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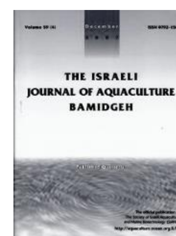

Response of Antioxidant Enzymes and Digestive Enzymes to Temperature Stress in *Lates Calcarifer* Larvae

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Abstract

Acute temperature stress in 15-day post-hatch (DPH) *Lates calcarifer* larvae was studied using the semi-static bioassay method to understand the physical response of fish larvae to low temperature. In this study, 20°C, 22°C, 24°C, and 26°C were tested and 28°C was the control. Antioxidant enzyme activity including superoxide dismutase (SOD), peroxidase (POD), catalase, acid phosphatase (ACP), glutathione peroxidase (GSH-Px) and digestive enzymes including lipase, amylase, trypsin, pepsin were used as the physical indicators. SOD activities were elevated to scavenge excess reactive oxygen species (ROS) after exposure to lower temperatures, and their activities increased dramatically in relation to the increasing residue time. POD enzyme activity maintained a low level in the early stage of cold exposure and increased to a high level in relation to exposure time. Catalase, GSH-Px, and ACP activities were elevated in fish after exposure to lower temperatures compared to those in fish maintained at 28°C. Lipase activity decreased after exposure to lower temperatures and modestly elevated over time. The highest amylase activity was observed in the control group. However, trypsin and pepsin activity was elevated with the decrease of environmental temperature. Our results indicate that lower temperature significantly affects activity of antioxidant enzymes and digestive enzymes in barramundi larvae. Barramundi larvae exhibited a certain tolerance of low temperature for a short period. Results from the present study will improve our understanding of the physical response of warm water fish to low temperatures and can therefore guide hatchery practices for *Lates calcarifer*.

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Introduction

In aquaculture, temperature is one of the major environmental factors modifying the physiological and biochemical parameters of aquatic organisms (Brett, 1971). The growth and survival of poikilothermal teleosts is limited and controlled by fluctuations in ambient temperature. Water temperature regulates the capacity of fish to obtain oxygen and alter the oxygen consumption rate. Optimal temperature ranges of ectotherms depend on developmental stage, genetics, and thermal history, and vary widely among species (Beitinger et al, 2000). Rapid decreases in temperature have the potential to cause a cascade of physiological, behavioral, and fitness responses for fish. This is called "cold shock", and ultimately affects the organism's health and fitness or may result in mortality (Van den Burg et al., 2005; Donaldson et al., 2008). Previous studies demonstrated that a sharp alteration in temperature caused oxidative stress in organisms like goldfish (Lushchak and Bagnyukova, 2006). When production rate of ROS exceeds its removal, oxidative stress occurs. Oxidative stress is crucial to membrane integrity and may cause disturbance of cellular homeostasis and subcellular structures (Halliwell and Gutteridge, 1989). If sharp stress occurs, oxidative damage may promote cell death and eventually limit the survival of an organism under stressful conditions. To cope with temperature fluctuations, Teleostei species have developed specific behavioral and physiological defense capabilities (Prosser and Heath, 1991). These adaptive mechanisms enable fish to survive through adaptation to temperature-stressed environments (Hazel and Prosser, 1974).

The front line of the defense mechanism in fish is the usage of antioxidant compounds such as vitamin E, vitamin C, glutathione, uric acid, and carotenoids (Rosa et al, 2005). Together with these antioxidants, diverse antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidases (GSH-Px), directly detoxify harmful xenobiotics or ROS and other compounds involved in oxidative damage to cellular structures and ROS generation, preventing the cascade of oxidant reactions. Antioxidant enzymes are critical for the process of counteracting oxygen toxicity when the supply of other antioxidant compounds is scarce or depleted (Ahmad et al, 2004). Antioxidant enzymes are widely chosen as biomarkers of marine contaminants, and research on antioxidant responses to environmental stress such as cold stress is tantamount. Therefore, in this study we explored the response of antioxidant enzymes from barramundi larvae to environmental temperature.

Some studies have indicated that environmental temperature appears to alter the physiology of aquatic organisms, including growth, propagation, osmoregulation, metabolic rate, food intake, digestive enzyme activity, and feed conversion efficiency, which are closely related to the growth performance of fish (Das et al,2004; Lushchak and Bagnyukova, 2006; Olaf et al, 2005). As part of the metabolic regulatory mechanisms, the digestive enzymes are widely used as indicators of fish physiological state and in studies as markers of fish larval development. Proteinases catalyze the hydrolytic degradation of protein molecules and play an essential role in the growth and survival of living organisms (Klomklao et al, 2006). Lipase activity is essential to lipid metabolism, involving an important reaction of energy supply (Elsayed, 2015). Amylase is the key enzyme carbohydrate digestion, which is associated with carbohydrate metabolism in food utilization and energy supply (Al-Tameemi et al, 2010). Without sufficient levels of enzymes in the digestive system, food particles cannot be thoroughly digested. The activity of digestive enzymes is expected to be affected by external factors that modify metabolic functions, such as temperature and PH (Hochachka and Somero, 2002; Xu et al, 2004). So far, the influence of temperature stress on digestive efficiency and enzymatic activity of marine organisms has been studied in many invertebrate organisms（Elsayed, 2015; Lushchak and Bagnyukova, 2006; Xie et al, 2015). Such analysis can directly reflect the response of the digestive process to temperature stress (Ueberschär,1988; Ma et al., 2014c, Ma et al., 2013).

Barramundi *Lates calcarifer* is a euryhaline teleost found in tropical and subtropical estuarine areas and native to the Indo-Pacific region and has been considered a high value commercial fishery and aquaculture species due to high-flesh quality and high growth rate (Chou and Lee, 1997). Culture of this species has been well established with significant industries of cage aquaculture in Indonesia, Australia, Malaysia, Philippines,

Thailand and Taiwan (Tian and Qin, 2003), and large-scale commercial farming cultured either in natural watercourses or in artificial ponds and reservoirs. Since 1980s, there has been a considerable effort to improve culture of this species. As a tropical species, temperature can significantly affect its growth and survival. However, little is known regarding the influence of acute changes in temperature on antioxidant enzymes and digestive enzymes in barramundi larvae. The primary objective of this work was to examine the effects of acute cold stress on antioxidant enzymes and digestive enzymes in larval barramundi, aiming to provide a scientific basis for the management of aquaculture water environment.

Materials and Methods

Eggs and larval fish rearing

Fertilized eggs of barramundi from the same batch were obtained from Tropical Fisheries Research and Development Center, South China Sea Fisheries Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Science, Lingshui Town. Upon arrival, eggs were stocked into five 500 L fiberglass incubators. On 3 DPH, fish larvae were transferred to twelve 1000 L larval rearing tanks. The rearing temperature was maintained at 29.0 ± 1.0°C, pH was 8.0-8.2 and the salinity was 33 ± 0.8‰ throughout the experiment. The light regime was set to 14/10-h light/dark and light intensity was maintained at 2000 lux. In each tank, two air stones were used to maintain dissolved oxygen > 6.5 mg/L. The nitrite content was maintained < 0.03 mg/L. Water was changed 2 times per day with a daily exchange rate of 50% during the larval rearing period

Rotifers (*Brachionus rotundiformis*) at a density of 10-20 ind/ml were used to feed the larvae from 2 DPH to 9 DPH (4.50±0.10 mm). *Artemia nauplii* were introduced at 5 nauplii/mL on 7 DPH, and the feeding density increased at 50% daily. *Artemia nauplii* and rotifers were enriched with DHA Protein Selco (INVE Aquaculture, Salt Lake City, UT, USA) following the manufacturer's instruction before they were added into the larval rearing tanks. Instant microalgal paste (*Nannochloropsis sp.* Qingdao hongbang biotechnology Co. LTD) was also added into larval fish tanks to create a green-water background. The outlet screen, residual bait and excreta were cleaned daily to decrease interference and reduce clogging.

Experimental design

Acute cold stress on 15 DPH barramundi was conducted in a semi-static 96-h experiment that involved a strict pH 8.1 regime. The experiment consisted of five different temperatures: 20°C, 22°C, 24°C, and 26°C, and 28°C was used as the control temperature. Temperatures were adjusted using a water cooler (HXLS, Dalian Huixin Titanium Equipment development Co., Ltd, China), starting at 28°C and decreasing at 2°C intervals until the final target temperatures were reached. The experiment was conducted in 50 L aerated glass tanks. Each treatment had three replicates with 120 experimental larvae for a total of 360 larvae per treatment. Five samples were collected from every tank at 24, 48, 72, and 96h.

Sample processing procedure

After sampling, the specimens were thoroughly rinsed in distilled water to remove external salt, and then immediately stored in liquid nitrogen. The whole frozen fish was thawed, weighed, and homogenized for enzymatic assays, using a glass homogenizer on ice in 0.2 M NaCl (w/v) (Gawlicka et al, 2000). The homogenates were centrifuged at 13, 3000×g for 10 min at 2°C. Then, the aqueous supernatant was collected and incubated in the enzyme substrate under 25°C or 37°C and read on a spectrophotometer (UV-1800BPC, LiuYi Biotechnology co., Ltd, China) at the target wavelength. Enzyme activities were carried out in triplicates.

Measurement of antioxidant enzymes

Peroxidase (E.C. 1.11.1.7) activity was assayed by peroxidase assay kit (Catalog No.A084; Jiancheng Bioengineering Institute, Nanjing, China). In the assay, peroxidase can catalyze the reaction of hydrogen peroxide, the enzyme activity of peroxidase was obtained by measuring the change of absorbance at 420nm. Superoxide dismutase (E.C.

1.15.1.1) activity was measured using a superoxide dismutase activity assay kit (Catalog No. A001-1; Jiancheng Bioengineering Institute, Nanjing, China). The superoxide dismutase activity was determined by the xanthine oxidase method (hydroxylamine). Catalase (E.C. 1.15.1.1) activity was measured using a catalase activity assay kit (Catalog No. A007-1; Jiancheng Bioengineering Institute, Nanjing, China). Catalase can decompose H_2O_2 and this reaction can be quickly suspended by adding ammonium molybdate, the rest of H_2O_2 combine with ammonium molybdate to produce a pale-yellow complex compound, which is detected at 405nm. Glutathione peroxidase (E.C. 1.11.1.9) activity was measured using a glutathione peroxidase activity assay kit (Catalog No. A005; Jiancheng Bioengineering Institute, Nanjing, China). In the assay, glutathione peroxidase can catalyze the reaction of reduced glutathione to hydrogen peroxide. Acid phosphatase (E.C. 3.1.3.2) activity was measured using an acid phosphatase activity assay kit (Catalog No. A060-2; Jiancheng Bioengineering Institute, Nanjing, China). Acid phosphatase can catalyze the decomposition reaction of disodium phenyl phosphate, free phenol and phosphoric acid produced. In alkaline condition, the reaction of phenol and 4-aminoantipyrin generate red quinone derivatives. The acid phosphatase enzyme activity is detected according to the red shades at 520nm.

Measurement of digestive enzymes

Trypsin (E.C. 3.4.21.4) activity was measured using a trypsin activity colorimetric assay kit (Catalog No. A080-2; Jiancheng Bioengineering Institute, Nanjing, China). In the assay, trypsin can hydrolyze the ester chain of arginine ethyl ester and increase the absorbance value at 253nm. Trypsin activity can be calculated according to the change of absorbance. Pepsin (E.C. 3.4.23.1) activity was measured using a pepsin activity assay kit (Catalog No. A080-1; Jiancheng Bioengineering Institute, Nanjing, China). In the assay, pepsin can hydrolyze protein to produce amino acids contained phenol, which can reduce phenol reagent to produce substances with blue color. The activity of α-amylase (E.C. 3.2.1.1) was measured using an amylase activity assay kit (Catalog No.C016; Jiancheng Bioengineering Institute, Nanjing, China). In the assay, iodine solution combined with unhydrolyzed starch into blue complex. Amylase activity can be calculated according to the absorbance value at 660 nm. Lipase (E.C. 3.2.1.1) activity was assayed using a lipase assay kit (Catalog No. A054-2; Jiancheng Bioengineering Institute, Nanjing, China). This assay is according to methyl halide substrate method measured at 570 nm. The specific activity was expressed as units per milligram of protein (U/mg protein). Soluble protein of crude enzyme extracts was quantified by Bicinchoninic Acid method (Walker J.M., 2002) using the bicinchoninic acid protein assay kit (Catalog No. A045-4; Jiancheng Bioengineering Institute, Nanjing, China).

Statistical analysis

The data in this paper were expressed as mean ± SD and tested by one-way ANOVA (PASW Statistics 18.0, Chicago, SPSS Inc.). When a significant treatment effect was found, Tukey's test was performed for multiple range comparisons with the level of significant difference set at $P < 0.05$. All the data were tested for normality, homogeneity and independence to satisfy the assumptions of ANOVA.

Results

Acute temperature stress effects to antioxidant enzymes

SOD activity

Acute temperature stress significantly affected the SOD activity of fish larvae ($P < 0.05$, Fig.1). After 48h exposure, the SOD activity of fish from 22°C and 24°C was significantly higher than fish from control group ($P < 0.05$). Meanwhile, SOD activity of fish from 26°C was significantly lower than control group ($P < 0.05$). However, the SOD activity was not significantly different between fish held in 20°C and the control group ($P > 0.05$). After 72h exposure, the highest SOD activity was observed in 24°C group ($P < 0.05$), while the SOD activity was not significantly different between 20°C and the control group ($P > 0.05$). By the end of this study, the SOD enzyme activity in 22°C treatment group exhibited a sharp increasing trend and was significantly higher than other treatments ($P < 0.05$). No significant difference ($P > 0.05$) was found between the 20°C and 28°C groups.

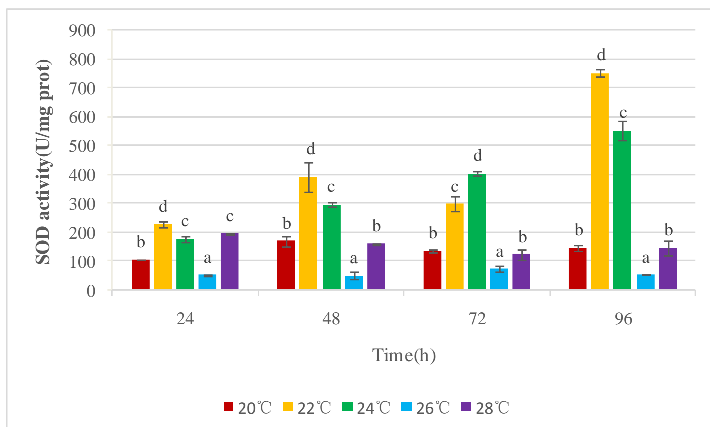

Fig.1 Effect of temperature on SOD activity of larval *L. calcarifer*

POD activity

After 24h exposure, the highest POD enzyme activity was observed in the 24°C group and was significantly higher than the 20°C group ($P < 0.05$, Fig. 2). No differences were recorded between other temperature treatments ($P > 0.05$). After 48h exposure, the POD activity in 24°C was significantly higher than other groups ($P < 0.05$). When exposure time reached 72h, the highest POD activity was found in the 24°C treatment group. The lowest POD activity was observed in the 22°C and 26°C groups. After 96h exposure, the highest POD activity was observed in 24°C group, and the lowest activity was observed in 22°C and 26°C groups.

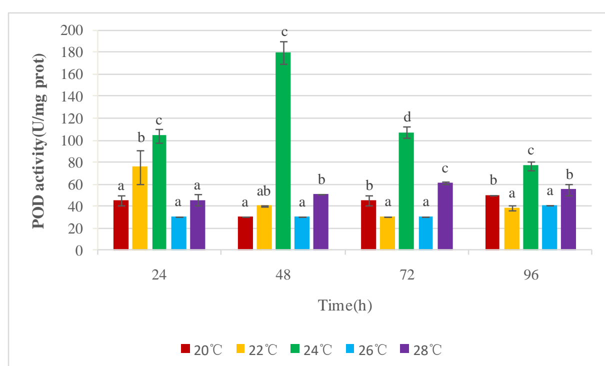

Fig.2 Effect of temperature on POD activity of larval *L. calcarifer*

Catalase activity

Temperature significantly affected catalase activity in barramundi larvae ($P < 0.05$, Fig.3). After 24h exposure, the highest catalase activity was observed in 24°C group ($P < 0.05$), and the lowest activity was found in the 22°C group. When exposure time reached 48h, the highest catalase activity was observed in the 20°C group, and lowest activity was found in the 26°C group. After 72h exposure, the highest catalase activity was observed in fish held in 24°C, and the lowest activity was observed in 26°C and 28°C groups. After 96h exposure, the highest catalase activity was observed in 24°C group, and the lowest activity was found in 26°C group.

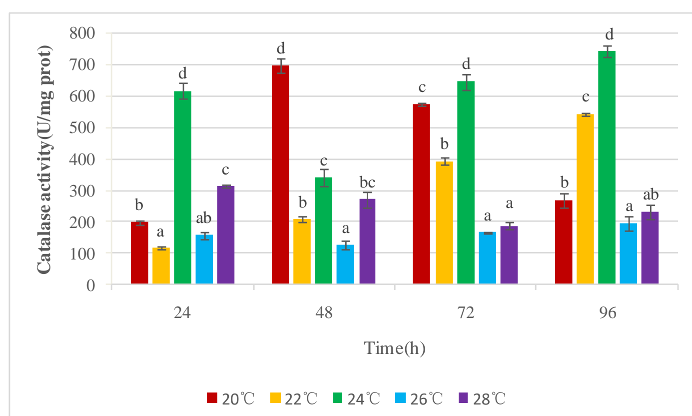

Fig.3 Effect of temperature on CAT activity of larval *L. calcarifer*.

<u>ACP activity</u>

Temperature significantly affected ACP activity in fish larvae ($P < 0.05$, Fig.4). After 24h exposure, the highest ACP activity was observed in the 20°C and 28°C groups, and the lowest ACP activity was observed in the 22°C and 26°C groups. After 48h exposure, ACP activity of fish from 22°C and 26°C groups was not significantly different ($P > 0.05$) but was significantly lower ($P < 0.05$) than those from the 20°C, 24°C, and 28°C groups. After 72h exposure, the highest ACP activity was observed in fish held in 24°C, and lowest ACP activity was found in 22°C group. At 96h, ACP activity was not significantly different between 24°C and 26°C groups, but was significantly lower than fish from 20°C, 22°C, and 28°C groups ($P < 0.05$)

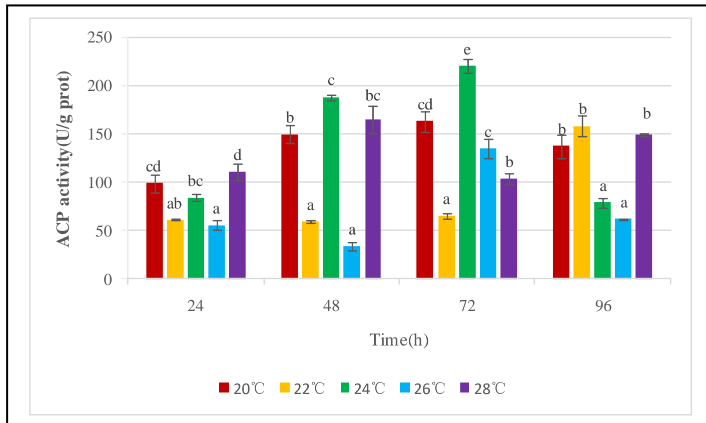

Fig.4 Effect of temperature on ACP activity of larval *L. calcarifer*.

<u>GSH-Px activity</u>

Temperature significantly ($P < 0.05$) affected GSH-Px activity of fish (Fig. 5). Throughout the experimental period, the highest GSH-Px activity was observed in 20°C group ($P < 0.05$). Before 96h, GSH-Px activity in fish decreased with increasing exposure temperatures. After 96h exposure, GSH-Px activity was not significantly different in fish from 22°C, 24°C, 26°C, and 28°C groups ($P > 0.05$).

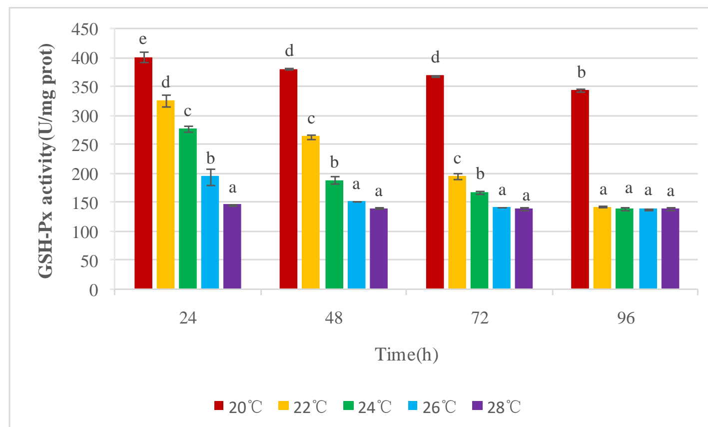

Fig.5 Effect of temperature on GSH-Px activity of larval *L. calcarifer*.

Acute temperature stress effects on digestive enzymes

<u>Lipase activity</u>

Temperature significantly affected the lipase activity of fish in this study ($P < 0.05$, Fig.6). After 24h exposure, the highest lipase activity was observed in 28°C group, and lowest activity was observed in 22°C group. At 72h and 96h, the lowest lipase activity was found in 24°C group, and the highest lipase activity was observed in 20°C group ($P < 0.05$).

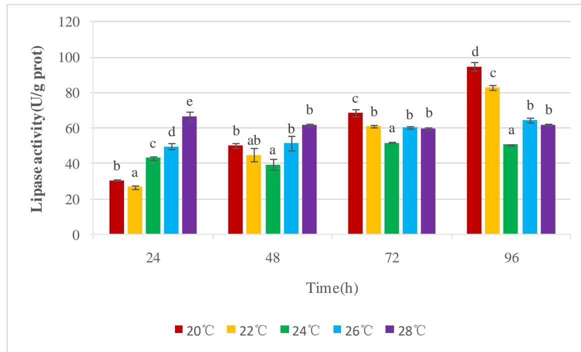

Fig.6 Effect of temperature on LPS activity of larval *L. calcarifer*.

Amylase activity

In this study, amylase activity of the fish was significantly affected by the temperature ($P < 0.05$, Fig.7). During the experimental period, amylase activity of fish increased with the increasing exposure temperatures. The lowest amylase activity was observed in 20°C group, and highest activity was found in 28°C group ($P < 0.05$).

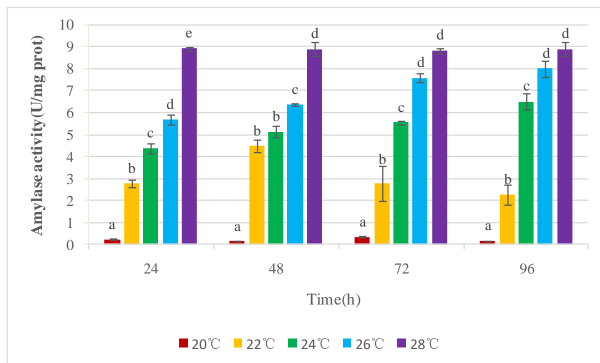

Fig.7 Effect of temperature on amylase activity of larval *L. calcarifer*.

Trypsin activity

The trypsin activity of fish was significantly affected by the exposure temperature ($P < 0.05$, Fig. 8). Throughout the experimental period, the lowest trypsin activity of fish was found in 28°C group. At 24 and 48h, the highest trypsin activity was found in 20°C group. At 72h, trypsin activity of fish from 20°C, 22°C, and 24°C groups was not significantly different ($P > 0.05$), but significantly higher than those from 26°C group ($P < 0.05$). At 96h, the highest trypsin activity was observed in 22°C group ($P < 0.05$).

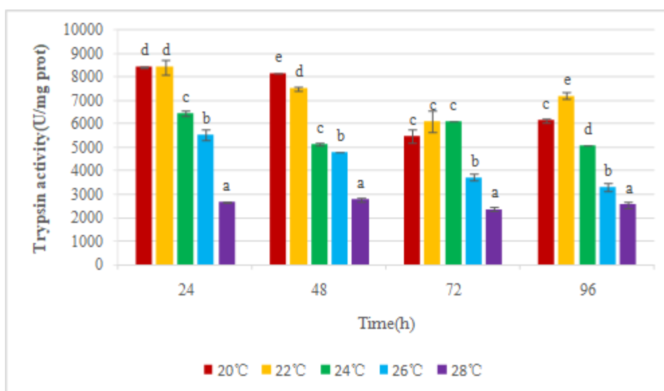

Fig.8 Effect of temperature on trypsin activity of larval *L. calcarifer*.

Pepsin

Pepsin activity was significantly affected by exposure temperature ($P < 0.05$, Fig. 9). At 24h, pepsin activity decreased with increasing exposure temperature. From 72h, pepsin activity was significantly different in fish from 20°C, 22°C, and 24°C groups, but was significantly higher than those from 26°C and 28°C groups ($P < 0.05$).

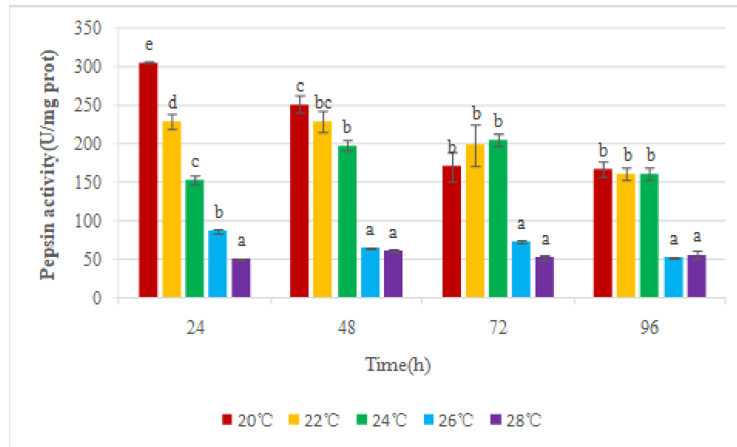

Fig.9 Effect of temperature on pepsin activity of larval *L. calcarifer*.

Discussion

Changes in the environment can cause oxidative stress in aquatic animals (Lushchak and Bagnyukova, 2006). The sharp temperature change which leads to metabolic variation combined with the changes of oxygen consumption initiates oxidative stress (Halliwell and Gutteridge, 1989). Oxidative stress is used for describing states where the balance between elimination and generation of reactive oxygen species (ROS) is disturbed in favor of the latter (Jacob, 1995). Responding to these problems, cells subjected to oxidative stress increase antioxidant defenses particularly the so-called antioxidant and associated enzymes (Heise et al., 2006). The consequence of stress events to the organisms depends on the ability of an individual to quickly raise antioxidant potential in response to oxidative stress. Sharp temperature change is regarded one of the main environmental factors that induce fish stress. ROS levels are controlled by collaborative action of antioxidant defense mechanisms under normal conditions (Ching et al, 2009; Jia et al, 2014; Jia et al, 2015; Hegazi et al. 2010). Therefore, activity of antioxidant enzymes may be good indicators of oxidative stress and adaptive responses to clean up the generated ROS. Previous studies have confirmed that acute temperature stress induced the generation of ROS (Chatterjee et al, 2004). In response to the overgeneration of ROS, SOD and CAT provide the first line of defense for eliminating ROS (Farombi et al, 2007). In this study, SOD activity of barramundi was elevated to scavenge excessive ROS after exposure to lower temperature. Furthermore, SOD activity increased dramatically with increased exposure time. For example, the 22°C activity level after 96h exposure was approximately 5-times higher than the control group at 28°C. However, SOD activity was found to decrease at 20°C and 26°C compared with the 28°C group. It is possible that substantial temperature changes cause irreversible damage to SOD enzyme system to fail to scavenge extremely high levels of ROS. In *Trachinotus ovatus*, SOD activity was significantly higher than in the control group larvae after 24h low temperature stress (Liu et al, 2013). In *Zoarces viviparous*, a significantly increasing trend of the specific activity of SOD was observed in the liver under acute low-temperature stress to 1°C (Heise et al, 2006). In *Scophthalmus maximus*, the specific activity of SOD was significantly higher than other groups in the lowest temperature treatment group of 17°C (Guo et al, 2012). The results from our current research together with previous published data demonstrate that the specific SOD activity increased to a high level after acute cold stress, while present at a low level under extreme cold exposure. Results of the present study also suggest that specific activity of SOD in barramundi is triggered by external factors. In the present study, POD enzymeactivity in the 24°C group exhibited a progressive increase followed by a decreased trend, and then reached the highest enzyme activity at 48h. Specific POD activity remained constant and then slightly decreased followed by an increasing trend at 20°C and 22°C. The results suggest that POD enzyme activities are presented at a low level in the early stage of cold exposure, and its activity increases to a high level over time.

Catalase is one of the key antioxidant enzymes for the removal of superoxide anion produced by SOD. Our results show that the catalase enzyme activity exhibited a trend of progressive decrease followed by an increasing trend at 24°C. This pattern of catalase activities in barramundi is similar to *Siganus guttatas*, in which the catalase enzyme activity of the14°C group decreased to a low level and then increased to be significantly higher than the control group (Song et al, 2015). However, other temperature groups of *Siganus guttatas* exhibited a trend of increase and then decrease. Similarly, barramundi showed a trend of a significant increase followed by a sharp decrease at 20°C. The increase of catalase activity may be related to excessive production of ROS under cold stress, which is consistent with the finding that excessive ROS induces the increase of antioxidant enzyme activities (Li et al, 2003).

The GSH-Px activity, an endogenous hydrogen peroxide, can scavenge H_2O_2 with glutathione as a substrate and remove lipid peroxides showing a similar function with catalase (Chatterjee et al, 2004). In *Penaeus monodon*, GSH-Px and catalase activities play complementary roles in the process of scavenging free radicals, but also exhibit a certain degree of competition (Wang et al, 2008). In the present study, the activities of GSH-Px of barramundi were elevated to scavenge excessive ROS after exposure to lower temperature compared to the fish maintained at 28°C, the GSH-Px activity was found to be significantly higher at 20°C. Furthermore, the GSH-Px activities gradually return to the same level as the 28°C group over time. Results of this study suggest that cold stress induced the larval barramundi to produce the GSH-Px activity to scavenge excess ROS. ACP activity widely exists in the organism, can open the phosphate ester bond and modify surface structure of the exogenous substances so that blood cells can modify the exogenous substances more precisely. ACP activity plays an important role in body defense system, immune regulation, ion secretion and other important physiological functions (Foss et al., 2009; Paust et al, 2011). In this study, the ACP activities of larval barramundi were elevated after exposure to lower temperature. This may suggest that individuals produce a higher level of ACP activities to increase metabolic adaptation under temperature stress.

Digestive enzyme activity is a good indicator of digestive capacity and directly reflects the nutritional status of fish (Deng et al, 2010). The increase of digestive enzyme activities is known to be related to enzyme synthesis and secretion in fish (Beccaria et al, 1991). Meanwhile, digestive system developmental changes are associated with food assimilation of fish (Gisbert et al, 2004). In this study, lipase activities were decreased after exposure to lower temperatures, however, were modestly elevated over time. Amylase activity in the28°C group were significantly higher than those of the other groups. In the early stage, the declining of lipase and amylase activities suggest that larval barramundi decreased food intake of fats and starchy foods under sharp lower temperature stress, but as the stress time went on, individuals increased food take of fats to satisfy thermodynamic demands for metabolic requirements and the demand for urgent energy utilization. With the help of secreted hydrochloric acid, pepsin appears to be responsible for the early stage of protein digestion in breaking down the large-chain polypeptides chains in the stomach (Tengjaroenkul et al, 2000). Our results show that the trypsin and pepsin activities were elevated in relation to the decrease of temperatures. Trypsin and pepsin activity affect absorption efficiency and metabolic levels of protein substance (Xu et al, 2011). This may suggest that individuals need to produce more trypsin and pepsin to take in additional proteinaceous food to supply the energy consumed, which is an important strategy to survive under acute environmental stress.

In conclusion, the decrease of temperature significantly affected antioxidant enzymes activity and digestive enzymes of larval barramundi. Larval barramundi exhibited a certain tolerance of low temperature in a short period of time. These results provide evidence to further our understanding of the impact of temperature on this species and can guide barramundi larvae culture practice in the future.

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