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Impact of Temperature Stress on Oxygen and Energy Metabolism in the Hepatopancreas of the Black Tiger Shrimp, *Penaeus monodon* (Crustacea: Decapoda: Penaeidae)

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Authors' Contribution
SGJ, SJ and FLZ designed the study. QBY and JHH executed the study. LSY, QBY and SJ implemented the study and analysed the samples. FLZ, SJ and SGJ drafted the manuscript.

Key words
***Penaeus monodon*, Temperature stress, Oxygen metabolism, Energy metabolism.**

ABSTRACT

The impacts of temperature on oxygen and energy metabolism in the hepatopancreas of the black tiger shrimp (*Penaeus monodon*) were investigated via the effects of thermal stress on superoxide anion (O_2^-) production, superoxide dismutase (SOD) activity, glutathione (GSH) and adenosine triphosphate (ATP) concentration, as well as nitric oxide synthase (NOS) activity and nitric oxide (NO) concentration catalyzed by NOS. Based on biochemical analysis of the above, results showed that O_2^- production could be induced significantly after cold stress at 15°C and heat stress above 30°C. SOD activity showed a similar changing profile as the concentration of O_2^- after thermal stress between 15°C and 30°C, and GSH concentration increased significantly under both high and low temperature. The NOS activity and concentration of NO catalyzed by NOS increased significantly after heat stress. The ATP concentration increased significantly after both low and high temperature stress, but returned to the control level after 8 h of recovery. Thus, low and high temperature stress could lead to oxygen metabolism disorder in the hepatopancreas of *P. monodon*, which might induce antioxidant enzyme responses. Our findings suggest that SOD and GSH might play different roles in the response of the shrimp to low and high temperature, whereas NO might play an important role in the induction of many signaling pathways in response to thermal stress. Additional ATP was also produced after cold and heat stress, suggesting that more energy was required to cope with temperature extremes. Our findings indicate that temperature stress led to oxygen metabolism disorder, possibly due to the temperature being beyond the oxygen- and capacity-limitation of thermal tolerance of *P. monodon*. Oxygen metabolism disruption might also impact energy metabolism and other physiological activities in *P. monodon*.

INTRODUCTION

The black tiger shrimp, *Penaeus monodon* (Crustacea: Decapoda: Penaeidae) is an economically and globally important marine species, and very important in the aquaculture industries of Taiwan and of Fujian, Guangdong, and Hainan provinces in China (Jiang *et al*., 2018). *Penaeus monodon* has adapted to a wide range of temperatures, but extreme changes can have a significant impact on them (Leelatanawit *et al*., 2017). At certain temperatures, shrimp can experience stress, physiological disturbance, poor adaptation to environmental change, as well as decreased immunity, increased risk of infection from pathogens, and mass death (Yang *et al*., 2011). Previous research has shown that oxygen and energy metabolism and growth are closely related to temperature in shrimp species (Zhou *et al*., 2010; Wang *et al*., 2006). For example, within a certain temperature range, the body weight and energy of *Fenneropenaeus chinensis* has been shown to increase with an increase in temperature (Tian *et al*., 2004). The growth and metabolism of *Litopenaeus vannamei* is also reported to be closely related to temperature (Yang *et al*., 2011). In addition, temperature extremes have been shown to not only slow down the growth of *P. monodon*, but also decrease their disease resistance and directly cause their death (Deering *et al*., 1995).

When the temperature exceeds the oxygen and heat tolerance limits of an animal, its metabolism can change from aerobic to hypoxic, which can, in turn, produce a significant impact on many physiological activities of the organism (Anestis *et al*., 2008). Studies have shown that temperature is closely related to the growth of *P. monodon* (Deering *et al*., 1995); however, the demand for oxygen in shrimp metabolism is very strong and sensitive to

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hypoxia (Wang *et al*., 2012). Therefore, the relationship between ambient temperature and shrimp oxygen and energy metabolism needs to be further studied. The hepatopancreas is one of the most important organs in shrimp species, and is critical for the synthesis of many large molecules required for life, as well as in the synthesis of other material and in catabolic metabolism (Dall *et al*., 1983). Although it is a significant metabolic organ, the relationship between temperature stress and oxygen and energy metabolism in the hepatopancreas of shrimp is not yet clear.

In this paper, we studied the effect of temperature stress on superoxide anion (O_2^-) production, superoxide dismutase (SOD) activity, glutathione (GSH) and adenosine triphosphate (ATP) concentration, as well as nitric oxide synthase (NOS) activity and the concentration of nitric oxide (NO) catalyzed by NOS in the hepatopancreas of *P. monodon*. The aim of this study was to understand the effect of temperature stress on the oxygen and energy metabolism of *P. monodon*, and provide a theoretical basis for its healthy breeding.

MATERIALS AND METHODS

Experimental shrimp

The body lengths and weights of the experimental shrimp were (10 ± 0.8) cm and (16.5 ± 0.6) g, respectively. All experimental animals (n = 90) were selected from the same family (same parents). Prior to the start of the trial, animals were held in sea water at a temperature of 25°C for 7 d at a density of one shrimp per liter. During the holding period, one third of the volume of fresh seawater was replaced. Appropriate bait food was fed to the shrimp regularly, with dead shrimp, bait, and excreta removed in a timely manner. At the beginning of the feeding trial, the shrimp were starved for 12 h. During the holding period, pH ranged from 7.8 to 8.1 and salinity was 25PSU. Dissolved oxygen was not less than 6.0 mg·L^{-1} and ammonia nitrogen was maintained at less than 0.02 mg·L^{-1}.

Temperature stress and sampling

Each experiment was performed in a large processing water bath container (100 L). During the experimental period, the natural temperature of the sea water was maintained at about 23°C±0.5°C. For the temperature stress experiments, the temperature was set at 15, 20, 25 (control), 30, or 35°C. Linear temperature increases or decreases were used to produce temperature stress. For the 15°C and 20°C temperature groups, cooled water from a water chiller (Kw-HXP-001A, Shenzhen Hongxin Refrigeration Equipment Co., Ltd., Shenzhen, Guangdong, China) was used to decrease the water temperature in the container. For the 30°C and 35°C temperature groups, heating rods (WN-B30E, Foshan Weinuo Refrigeration Equipment Co., Ltd., Foshan, Guangdong, China) were used to increase the water temperature in the container. The shrimp were transferred to the water bath container under each set temperature for 8 h, and then rapidly transferred to sea water at 25°C and again sampled after 8 h. At each sampling time point, the hepatopancreas of five shrimp were randomly selected and mixed into one sample on ice. The samples were then quickly placed into a liquid nitrogen tank and transferred to a refrigerator at -80°C. A total of 90 shrimp were divided into three parallel groups (n = 3).

Preparation of hepatopancreas extracts

50 mmol·L^{-1} Tris-HCl homogenization buffer (pH 7.4) (Almeida *et al*., 2005) was added to 100 mg of hepatopancreas tissue according to a tissue mass: homogenization buffer ratio of 1:9 (W:V). The sample was then homogenized and centrifuged at 12,500 r·min^{-1} for 15 min at 4°C, with the supernatant then diluted to the appropriate concentration for the determination of the physiological and biochemical indicators.

Determination of total protein content

Total protein content in the supernatant of the tissue homogenate was determined by the Bradford method. A standard protein curve was constructed, with bovine serum albumin (BSA) used as the standard (Bradford, 1976).

Determination of superoxide anion O_2^- content

The content of O_2^- in the samples was determined according to Drossos *et al*. (1995), with some modification. Briefly, each sample was placed in Krebs buffer and then an ice bath, after which 15 μmol·L^{-1} CytC (C2506, Sigma, USA) was added, with the sample then reacted in a water bath at 37°C for 4 min, and centrifuged at 12,500 r·min^{-1} for 10 min at 4°C. After the supernatant was obtained, 100 μL of 3 mmol·L^{-1} N-ethylmaleimide (E3876, Sigma, USA) was added immediately. Once the reaction was terminated, light absorption intensity was measured at 550 nm wavelength (A_{550}). The O_2^- content was quantified according to the change in the molar extinction coefficient between high-iron cytochrome c and ferrous cytochrome C. The following equation was used to determine O_2^- generation:

$$\text{Superoxide anion generation (nmol/min·mg)} = 21 / (\Delta\text{OD550 nm} \times \text{Reaction time} \times \text{Total protein concentration})$$

Determination of SOD activity

The SOD activity was measured using the SOD WST-1 method (Liu *et al*., 2011). The supernatant was diluted a

number of times for the determination of SOD activity, in accordance with the Nanjing Jian Cheng (A001-3, China) kit instructions. Enzyme activity was defined as the amount of enzyme corresponding to SOD activity when the SOD inhibition rate reached 50% in the reaction system. The calculation formulas used are as follows:

$$\text{SOD inhibition rate (\%)} = [1 - (A_{\text{Experiment}} - A_{\text{Experimental blank}}) / (A_{\text{Control}} - A_{\text{Control blank}})] \times 100\%$$

$$\text{SOD enzyme activity (U/mg)} = (\text{SOD Inhibition rate} / 50\%) \times \text{dilution ratio} / [\text{Sample protein concentration (mg·mL}^{-1})]$$

Determination of GSH content

The GSH content was determined according to Sedlak *et al*. (1968). The GSH content (mg·mL^{-1}) of total protein in the unit mass was calculated using a standard curve drawn by the GSH standard (Amresco, USA).

NOS activity determination

Total NOS activity was measured using NOS commercial kits (Jiancheng Institute of Biotechnology, Nanjing, China, A014-2), as per the manufacturer's instructions. NOS activity was determined by NO production catalyzed by NOS using the following equation:

$$\text{Total NOS activity (U / mg)} = [(\text{A test-A control}) / (38.3\times10^{-6}\times \text{total protein concentration})] \times[(2.41 + a) / a] \times [1 / (1 \times 15)]$$

Where, 38.3×10^{-6} is the nano-molar extinction coefficient, 2.4 is the total volume of reagents (mL), a is the sample volume (mL), 1 is the colorimetric optical path (cm), and 15 is the reaction time (min).

Determination of NO content

The determination of NO content was based on the Griess method (Ding *et al*. 1988), with some modification. Standard sodium nitrite was prepared in standard solutions of different concentrations, and the standard curve was drawn to calculate the NO content in total protein.

Determination of ATP content

The determination of ATP content was based on the creatine kinase method (Chen and Sun, 2002), with some modification. ATP content was defined as ATP content per unit of total protein (mmol/g), and calculated using the following equation:

$$\text{ATP (mmol/g)} = (A_{700}\,U - A_{700}\,U_B) / [(A_{700}S - A_{700}S_B) \times \text{total protein concentration}] \times 5 \times 10^5$$

Statistical analysis

Results are presented as means ± SEM of three replicates. All data were subjected to one-way analysis of variance (ANOVA). When there were significant differences, the group means were further compared with Duncan's multiple-range test. All statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Michigan Avenue, Chicago, IL, USA) for Windows.

RESULTS

O_2^- production and antioxidant enzyme activity

The effect of temperature stress on O_2^- content in hepatopancreas of *P. monodon* is shown in Figure 1A. The content of O_2^- increased significantly in the experimental group at 15°C, and was 1.49 times (P<0.05) higher than that in the control group after 8 h of stress. When the temperature was returned to the control level for 8 h, the O_2^- content also returned to the control group level. There was no significant difference in O_2^- content between the control group (P>0.05) after 8 h at 20°C and 8 h after stress recovery. The content of O_2^- increased significantly at 30°C, and was 1.38 times (P<0.05) higher than that in the control group after 8 h stress. When the temperature was returned to the control level for 8 h, the O_2^- content also returned to the control group level. After 8 h of stress at 35°C, the content of O_2^- was 2.64 times (P<0.05) higher than that in the control group, and was still 1.13 times higher after the temperature was returned to the control level for 8 h, though the difference was not significant (P>0.05).

The effect of temperature stress on SOD activity in the hepatopancreas of *P. monodon* is shown in Figure 1B. Low temperature stress at 15°C resulted in a significant increase in SOD activity ($P < 0.05$), which was 1.46 times higher than that in the control group. With the increase in temperature, the activity of SOD was only 53% of that in the control group ($P < 0.05$). When stressed at 20°C and 30°C for 8 h, SOD activity was significantly increased ($P < 0.05$), and was 1.29 and 2.47 higher times than that in the control group, respectively. When the temperature was returned to the control level, SOD activity was restored to the control group level ($P > 0.05$). When stressed at 35°C for 8 h, SOD activity was 2.12 times higher than that in the control group; however, when the temperature was returned to the control level for 8 h, SOD activity was significantly lower, only reaching 48% of that in the control group ($P < 0.05$).

The effect of temperature stress on GSH content in the hepatopancreas of *P. monodon* is shown in Figure 1C. The content of GSH in the experimental group significantly increased after 15°C stress for 8 h, and was 2.31 times ($P < 0.05$) higher than that in the control group. When the

temperature was returned to the control level for 8 h, the GSH content was restored to the control group level ($P > 0.05$). At 20°C, no significant changes in GSH content were observed. However, GSH content in the experimental group significantly increased under 30°C stress for 8 h, and was 2.45 times ($P < 0.05$) higher than that in the control group. When the temperature was returned to the control level for 8 h, the GSH content was restored to the control group level ($P > 0.05$). Under 35°C stress for 8 h, there was no significant difference in the content of GSH between the experimental and control group; however, the content of GSH gradually increased with decreasing temperature, and when the temperature in the experimental group was returned to the control temperature for 8 h, the GSH content was 1.89 times ($P <0.05$) higher than that in the control group.

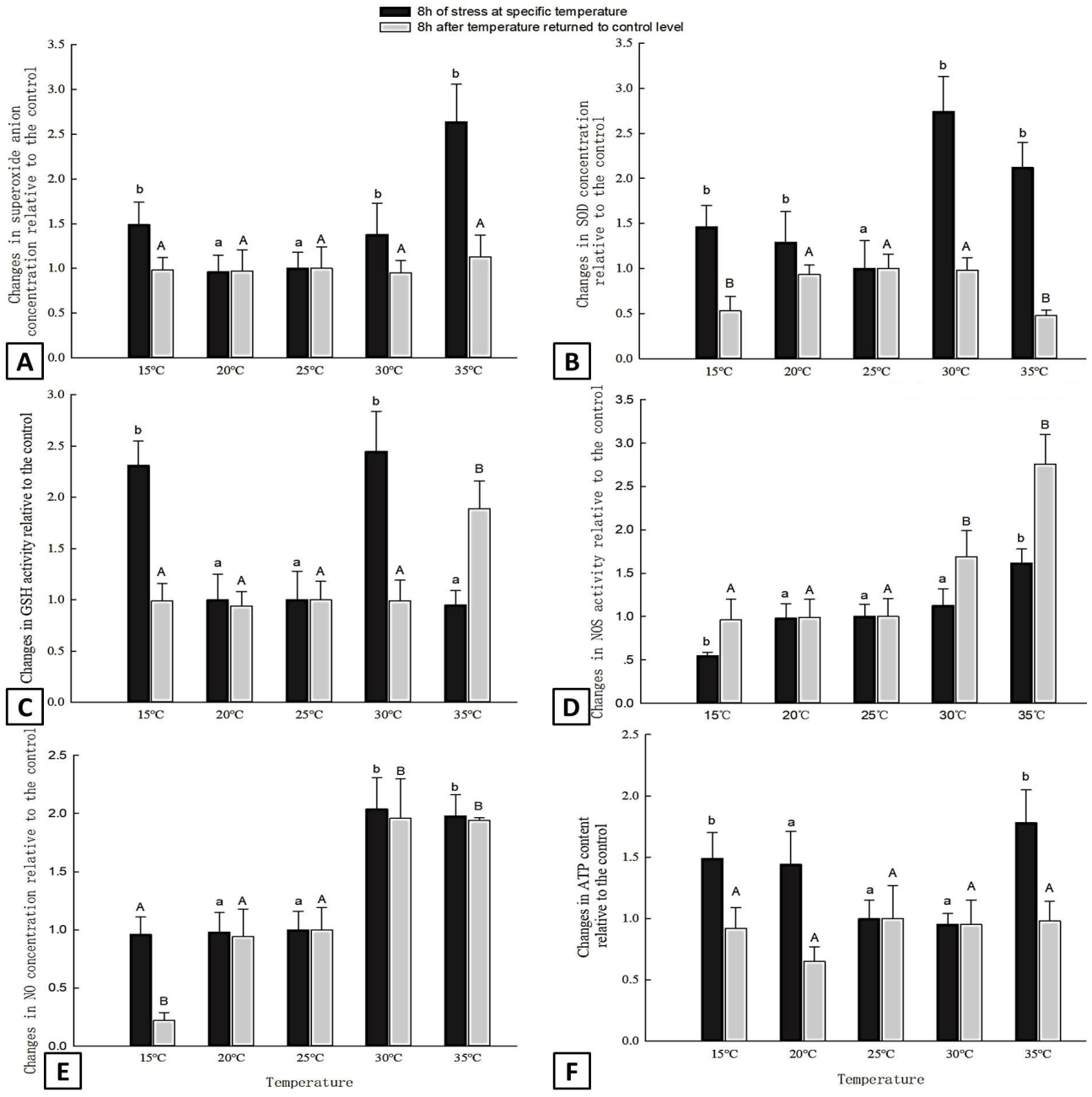

Fig. 1. Effects of different temperatures on O_2^- (A), SOD (B), GSH (C), NOS (D), NO (E) and ATP content (F) in the hepatopancreas of *P. monodon*. Different letters within each bar indicate significant differences ($P < 0.05$). Data are compared in the same series.

NOS activity and NO content

The effect of temperature stress on NOS activity in the hepatopancreas of *P. monodon* is shown in Figure 1D. When the shrimp were stressed at a low temperature of 15°C for 8 h, NOS activity decreased significantly and only reached 55% of that in the control group ($P < 0.05$). When the temperature was returned to the control level for 8 h, the activity was also restored to the control group level ($P > 0.05$). At 20°C, no significant changes in NOS activity were observed. However, when the shrimp were stressed at high temperatures of 30°C and 35°C for 8 h, the NOS activity increased and was 1.13 ($P > 0.05$) and 1.62 ($P < 0.05$) times higher than that in the control group, respectively. When the temperature was returned to the control level for 8 h, the activity of NOS continued to increase and was 1.69 and 2.76 times ($P < 0.05$) higher than that in the control group, respectively.

The effect of temperature stress on NO content in the hepatopancreas of *P. monodon* is shown in Figure 1E. When the shrimp were stressed at the lower temperatures of 15°C and 20°C for 8 h, no significant differences in NO content were observed between the experimental and control groups. However, when the temperature in the experimental group was returned to the control temperature for 8 h, the NO content was 0.22 ($P < 0.05$) times higher than that in the control group. When the shrimp were stressed at 30°C and 35°C for 8 h, the contents of NO were significantly increased ($P < 0.05$) and were 2.04 and 1.98 times higher than that in the control group, respectively. When the temperature was returned to the control level, the contents of NO remained at high levels, and were 1.96 ($P < 0.05$) and 1.94 ($P < 0.05$) times higher than that in the control group, respectively.

ATP content

The effect of temperature stress on ATP content in the hepatopancreas of *P. monodon* is shown in Figure 1F. The content of ATP in the experimental group was significantly increased after 15°C stress for 8 h, and was 1.49 times ($P < 0.05$) higher than that in the control group. When the temperature was returned to the control level for 8 h, the content was restored to the control group level ($P > 0.05$). The content of ATP in the experimental group was 1.44 ($P < 0.05$) times higher than that in the control group when the shrimp were stressed at 20°C. However, ATP content decreased with the increase in temperature. When the temperature was returned to the control level for 8 h, the ATP content was only 65% of that in the control group ($P < 0.05$). At 30°C stress, no significant changes were observed in ATP content; however, the ATP content in the experimental group was significantly increased after 35°C stress for 8 h, and was 1.78 times ($P < 0.05$) higher than that in the control group. When the temperature was returned to the control level for 8 h, the content was restored to the control group level ($P > 0.05$).

DISCUSSION

Many physiological, biochemical, and metabolic changes in aquatic animals, especially lower aquatic animals, are closely related to environmental conditions. Aquatic animals initiate the body's compensatory adaptation function to resist and adapt to a variety of stresses (Lutterschmidt *et al*., 1997). When the stress response exceeds the adaptation limits of the body, tissue structures can be damaged. Temperature is one of the most important abiotic factors affecting the survival and growth of marine organisms (Lutterschmidt *et al*., 1997).

The optimum temperature range for *P. monodon* is 24-28°C, and when the water temperature drops below 15°C or increases above 30°C, the animals can experience environmental stress (Wyban and Sweeney, 1991). Deterioration in shrimp health when facing environmental stress could be related to stress state and physiological changes. To date, however, temperature stress impact on the hepatopancreas, an important synthesis and metabolic organ of shrimp species, has remained unclear. Some studies have shown that when seawater temperature suddenly changes, the metabolic rate of some lower marine organisms will accelerate, possibly due to the loss of metabolic components in cells due to sudden temperature changes (Zhou *et al*., 2010). Fluctuations in the temperature of ponds are often accompanied by changes in the concentration of dissolved oxygen. Therefore, the relationship between oxygen metabolism and temperature has become the focus of many studies on temperature stress physiology (Tu *et al*., 2013).

During normal aerobic metabolism and defensive reaction, reactive oxygen species (ROS) and reactive oxygen intermediates (ROI) are produced by organisms, with surplus ROS quickly removed by antioxidant enzymes under normal physiological conditions. Oxidative stress occurs when the balance of ROS production and consumption is broken. Superoxide anion is the most common form of ROS, and can further generate other types of ROS (Halliwell and Gutteridge, 1984). In the present study, we found that O_2^- content in the hepatopancreas of *P. monodon* significantly increased under temperature stress at 15°C. Shrimp are relatively sensitive to ambient temperature (Tu *et al*., 2013), suggesting that a sudden drop in temperature might result in a change in oxygen metabolism, thus leading to a significant increase in O_2^- content in the hepatopancreas. Zenteno *et al*. (2006) also found that O_2^- content increased in the hepatopancreas after

low temperature stimulation in *Litopenaeus vannamei*, but returned to the control group level when the temperature was reverted to the control level. However, it is still not clear whether this improvement was due to complete metabolic recovery or temporary recovery under a suitable temperature.

Under high temperature conditions, shrimp respiration accelerates and ROS production increases accordingly, which could lead to oxidative stress in the body (Wang *et al*., 2006). In the present study, the contents of O_2^- in all experimental groups were higher than that in the control. The content of O_2^- in the 35°C experimental group was 2.64 times higher than that in the control, revealing that high temperature stress could induce an increase in O_2^-. When the temperature was returned to the control level for 8 h, the O_2^- content did not increase correspondingly, thus conforming to the oxygen- and capacity-limitation of thermal tolerance law (Anestis *et al*., 2008).

SOD is a key enzyme in the biological antioxidant system, and it can directly remove excess free radicals in the body. In the present study, after 8 h of thermal stress at 30°C, the activity of SOD in the experimental group increased and was 2.74 times ($P < 0.05$) higher than that in the control group, suggesting that SOD might play an important function in the treatment of high temperature stress in *P. monodon*. After thermal stress at 15°C and 35°C, the activity of SOD decreased significantly when the temperature was returned to the control level, which might be due to the serious damage caused by the sudden change in temperature, and the difficultly of recovery in the short term. Zho *et al*. (2007) also showed that drastic changes in temperature decreased metabolic and tissue antioxidant enzyme activity in shrimp, thus increasing vulnerability to oxidative damage and decreasing disease resistance.

GSH is an important non-protein mercapto compound in body tissue, and can be combined with free radicals and heavy metals to protect the thiol enzyme and hemoglobin from oxidative damage (Pompella *et al*., 2003). When GSH is lacking or exhausted, the body is essentially poisoned, with the toxic effects associated with oxidative damage (Pompella *et al*., 2003). Thus, GSH content is considered an important factor to measure the body's antioxidant ability, and an obvious biomarker affected by abiotic factors (Pompella *et al*., 2003). In the present study, the content of GSH in the hepatopancreas of the shrimp was significantly increased after 8 h of stress at 15°C and 30°C ($P < 0.05$), suggesting that temperature changes led to metabolic changes. Specifically, more ROS were produced, resulting in higher antioxidant stress and the production of additional GSH to protect the animal from oxidative stress. When the temperature was very high (35°C), the shrimp exhibited metabolic disorder, resulting in unchanging GSH; however, when the temperature was returned to the control level, the effects of stress began to appear. Other studies on shrimp have also shown that high temperature stress can lead to an increase in GSH content in various tissue (Hermes and Zenteno, 2002; Rajagopal *et al*., 2005; Tu *et al*., 2013), suggesting that GSH plays an important role in protecting the body against oxidative damage caused by hypoxia.

NO is a second messenger molecule with a variety of physiological functions. Changes in its expression are closely related to immune signal transduction, muscle contraction, nerve signal transmission, and other important physiological processes (Soderhall, 1999). It can be combined with the O_2^- produced from oxygen metabolism, thus producing nitric oxide and playing a bactericidal function (Soderhall, 1999). NOS can produce NO by catalyzing L-arginine (L-Arg) and O_2 (Mayer and Andrew, 1998). Therefore, NOS activity and NO production are important indicators of oxidative metabolism in animals. In this study, we found that 30°C and 35°C stress led to an increase in NOS activity and NO content (produced by the catalysis of NOS) in the hepatopancreas of *P. monodon*. Temperature stress, especially high temperature stress, induced an increase in NOS activity and NO synthesis. This increase in NO might be related to its involvement in additional signal transmission to cope with temperature stress.

As one of the most important energy molecules, ATP plays important roles in various physiological and pathological processes within cells. As a critical environmental factor, temperature can significantly affect the metabolic rate of organisms, thereby affecting the level of energy metabolism in the body. Wang *et al*. (2012) found that the expressions of V-H ATPase and Na^+-K^+ ATPase in the hepatopancreas of *P. monodon* were up-regulated under 12°C temperature stimulation. Here, we found that ATP content increased in the hepatopancreas of *P. monodon* under both low and high temperature stress, though the content increased with increasing temperature. We speculated that at low temperatures, shrimp might need to synthesize more ATP to maintain body metabolism. However, under extreme high temperature conditions, ATP synthesis could be accelerated, but more energy might be consumed to maintain steady balance in *P. monodon*.

In conclusion, O_2^- content, SOD activity, GSH content, ATP content, NOS activity, and its catalytic NO production, were studied in the hepatopancreas of *P. monodon* under low and high temperature stress. The results showed that both low and high temperature stress caused an increase in ROS and corresponding antioxidant enzyme activity in the hepatopancreas of *P. monodon*, but different types of antioxidant enzymes might play different

roles in the resistance of shrimp to low or high temperature stress. High temperature stress led to a significant increase in NOS activity and NO production, with the body possibly requiring more NO for stress-related signal transmission. Low and high temperature stress induced a significant increase in ATP content, suggesting that the body might need more energy to maintain metabolic activity, respond to temperature stress, and repair damage caused by stress. When temperature exceeds a certain range, it can result in greater damage to the body. Here, however, the metabolic level in the shrimp remained unchanged, despite the increase in temperature, which could be related to oxygen and thermal tolerance in *P. monodon*.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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