



# Physiological and Transcriptional Responses to Acute and Chronic Thermal Stress in the Ark Shell *Scapharca subcrenata*

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Ark shells (*Scapharca subcrenata*) grown on the tidal flats are often exposed to high temperature stresses in summer. In order to better understand their adaption to extreme or natural high temperature, we first determined the 96-h upper lethal temperature of ark shell and then investigated their physiological and transcriptional responses to acute or chronic thermal stress at the 96-h upper median lethal temperature (32°C). A significantly higher cumulative mortality (52% in 96 h) was observed in the acute heating treatment (AHT) group than that (22% in 7 days) in the chronic heating treatment (CHT) group. The apoptosis and necrosis rates of hemocytes were increased significantly in a time-dependent manner under both thermal stress strategies. Activities of antioxidant enzymes [superoxide dismutase (SOD) and catalase (CAT)] increased dramatically in a short time followed by a quick decline and reached to a lower level within 12 h in the AHT group, but maintain relatively high levels over a long period in the CHT group. The contents of malondialdehyde (MDA) were increased significantly firstly and restored to the original later in both acute and chronic thermal stress. Moreover, expression of the genes related to heat shock proteins (HSPs; *HSP90*, *HSP70*, *HSP20*, and *sHSP*), apoptosis [TNF receptor-associated factor 6 (*TRAF6*), glucose regulated protein 78 kD (*GRP78*), and caspase-3 (*Casp-3*)] and antioxidant responses [glutathione S-transferase (*GST*) and multidrug resistance protein (*MRP*)] could be induced and up-regulated significantly by thermal stress, however, expression of *regucalcin* (*RGN*), metallothionein (*MT*), and *peroxiredoxin* (*PRX*) was down-regulated dramatically under the two heating treatments. These results suggested that anti-apoptotic system, antioxidant defense system and HSPs could play important roles in thermal tolerance of ark shells, and the heat-resistant ark shell strains could be selected continuously by properly chronic thermal stress.

**Keywords:** *Scapharca subcrenata*, thermal stress, apoptosis, antioxidant enzymes, gene expression response

## INTRODUCTION

In recent decades, the seawater temperature keeps rising with the acceleration of global warming (Hoegh-Guldberg et al., 2007), and in the worst cases, seawater temperature could increase by 4.5°C by the end of this century (IPCC, 2013). As a consequent, many intertidal marine mollusks are living at temperatures close to or just below their upper thermal limits (Stillman, 2003; Tomanek and Zuzow, 2010; Clark et al., 2021; Ning et al., 2021). Unlike terrestrial animals, marine organisms inhabiting nearshore are usually unable to shelter from high temperature environment (Burrows et al., 2019). Therefore, investigation of their biological responses to thermal stresses may provide insights into underlying the adaptive mechanisms of these intertidal mollusk to elevated temperatures.

Habitat temperature is a prominent stressor that can affect the survival, growth, development, reproduction, metabolism, immunity and geographical distribution of marine organisms (Chen et al., 2007; Anestis et al., 2010; Jiang et al., 2016; Yang et al., 2017; Monaco and McQuaid, 2019). For instance, thermal stress can induce overproduction of reactive oxygen species (ROS), which damages biological macromolecules (proteins, lipids, and DNA) and initiates a cascade of cellular defense events (Meng et al., 2014; Luo et al., 2014). Moreover, the physiological performance and gene expression of marine organisms can be disorganized by thermal stress (Clark et al., 2018, 2021; Li et al., 2020; Shi et al., 2020). It has been reported that enzymatic defense system, antioxidant system and apoptosis pathway of marine mollusk can be activated to counteract the adverse effect of oxidative stress induced by temperature changes (Zhang et al., 2012; Zhou et al., 2019; Ning et al., 2021). However, intertidal organisms have poor capacities in acclimation to rapid climate warming (Stillman, 2003). For instance, the growth and gonad development of *Crassostrea gigas* can be inhibited by thermal discharge from a nuclear power plant (Dong et al., 2018). It is thus necessary to investigate the thermo-resistant mechanisms of intertidal organisms in response to elevating temperatures.

The ark shells, *Scapharca subcrenata*, are economically important species and widely distributed in the coastal waters of China, Japan, and Korea (Chen et al., 2009). In the past decades, the wild resources of ark shells in China have suffered serious damage owing to overfishing and ecological changes, resulting in the decline of genetic diversity and stress resistance. Moreover, cultured ark shells, especially those cultured in outdoor ponds in China, are vulnerable to high temperature and their growth and survival are often threatened severely by continuous or extreme high temperature in summers (Lin J. L. et al., 2020; Ning et al., 2021). The seawater temperature of outdoor shallow ponds in Northern China can reach as high as 33.5°C in summer (Ning et al., 2015), thus pond cultured ark shells are often exposed to acute or chronic high temperature stress during this period. Therefore, in this study, we investigated the effects of acute or chronic thermal stress on apoptosis, physiological performance, and gene expression of ark shells, which would help to understand the thermal tolerance and contribute to selection of heat-resistant strains in ark shells.

## MATERIALS AND METHODS

### Experimental Animals and Determination of the Upper Median Lethal Temperature

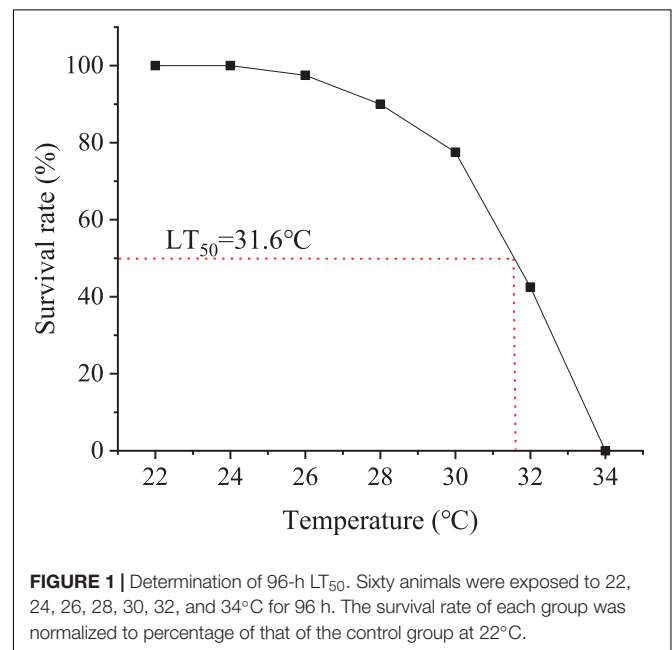
Healthy ark shells ( $23.81 \pm 2.05$  mm in shell-length,  $19.42 \pm 1.63$  mm in shell-height,  $15.68 \pm 1.05$  mm in shell-width, and  $5.10 \pm 1.26$  g in whole weight;  $n = 30$ ) were collected from the intertidal flats of Dongying, China ( $38^{\circ}08' N$ ,  $118^{\circ}17' E$ ). The ark shells were reared in 100-l tanks containing filtered seawater (temperature  $18 \pm 0.5^{\circ}C$ , salinity  $28 \pm 1$  and pH  $8.13 \pm 0.02$ ), fed with a concentrated microalgae diet and the seawater was changed twice a day in the lab. After acclimation for 1 week, 420 healthy ark shells were randomly divided into six groups, which were heated to 22, 24, 26, 28, 30, 32, and  $34^{\circ}C$ , respectively, at a rate of  $1^{\circ}C/h$  and maintained at that temperature for 96 h. The animals were checked once every 6 h and those failed to react to mechanical touch and cannot recover within 1 h in the optimum water temperature were considered as dead and picked out. The water in the tanks were replaced twice every day with clean seawater preheated to the designated temperatures. The 96-h upper  $LT_{50}$  was determined according to the two-point method as described in Liu et al. (2007) (Figure 1).

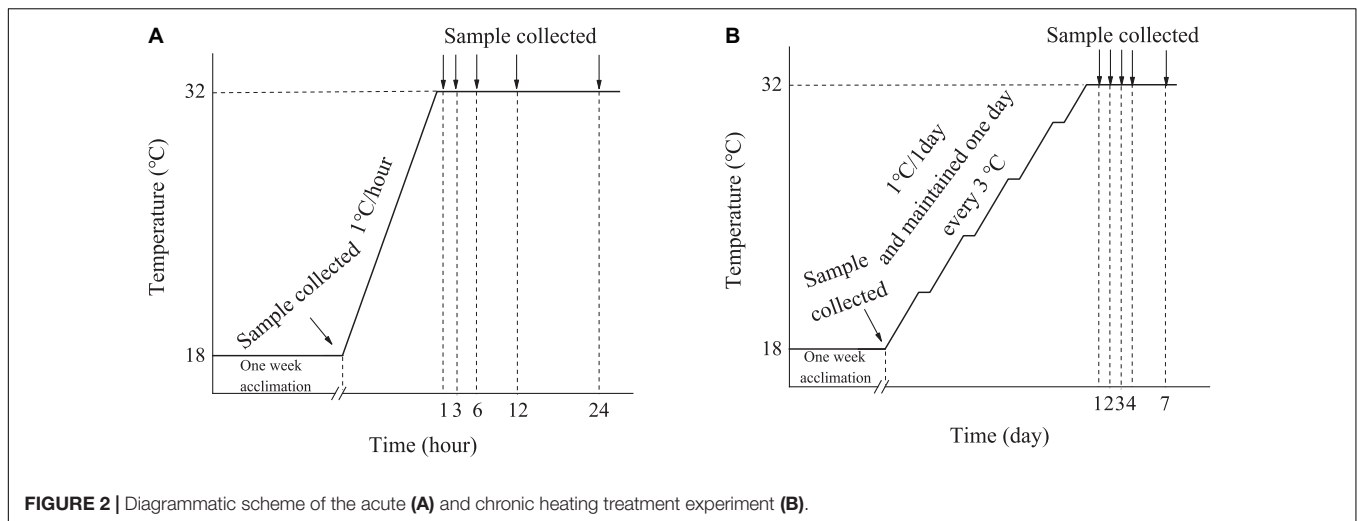
### Setup of Acute Heating Treatment and Chronic Heating Treatment

Prior to the experiment, 480 healthy ark shells were randomly selected and divided equally into two experimental groups, one for acute heating treatment (AHT) and another for chronic heating treatment (CHT).

#### Acute Heating Treatment

The seawater in the tank holding the ark shells was heated rapidly from 18 to  $32^{\circ}C$  at a rate of  $1^{\circ}C/h$ . Six animals were randomly





sampled at 1, 3, 6, 12, and 24 h after the water reached 32°C (Figure 2A). Six animals maintained at 18°C were sampled at the beginning of the experiment and used as a control. The animals were not fed during the experiment.

### Chronic Heating Treatment

The seawater in the tank holding the ark shells was heated slowly from 18 to 32°C at a rate of 1°C/day, and maintained one day every 3°C. Six animals were randomly sampled at Day 1, 2, 3, 4, and 7 after the water reached 32°C (Figure 2B). Six animals maintained at 18°C were sampled at the beginning of the experiment and used as a control. During the experiments, the animals were fed with concentrated microalgae diet twice a day, and the seawater was changed 2 h after each feeding.

The sampled ark shells were dissected immediately to collect their hemocytes. In brief, 0.5 ml of hemolymph was harvested with a sterile syringe pre-filled with an equal volume of precooled anticoagulant buffer (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA-Na<sub>2</sub>, pH 7.0), and filtered with a 300 mesh sieve. The hemocytes were saved in a mixture of ice water at 0°C and collected after centrifugation at 1,000 × g at 4°C for 5 min and eventually stored at (80°C until further analyses. Meanwhile, the gill tissues from the same animal were collected and cleaned thoroughly with phosphate buffered saline (PBS, PH 7.4) and stored at -80°C until further analyses. Hemocytes were selected as they are the main components for cellular immune response and non-specific defense mechanism, as well as play a key role in the heat stress response of mollusks (Anestis et al., 2010). Gills are often chosen in analysis of thermal stress response mechanism in aquatic animals as gills, with large surface area, are directly exposed to a variety of environmental factors (Lim et al., 2016).

### Determination of Apoptosis and Necrosis Rate of Hemocytes

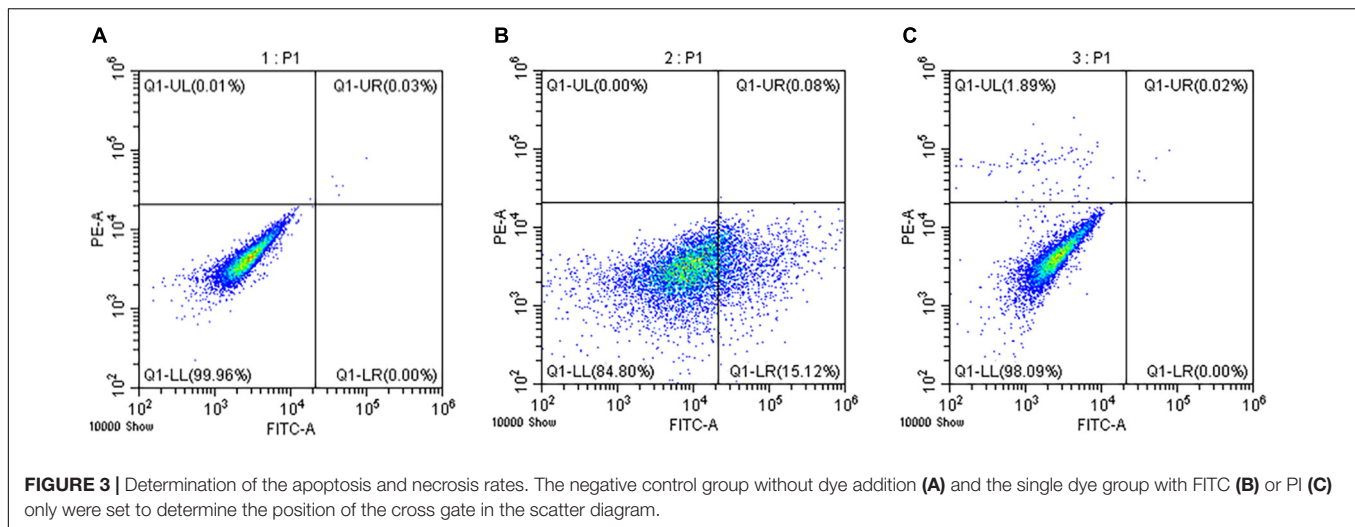
The hemocytes were resuspended in 195 μl 1 × binding buffer solution at a final concentration of 2.5–5.0 × 10<sup>5</sup> cells/ml,

and stained with 5 μl Annexin V-Fluorescein Isothiocyanate (FITC) and 10 μl Propidium Iodide (PI) dyes at 20°C in dark for 20 min. The cell suspensions were analyzed by the flow cytometry (CytoFLEX, United States). The negative control group without dye addition and the single dye control group with only FITC or PI added were set to determine the position of the cross gate in the scatter diagram (Figure 3). The apoptosis rate of ark shell hemocytes in each sample was represented as the percentage of the number of FITC positive and PI negative cells in the total number of cells. The necrosis rate of the hemocytes in each sample was expressed as the percentage of the number of FITC positive and PI positive cells in the total number of cells (Schutte et al., 1998).

### Enzyme Activity Assays

Prior to analyses, the frozen gill samples were thawed on ice. About 100 mg gill tissues were homogenized in nine volume of ice-cold PBS buffer (0.01M, pH 7.4) using a homogenizer (T10 Ultra Turrax basic, Germany). After homogenization, the extract was centrifuged at 10,000 × g for 30 min at 4°C, and stored at 4°C until analyzed.

The activities of superoxide dismutase (SOD) and catalase (CAT) and the content of malondialdehyde (MDA) were selected to evaluate the physiological responses to thermal stress. The activities of SOD and CAT were measured using a commercial kit (Nanjing Jiancheng Chemical Industries, China). The activity was expressed as units per mg protein (U mg prot<sup>-1</sup>). The content of MDA was measured spectrophotometrically with the thiobarbituric acid method according to Esterbauer and Cheeseman (1990) with a kit from the Nanjing Jiancheng Chemical Industries (China). Briefly, gill extract was added to an equal volume of 1% thiobarbituric acid in a 95°C water bath for 40 min. After cooling down on ice, thiobarbituric acid reactive substance was centrifuged at 2,000 × g for 10 min and the supernatant was measured at 532 nm against a blank control consisting of anhydrous ethanol mixed with 1% thiobarbituric acid. Tetraethoxypropane



was used as a standard. MDA content was expressed as  $\text{nmol mg prot}^{-1}$ .

## RNA Extraction and cDNA Synthesis

Total RNA was extracted from the frozen gill samples using TRIzol reagent (Invitrogen, United States), and genomic DNA was removed using RNase-free DNase I (TaKaRa, China). The quantity and purity of the RNA samples were determined by the Nanodrop 2000 device (Thermo, United States), and RNA quality was analyzed using 1% agarose gel electrophoresis. The first-strand cDNA was synthesized for the quantitative real-time PCR (qRT-PCR) analyses using a PrimeScript™ first Strand cDNA Synthesis Kit (TaKaRa, China) with an oligo dT primer.

## Gene Expression Analyses

The expression levels of twelve selected genes involved in heat stress responses (Ning et al., 2021) were examined using qRT-PCR. The primers used for the qRT-PCR analyses were listed in Table 1. 18S rRNA was employed as the reference gene. Reactions were carried out on the QuantStudio™ 5 Real-Time PCR Instrument (Applied Biosystems) using SYBR green II as fluorescent dye. PCR amplifications were conducted in 20- $\mu\text{l}$  reaction mixtures containing 10  $\mu\text{l}$  of 2 SYBR Premix Ex Taq (TaKaRa), 1.2  $\mu\text{l}$  50  $\times$  ROX Reference Dye, 2  $\mu\text{l}$  of the diluted cDNA, 0.4  $\mu\text{l}$  of each primer (10  $\mu\text{M}$ ), and 6  $\mu\text{l}$  of sterile distilled H<sub>2</sub>O. The PCR programs were 95°C for 60 s, followed by 40 cycles of 95°C for 5 s and 60°C for 15 s. Melting curve analysis was performed following each PCR reaction to confirm the single amplification product. All reactions were performed in triplicates. The obtained results were analyzed by the  $2^{-\Delta\Delta\text{Ct}}$  methods (Livak and Schmittgen, 2001).

## Statistical Analyses

Statistical analyses were processed using SPSS 17.0 software. Significant differences were determined via one-way analysis of variance and Duncan test. Differences were considered statistically significant at  $P < 0.05$  (\*).

**TABLE 1** | Primers used in this study.

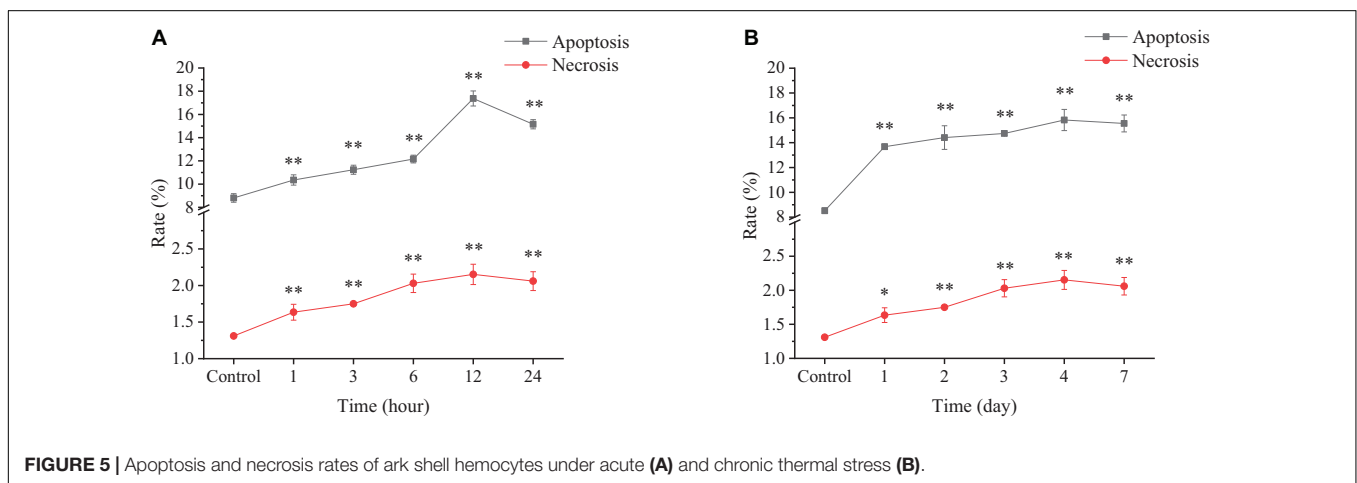
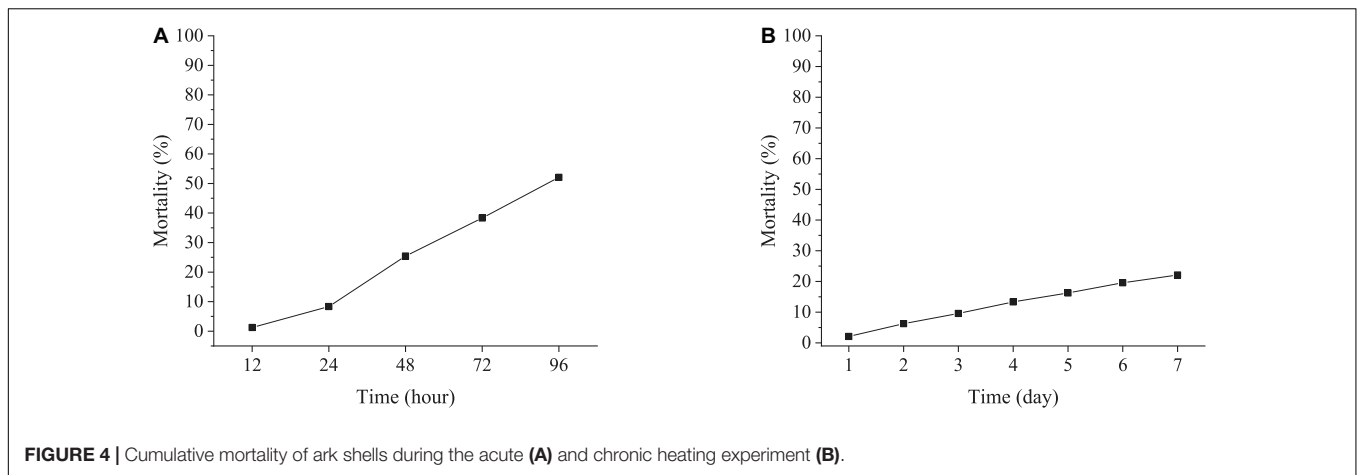
Primer name	Primer sequence (5'-3')	Ta/°C	Product length (bp)
sHSP-F	ACCAGTGTTTTGGTGGGGAC	60	137
sHSP-R	CAGCTCTAGCAATGGGGTCA		
HSP20-F	GGCGGGGCATGTATGTAAGA	60	133
HSP20-R	ACGTGGATCTCCTCTGGTGT		
HSP70-F	AGTGTGAGACAAGCGACAACA	60	121
HSP70-R	CCACACCAGCCAGTGAGTT		
HSP90-F	TGGAATCGTCATCCTCGTCTC	60	135
HSP90-R	TACATGCCAGCAGAATCCACA		
MRP-F	TCATCCGGGCATTTCGTCT	60	118
MRP-R	AGCCTTAGTCCCATCCACCT		
Prx-F	AAGTTCCCGTCTGTGGAGGT	60	116
Prx-R	CACCCAGGTGTAATGCTCC		
MT-F	GCTGTACCTTGAACAGCTTGAG	60	119
MT-R	TGCACTTAGCAGGATCTCACC		
RGN-F	GGATCTTGGTGGACCCGATG	60	95
RGN-R	TTGGGTCAAACGGACGACT		
GST-F	TGAACAGAAAGGTGCGGAGT	60	172
GST-R	GCCAGTCTGGAATGCTTCGT		
Casp-F	CTATGCCACAGTTCGGGTT	60	159
Casp-R	TTAGCATTGGCTCTCCCCAC		
TRAF-F	AGCCAGCCATTTACACGGA	60	195
TRAF-R	ACGTCCTCCAGTCTCATGGT		
GRP78-F	GCGGATCCACCAGAATTCCA	60	142
GRP78-R	TCCTGTTCGCCACCCAAATC		
18S-F	CTTTCAAATGTCTGCCCTATCAACT	59	195
18S-R	TCCCATTATGTTATTTTCGTCACT		

Ta indicates annealing temperature.

## RESULTS

### The Cumulative Mortality of Ark Shells Under Acute and Chronic Thermal Stress

In the AHT, the cumulative mortality of ark shells showed a linear increase with time, and reached 52% at 96 h (Figure 4A).



Moreover, no feeding and thus no feces were observed during the AHT experiment. The seawater in the tank often turned turbid as the animals died.

In the CHT group, the cumulative mortality was much lower than that in the AHT, with a cumulative mortality rate of only 22% by 7 days (Figure 4B). The ark shells fed normally and lots of feces were observed during the experiment.

## The Apoptosis and Necrosis of Hemocytes Under Acute and Chronic Thermal Stress

The apoptosis and necrosis of ark shell hemocytes were assessed by Annexin V-FITC and PI staining followed by analyses with flow cytometry. In the AHT, the apoptosis rate of hemocytes increased significantly at a short time and showed a linear increase with the prolonging of thermal stress, with the highest value (17.38%) at 12 h. Similar trend was also observed for the necrosis rate of hemocytes during the AHT (Figure 5A).

In the CHT group, the apoptosis rate increased significantly at day 1 and sustained steadily high over the whole period of the experiment. The necrosis rate showed a linear increase

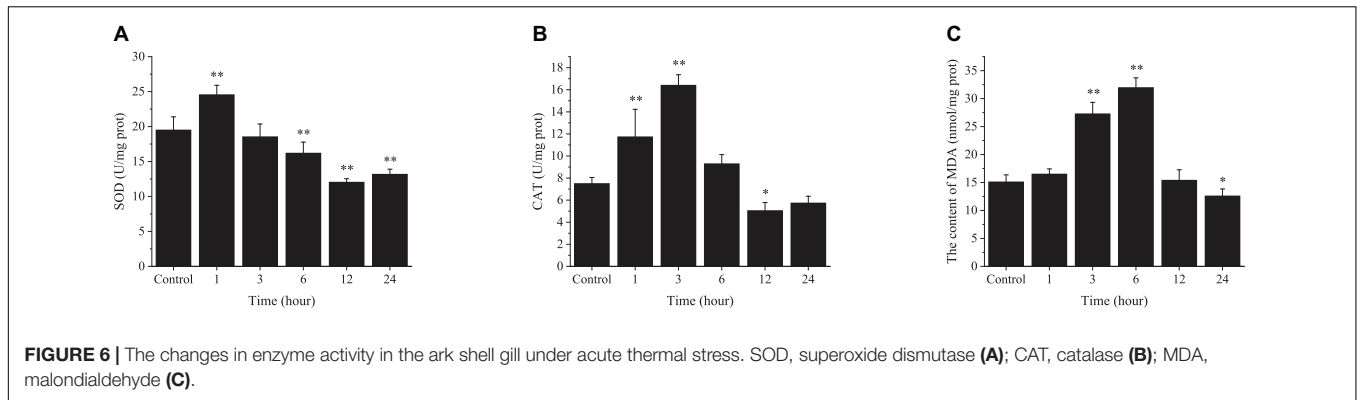
within first 4 days and decreased a bit at the end of the experiment (Figure 5B).

## Changes in Enzyme Activity in the Ark Shell Gill Under Acute Thermal Stress

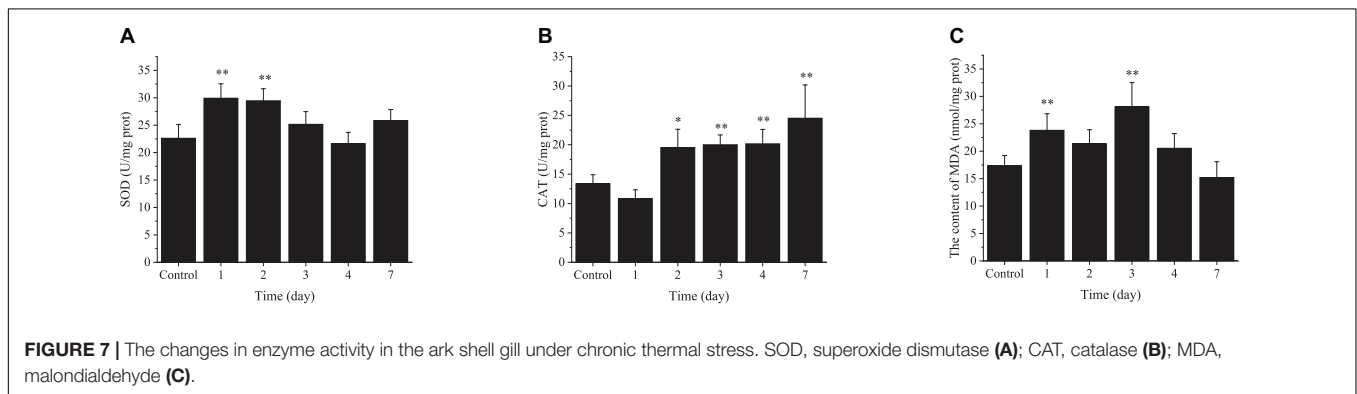
In the AHT, SOD activity increased significantly at 1 h (24.53 U mg prot<sup>-1</sup>) followed by a continuous decline over the experimental period. Moreover, the SOD activity became significantly lower than the control group after 6 h (Figure 6A). The CAT activity increased significantly after exposure to high temperature stress, reaching a peak value of 16.40 U mg prot<sup>-1</sup> at 3 h, followed by a sharp decrease and became significantly lower than the control group at 12 h (Figure 6B). The content of MDA also increased significantly with time and reached the highest value of 31.94 U mg prot<sup>-1</sup> at 6 h, followed by a continuous decline (Figure 6C).

## The Changes in Enzyme Activity in the Ark Shell Gill Under Chronic Thermal Stress

In the CHT, SOD activity was increased significantly at day 1 and day 2, and restored to the initial level after day 3 (Figure 7A). CAT



**FIGURE 6 |** The changes in enzyme activity in the ark shell gill under acute thermal stress. SOD, superoxide dismutase (A); CAT, catalase (B); MDA, malondialdehyde (C).



**FIGURE 7 |** The changes in enzyme activity in the ark shell gill under chronic thermal stress. SOD, superoxide dismutase (A); CAT, catalase (B); MDA, malondialdehyde (C).

activity was increased significantly at day 2 and remained elevated until the end of the experiment (Figure 7B). The content of MDA was increased significantly at day 1 and day 3, and returned to the initial level at day 4 (Figure 7C).

dramatically at day 1 and day 2, but decreased sharply after day 3 and remained down-regulated until the end of the experiment.

## Transcriptional Response in the Ark Shell Gills to Acute Thermal Stress

As shown in Figure 8, the expression of selected genes related to stress proteins (*HSP90*, *HSP70*, *HSP20*, and *sHSP*), apoptosis [TNF receptor-associated factor (*TRAF*), glucose regulated protein 78 kD (*GRP78*), and caspase-3 (*Casp-3*)] and antioxidative [glutathione S-transferase (*GST*) and multidrug resistance protein (*MRP*)] were up-regulated significantly under acute thermal stress, with peak values observed at 3 or 6 h. However, compared with the control groups, the expression of *peroxiredoxin* (*PRX*), *regucalcin* (*RGN*), and metallothionein (*MT*) was down-regulated significantly in the AHT.

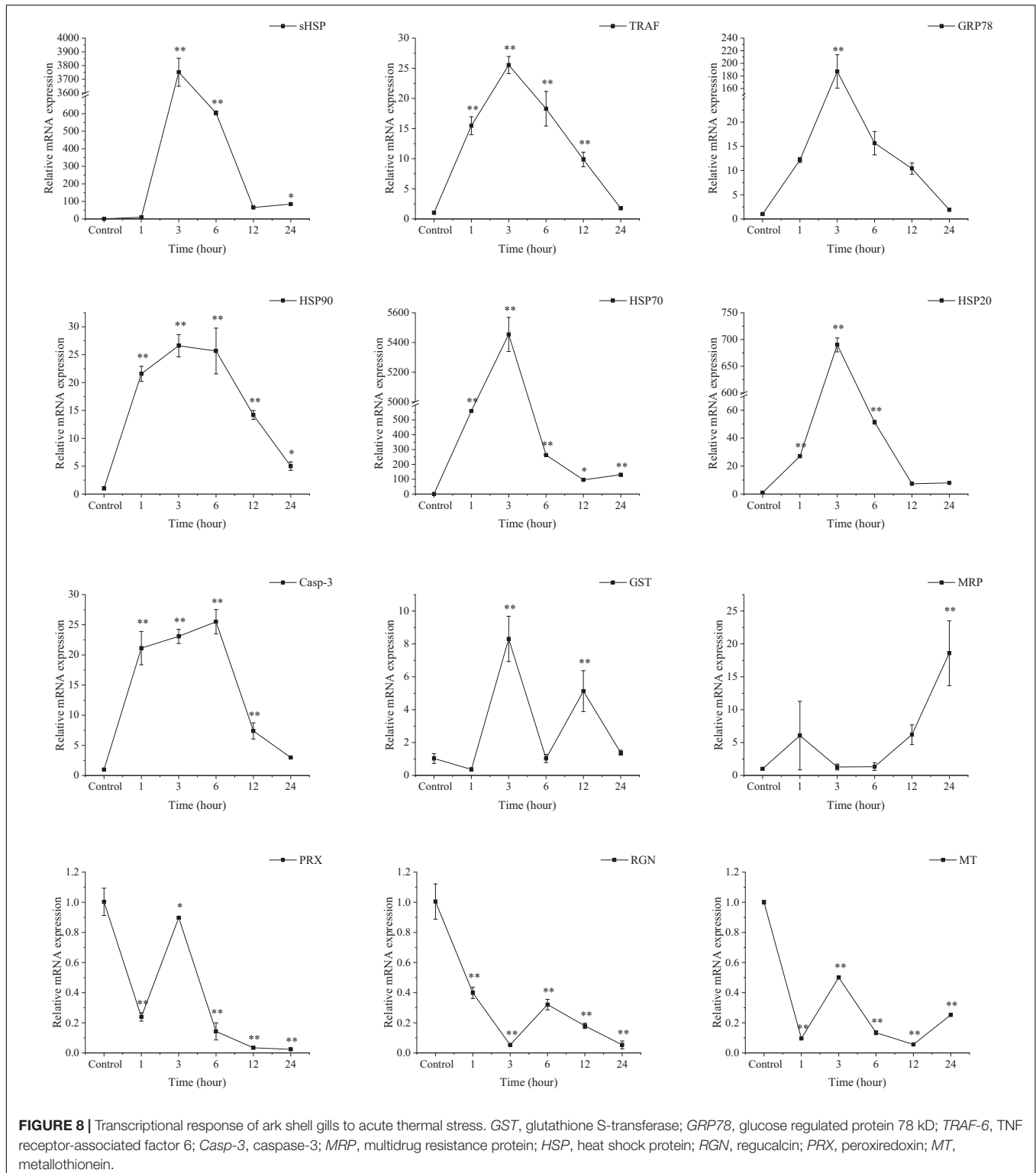
## Transcriptional Response of Ark Shell Gills to Chronic Thermal Stress

As depicted in Figure 9, the expression of the selected genes involved in stress proteins (*HSP90*, *HSP70*, *HSP20*, and *sHSP*), apoptosis (*TRAF*, *GRP78*, and *Casp-3*) and antioxidative (*GST* and *MRP*) were up-regulated significantly during the CHT experiment, with peak values mainly at day 2. However, the expression of *PRX* and *RGN* was down-regulated significantly in the CHT. Noticeably, the expression of *MT* was increased

## DISCUSSION

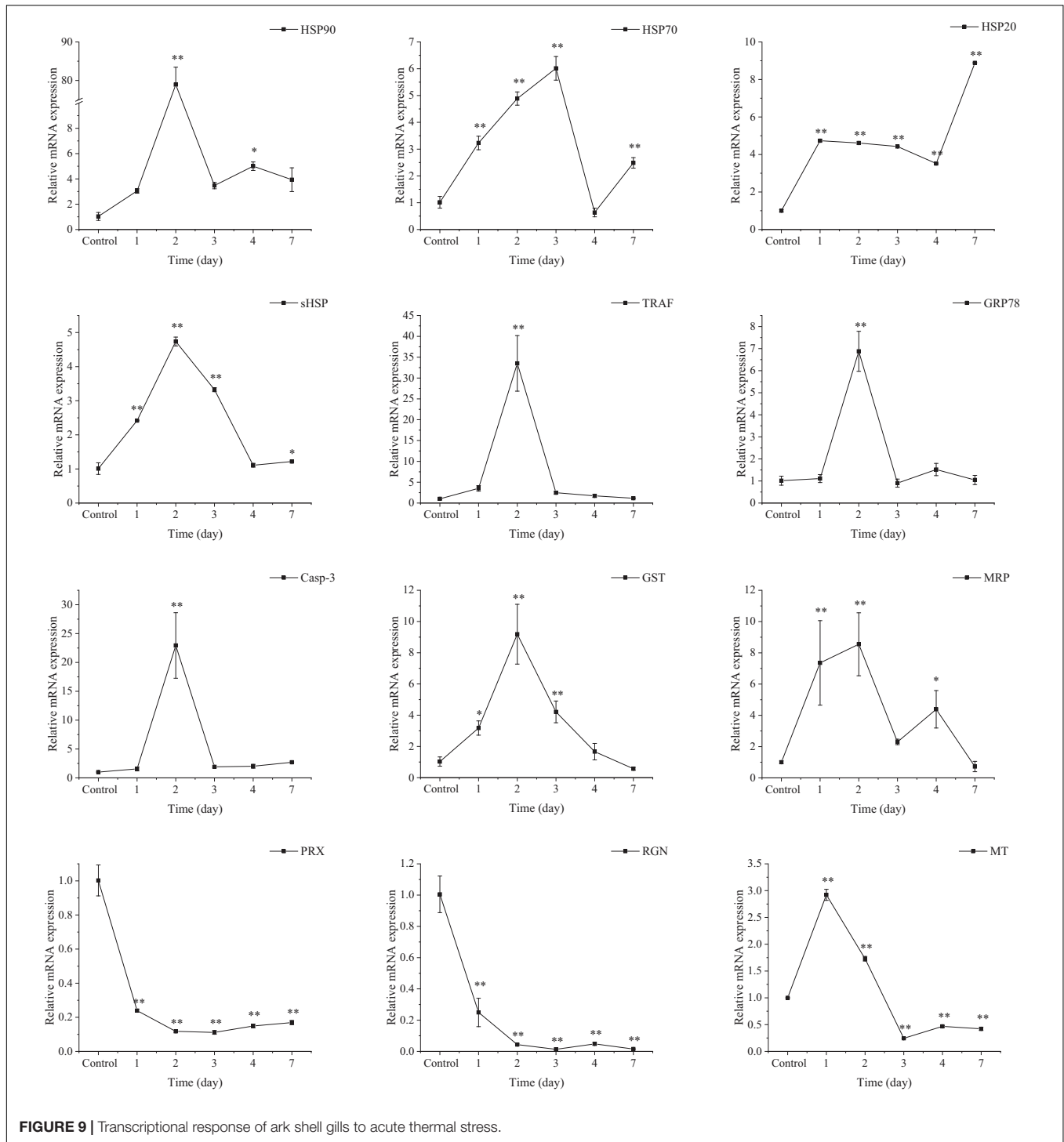
Temperature, as an important abiotic factor, can substantially affect the physiological responses in marine organisms (Windisch et al., 2011; Dong et al., 2019). In this study, the 96-h upper  $LT_{50}$  of ark shells was determined to be 31.6°C, based upon acute temperature tolerance experiments. However, the highest temperature in outdoor ponds or tidal flats in summer is far beyond this temperature, indicating that the cultured ark shells might be subjected to severe thermal stress. Moreover, the high temperature stress could last for up to over 1 week, suggesting that the ark shells could suffer from a long-term thermal stress. Therefore, it is important to examine the responses of the ark shells to both acute and chronic thermal stress.

Apoptosis, also called programmed cell death, is a vital physiological protective mechanism of organisms in adaptive responses to environmental stress, which can be induced significantly by high temperature stress (Zhang et al., 2012; Cheng et al., 2018; Zhou et al., 2019). However, severe stress may also cause necrosis when the stress is beyond the tolerance of the animal. In consistent with previously published results in oyster (Yang et al., 2017), we also observed that the apoptosis and necrosis rates of hemocytes were increased significantly in a time-dependent manner under both acute and chronic thermal stress, suggesting the apoptosis process of hemocytes has been



activated and the stress injury gradually became irreparable with the prolonging of stress time and eventually caused necrosis in the ark shells. This is supported by the significant upregulation of *Casp-3*, a member of cysteine-dependent protease families

that plays important roles in the activation of apoptotic pathway (Qin et al., 2020) in the two thermal stress treatments. It is notable that higher apoptosis level was observed under the acute thermal stress than under the chronic stress, suggesting the ark



shells suffered more damage when exposed to acute temperature changes. The higher cumulative mortality rates of ark shells in the AHT also support this point.

In order to counteract the irreparable injuries caused by apoptosis, anti-apoptotic pathways could be activated immediately when organisms suffer from abiotic stresses. The expansion of inhibitors of apoptosis proteins in the oyster

genome and their response to various stresses are crucial for oysters in adaption to sessile life in the highly stressful intertidal zone (Zhang et al., 2012). Glucose regulated protein 78 kD (GRP78) is an essential molecular chaperone located in the endoplasmic reticulum (Melnick et al., 1994; Ni et al., 2011). It has been reported that *GRP78* could reduce apoptosis induced injuries by suppression of Caspase 7 activation



(Reddy et al., 2003). Tumor necrosis factor receptor-associated factors (TRAFs) TRAFs plays crucial roles in stress resistance. Among which, *TRAF6* is an important signaling molecule involving in cell proliferation and metabolism through a series of signal transduction (Rothe et al., 1995). As reported in recent study, *TRAF6* could suppress the apoptosis of oyster hemocytes through activation of *Pellino* (Lin Y. et al., 2020). In the present study, the expression of anti-apoptosis related genes (*GRP78* and *TRAF6*) was up-regulated significantly, indicating anti-apoptotic system also exists in ark shells in response to thermal stresses. This has been confirmed by our previous study according to the transcriptomic analyses (Ning et al., 2021).

Marine invertebrates are equipped with antioxidant defense system such as SOD and CAT to maintain the balance between normal physiological function and ROS accumulation (Lesser, 2006; Soldatov et al., 2007). SOD can catalyze the conversion of intracellular superoxide radicals into hydrogen peroxide ( $H_2O_2$ ) and molecule oxygen ( $O_2$ ), whereas CAT can convert the  $H_2O_2$  into  $O_2$  and  $H_2O$ . Therefore, the elevated activities of antioxidant enzymes are essential for animal to counteract oxidative damage induced by thermal stress (Cheng et al., 2018). In this study, the activities of SOD and CAT can be induced and increased significantly in response to both acute and chronic thermal stress, which was consistent with previous studies in Yesso scallop (Jiang et al., 2016) and Pacific oyster (Ding et al., 2020). However, the activities of SOD and CAT were decreased and lower than the initial level after 12 h under the acute thermal stress, suggesting the antioxidant ability could be repressed with the extension of acute thermal stress. This is consistent with the conclusion observed in giant clam, *Tridacna crocea* (Zhou et al., 2019), indicating a transition occurs from aerobic to anaerobic metabolism. Just as shown in our previous study, ark shells may suffer irreparable injuries due to oxygen limitation and metabolic dysregulation if the stress sustains (Ning et al., 2021).

Malondialdehyde content has been widely used as a marker for the extent of oxidative damage in marine organisms (Talmage and Gobler, 2011; Ferreira et al., 2019). In this study, the content of MDA was increased significantly firstly under the two thermal stress modes, suggesting the elevated seawater temperature induced oxidative damage in ark shells. However, the content of MDA was decreased and restored to the initial level subsequently, probably because the increased activity of antioxidant enzymes in a short time removed harmful substances induced by thermal stress (Wang et al., 2020). The enhanced activities of SOD and CAT were also observed under the two thermal stress modes in this study. The restored expression levels of most stress-related genes (*GST*, *GRP78*, *TRAF-6*, *Casp-3*, *MRP*, *sHSP*, *HSP70*, and *HSP90*) at day 4 also support the above viewpoint. Moreover, the higher MDA content under the acute thermal stress than under the chronic stress is consistent with the results found in Yesso scallop (Jiang et al., 2016), suggests that ark shells suffered more damage during the acute temperature changes.

Except for antioxidant enzymes, non-enzymatic antioxidant defense mechanisms also existed in marine invertebrates to prevent oxidative damage (Jiang et al., 2016). *Peroxiredoxin* is

widely existed in prokaryotes and eukaryotes, which plays a vital role in the elimination of metabolically produced peroxides (Rothe et al., 1995; Fujii and Ikeda, 2002; Papadia et al., 2008). *MT* is responsible for removing metal ion toxicity through combination with them, which is involved in scavenging free radicals and the process of stress resistance (Masters et al., 1994; Carginale et al., 2000). *RGN* has the function of maintaining the dynamic balance of  $Ca^{2+}$  in cells and overexpression of *RGN* could inhibit cell death and apoptosis caused by a variety of signaling factors (Laurentino et al., 2011). In marine mollusks, the anti-oxidative responses to thermal stress are complicated and often affected by the differences in temperature gradient and exposure duration (Ning et al., 2021). Similar results were also observed in this study, with expression of *GST* and *MRP* up-regulated significantly and *PRX*, *MT*, and *RGN* down-regulated in both thermal stress modes. In consistency with previous results observed in oyster (Lim et al., 2016) and ark shells (Ning et al., 2021), our results suggest that marine mollusks may consume more energy for their redox responses.

Heat shock proteins (HSPs) are a group of highly conserved proteins which play important roles in cellular homeostasis after exposure to drastic environmental changes (Kayhan and Duman, 2010). HSP70s and HSP90s are well-known stress proteins in response to thermal stress in many marine mollusks (Farcy et al., 2007; Ivanina et al., 2009; Kim et al., 2009; Lim et al., 2016). HSP20s (stress induced protein 1) could promote the regeneration of denatured proteins and prevent stress induced protein aggregation when suffered elevated temperature stress (Nover and Scharf, 1997; Ning et al., 2021). In this study, the expression of HSPs (*sHSP*, *HSP20*, *HSP70* and *HSP90*) were up-regulated significantly under both thermal stress modes. These results are consistent with our previous study (Ning et al., 2021), suggesting that the significant up-regulation of HSPs may help ark shells become more resistant to elevated seawater temperature. Moreover, the fact that the expression levels of HSPs (*sHSP*, *HSP20*, and *HSP70*) in the AHT were much higher than that in the CHT mode may imply that the ark shells were subjected to more severe cellular damage induced by acute thermal stress. Similar results were also observed in Yesso scallop (Jiang et al., 2016) and pufferfish, *Takifugu obscurus* (Cheng et al., 2018).

## CONCLUSION

In summary, the physiological performance and gene expression were affected strongly by both acute and chronic thermal stress in ark shells. The apoptosis and necrosis rates of hemocytes were stimulated significantly in a time-dependent manner, and the content of MDA was increased in gills, indicating elevated seawater temperature has caused oxidative stress to ark shells. Thermal stress also induced actions in the anti-apoptotic system, antioxidant defense system and HSPs to protect ark shells from oxidative damage. The activities of antioxidant enzyme and expression of most genes in the CHT could be restored to initial level, which did not occur in the AHT, suggesting that the damage

caused by rapid ambient temperature increase is irreversible, and thus more harmful to marine mollusks. Our results may provide more clues for management strategy of ark shell husbandry and further understanding of the mechanisms underlying the tolerance of ark shells to high temperature stress.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

DZ: investigation and roles/writing – original draft. JN: methodology, validation, software, and writing – review and editing. XL: supervision, formal analysis, and visualization. XW: validation and data curation. MC: software and data curation. BL: conceptualization and resources. JF: conceptualization and

resources. CW: funding acquisition, writing – review and editing, and project administration. All authors contributed to manuscript revision, read, and approved the submitted version.

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