only at 40°C and began after 36 h. The survival rates of clams from GuangXi (GX), JiangSu (JS), ShanDong (SD), LiaoNing (LN) populations at 40°C are shown in Fig. 2. Survival rates of all populations declined in relation to rising temperatures. Survival rate of GX was obviously higher than other populations (P<0.01). All clams from ShanDong and LiaoNing populations died under thermal stress after 72 h.

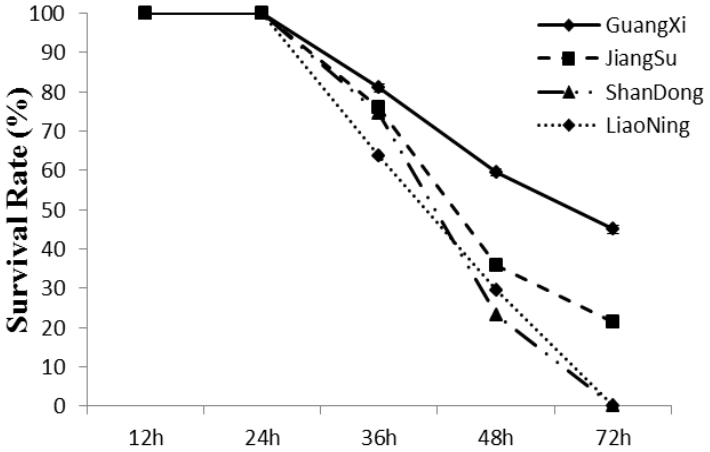

Fig. 2. The survival rates of four natural populations at 40°C.

Hepatic SOD and CAT activities revealed the antioxidant status of the clams from the different selected populations. SOD activities of these four populations were all similar to the control group (25°C) (Fig. 3a). At 35°C, SOD activity surpassed those measured at 25°C. The activities of GX and JS were especially high after 6 h and 72 h (Fig. 3b). At 37.5°C, SOD activities of these populations rose after 1 to 6 h, then declined from 12h to 24 h and gradually increased again after 36 h. SOD activities of GX were highest at 37.5°C and the peaks were detected at 6 h (Fig. 3c). At 40°C, the maximum values of SOD activities occurred at 1h, then the activities decreasing with the time (Fig. 3d).

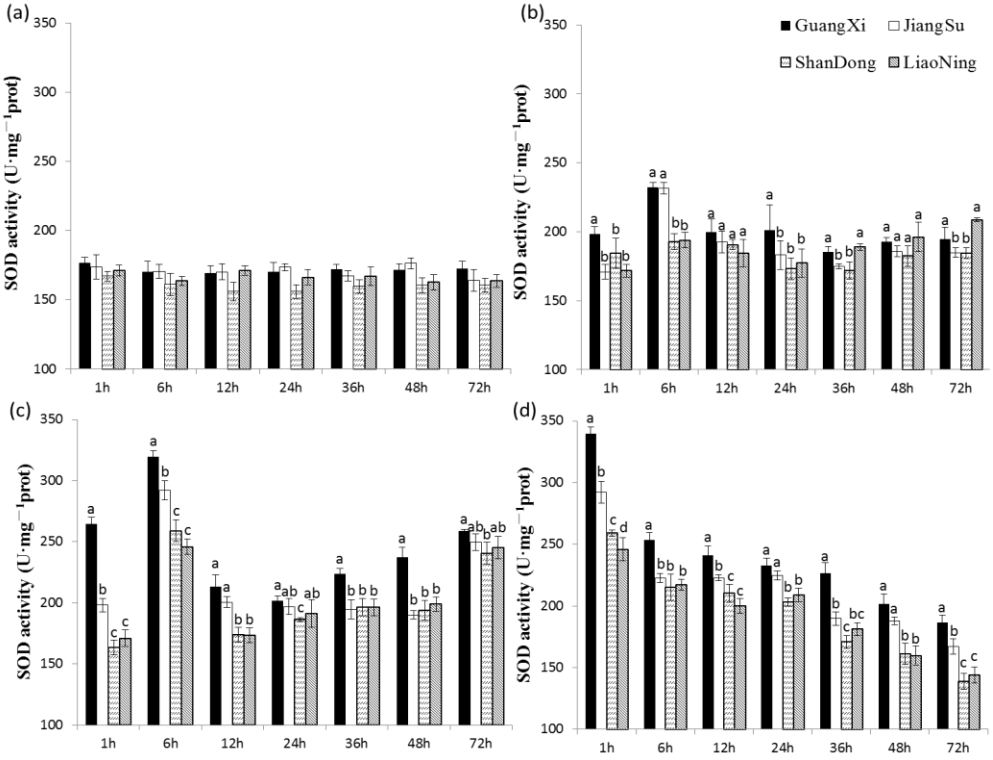

Fig. 3. SOD activities in the hepatopancreas of GuangXi, JiangSu, Shandong, LiaoNing natural populations at (a) 25°C, (b) 35°C, (c) 37.5°C, and (d) 40°C. Data are shown as mean±SD (n=3). Different letters indicate significant differences among different populations at the same time (P<0.05). Statistical analyses were performed with one-way ANOVA analysis.

Hepatopancreatic CAT activities showed a similar trend to SOD activities. At 25°C, there were no obvious differences among the four populations (Fig. 4a); hepatic CAT activities were up-regulated at 35°C compared to the control treatment (25°C) but there were no obvious differences among these geographical populations (P>0.05) (Fig. 4b). At 37.5°C, CAT activities of these populations rose from 1h and peaked after 12 h after which CAT activities declined slightly, then increased gradually again (Fig. 4c); Under 40°C, CAT activity peaked after 1 h, then declined (Fig. 4d).

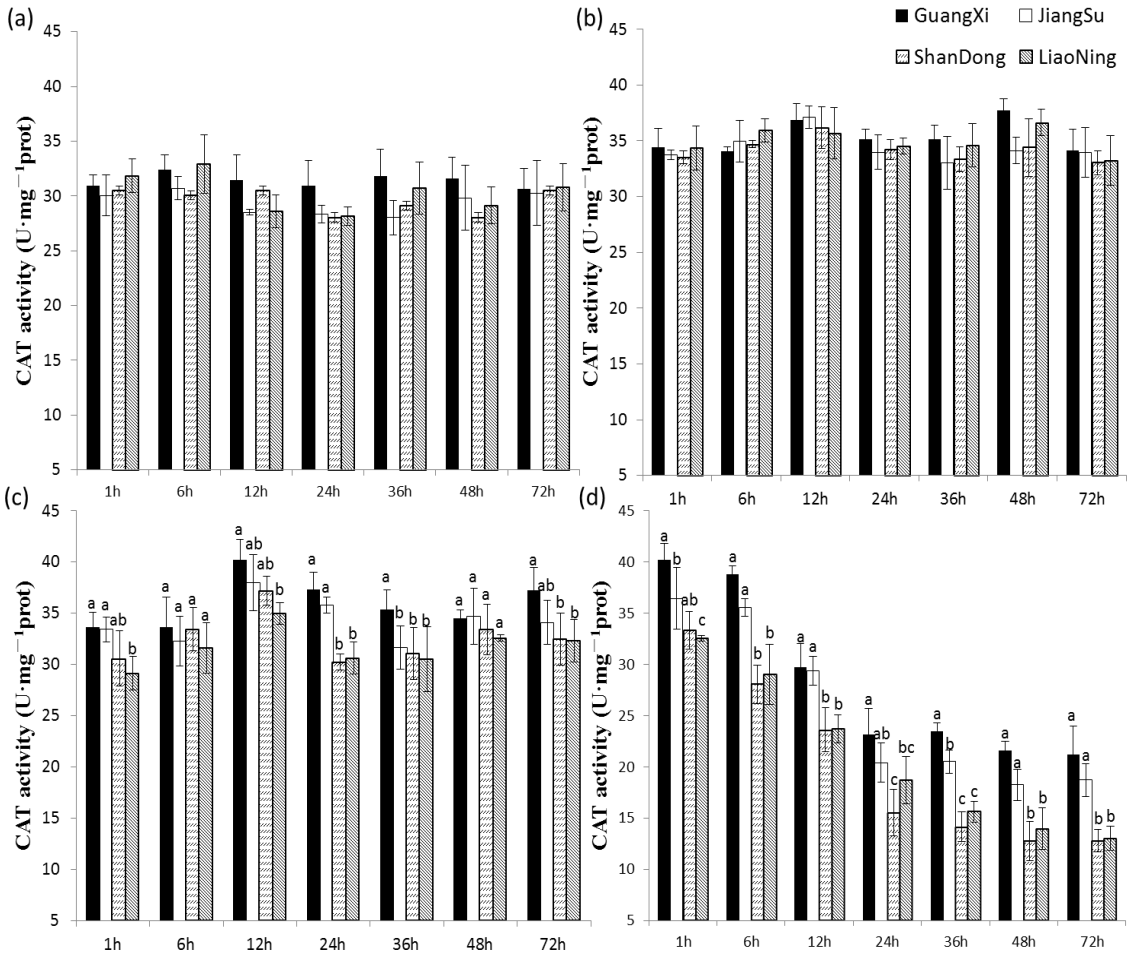

Fig. 4. CAT activities in the hepatopancreas of GuangXi, JiangSu, Shandong, LiaoNing natural populations at (a) 25°C, (b) 35°C, (c) 37.5°C, and (d) 40°C. Data are shown as mean±SD (n=3). Different letters indicate significant differences among different populations at the same time (P<0.05). Statistical analyses were performed with one-way ANOVA analysis.

To investigate the effects of high temperature on the clams, we analyzed the expression levels of Hsp gene in the hepatopancreas of four geographical populations under different temperatures at 72 h (Fig. 5). The Hsp expression levels of four populations were similar to the control group; the 35°C treatment showed an up-regulation of Hsp mRNA expression. The Hsp mRNA expressions of these populations increased at 37.5°C and the expression level of GX was significant highest (P<0.05). Under high temperature stress of 40°C, Hsp mRNA expression of GX were higher than other three populations (P<0.05) and still higher than the control of GX.

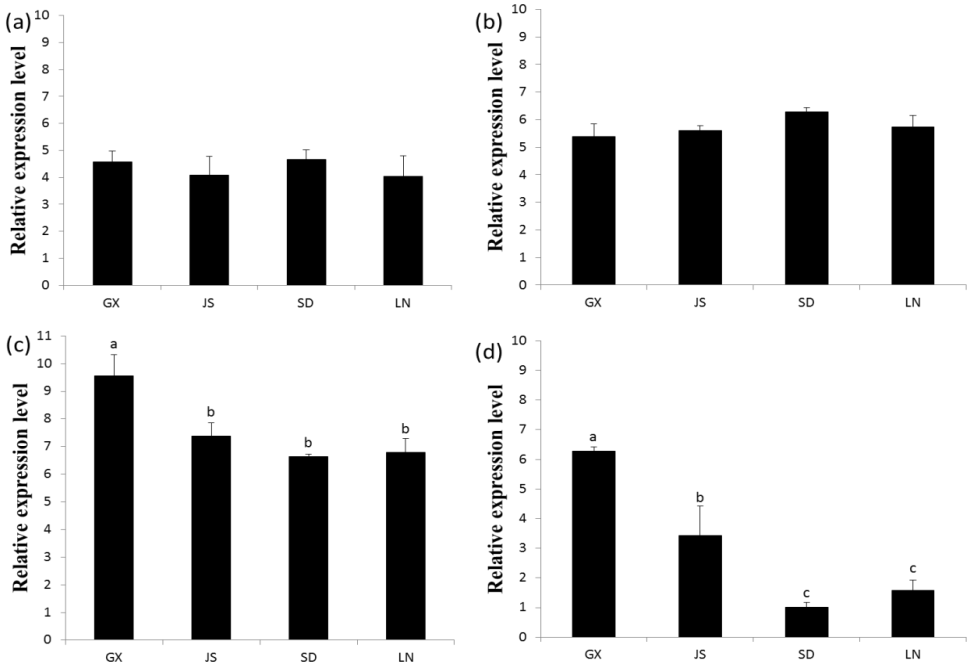

Fig. 5 The expression levels of *Hsp* gene from GuangXi (GX), JiangSu (JS), ShanDong (SD), LiaoNing (LN) natural populations at (a) 25°C, (b) 35°C, (c) 37.5°C, and (d) 40°C. Data are shown as mean±SD (n=3). Different letters indicate significant differences among the populations ($P<0.05$). Statistical analyses were performed with one-way ANOVA analysis.

Discussion

Various research studies of marine shellfish indicate that elevated temperatures have a negative influence on their health and can cause increased mortality (Hiebenthal et al., 2013; Verdelhos et al., 2015). In this study, differences in survival rates intuitively showed the tolerance to high temperature of clams from different natural populations. The high survival rate of *M. meretrix* from GX indicated that they tolerate high temperature better than others. We predicted that the geographical location and climate might be the reasons for the tolerance to high temperature. Substantial researche should be done to find the mechanism of high temperature resistance.

SOD and CAT are very important antioxidant enzymes that remove excessive free radicals and protect organisms against hydrogen peroxide (Freeman and Crapo., 1982; Radovanović et al., 2010). The results of the present study suggest that SOD of *M. meretrix* exhibiteds an increasing trend in relation to increasing temperatures (from 25°C to 37.5°C). A similar trend with CAT was observed when the temperature rose (from 25°C to 37.5°C). In pearl oysters *Pinctada fucata*, SOD and CAT activities increased with increasing stress time under high temperature stress (Meng et al., 2016). SOD activities increased when temperature rose in *Chamelea gallina* (Monari et al. 2007). The results indicated SOD and CAT play important roles in resisting elevated temperatures. Increased antioxidant abilities reflect the immune responses of shellfish to high temperatures. As SOD and CAT activities declined, the scavenging ability of free radicals in individuals also decreased and increased oxidative damage (Sun et al., 2016). In this study, decreasing SOD and CAT activities of the four populations were found at 40°C, but the GuangXi clams showed a higher antioxidant activity compared to the other three populations. The higher activities of GX suggested that the effects of high temperature on clams from GuangXi were lower than others.

Hsp gene expression is up-regulated by various physiological disturbances or stressors, such as oxygen radicals, toxins, high temperature, bacterial infection, and other stresses (Yenari et al., 1999; Srivastava, 2002). In *Paphia undulate*, high temperature caused up-regulated expressions of Hsp70 gene throughout the study period (Wu et al., 2014). When Hsp mRNA reached saturation, heat shock transcription factors lost the DNA binding activities which caused decrease of the expression levels of *Hsp* gene (Tachibana et al., 2002).

In our study, the *Mm-hsp* expression levels of clams from different populations after high temperature exposure were detected by qPCR. We found the Hsp expression levels of the four tested populations increased when the temperature rose (from 25°C to 37.5°C), then decreased at extremes of temperature (40°C). The results implied that the *Hsp* gene has an important role in responding to thermal stress. Hsp expression level of GX clams was significantly higher than that of the others under the exposure of thermal stress. Therefore, we speculate that GX clams possess better heat tolerance. Our findings preliminarily expound the responses of the saltwater clams from different geographical populations to high temperatures. GX clams in particular, will be used as key material for developing new breeding cultivars with high temperature tolerance. Further systematic and detailed studies are needed for both theory and practice.

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