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Full Length Research Paper

Effect of high water temperature on growth, survival and antioxidant enzyme activities in the Japanese flounder *Paralichthys olivaceus*

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To investigate the effect of high water temperature on growth performance and antioxidant enzyme activities of Japanese flounder (*Paralichthys olivaceus*), the juveniles were reared at 4 different temperatures: 25°C (control), 28, 30, and 32°C for a period of 18 days. The survival rate was 20.83 ± 2.20% at 32°C, which was significantly lower than that at other temperatures ($P < 0.05$). The water temperature significantly affected the specific growth rate (SGR), feeding rate (FR), and feed conversion efficiency (FCE) of juveniles. A quadratic regression model was fit to describe the relationship between the SGR, FR, FCE, and the water temperature (SGR: $r^2 = 0.94$; FR: $r^2 = 0.94$; FCE: $r^2 = 0.86$; $P < 0.01$). Additionally, superoxide dismutase (SOD) and catalase (CAT) activities in the liver were significantly influenced by high water temperature. Bivariate analysis revealed that the SOD and CAT activities at different temperatures were highly associated with growth performance of juveniles. The results of present study indicated that the temperature of 32°C may be the upper limit of Japanese flounder. The high water temperature has a significant effect on growth performance and antioxidant enzyme activities of the Japanese flounder and results in poor health condition of the Japanese flounder.

Key words: *Paralichthys olivaceus*, high water temperature stress, survival, growth, antioxidant enzymes.

INTRODUCTION

The Japanese flounder *Paralichthys olivaceus* is one of the most important marine culture species and widely cultured throughout the coastal areas of northern China. Because of the great economic benefits, aquaculture of this species has expanded to the coastal waters of southern China, that is, to regions such as Zhejiang, Fujian, and Guangxi Province, in recent years (Wang et al., 2005; Lin et al., 2005). However, the farming of the Japanese flounder in the southern regions has been thwarted by summer mortality for several years, thereby causing extensive economic losses (Lin et al., 2005). Similar patterns of mortality have also been reported for the hard clam (*Meretrix meretrix*) (Ho and Zheng, 1994), Zhikong scallop *Chlamys farreri* (Xiao et al., 2005), and Pacific oyster *Crassostrea gigas* (Degremont et al., 2007). In summer, the seawater temperature in southern China can increase up to 32°C for several weeks. The high water temperature is hypothesized to be one of the most important environmental factors that influences the summer mortality of the Japanese flounder by affecting its physiology (reproduction, energetic metabolism, growth, and immunity) (Seikai et al., 1986; Zheng et al., 2004; Lin et al., 2005; Dou et al., 2005). Water temperature is one of the most important factors affecting survival, growth, feeding, and other physiological performances in aquatic ectotherms (Van Weerd and Komen, 1998; Martinez-Alvarez et al., 2005; Mendez et al., 2009). Generally, growth increases with rising temperature. However, an increase in the water temperature beyond the optimum limits of a particular species accelerates the metabolic rate and subsequently increases oxygen consumption in fish. This adversely affects fish health and even results in death (Van Weerd and Komen, 1998). The increase in tissue oxygen consumption may also stimulate the production of reactive oxygen species (ROS), which may cause oxidative stress (Lesser, 2006). As an adaptive response

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to challenging environmental conditions, the cells subjected to high temperature increase their antioxidant defenses, particularly the antioxidant and associated enzymes (Martinez-Alvarez et al., 2005). For example, these ROS can be detoxified by an antioxidant defense-system, which includes the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and glutathione S-transferases (GST). Recent data suggest that changes in the levels of antioxidant enzyme activities can be used as possible biomarkers in some aquatic organisms (Taoka et al., 2006; Lushchak and Bagnyukova, 2006). These biomarkers can reflect the health of fish as a response to various environmental stressors (Suja et al., 2009; Cao et al., 2010).Previous investigations on the Japanese flounder, however, have focused on the effect of temperature on embryonic and larval development or on the optimal temperature for the growth of fry and juveniles (Seikai et al., 1986; Iwata et al., 1994; Fonds et al., 1995; Dou et al., 2005). Information regarding the performance of juveniles under stress induced by high water temperature is absent (Kikuchi and Takeda, 2001). In the present study, we examined the growth, feeding, and survival of juveniles reared at four temperatures (25, 28, 30, and 32°C) to investigate the effect of water temperature on growth performance of the Japanese flounder. Additionally, the activities of immune biomarkers, SOD and CAT, were measured to evaluate the health conditions of the Japanese flounder in response to temperature.

MATERIALS AND METHODS

Fish and rearing conditions

Juvenile fish (3-month-old) had been spawned in April 2009, from wild-caught and captive-reared broodstock from Zhoushan coastal water (Zhejiang Province, China). Temperature experiments were conducted at the Key Laboratory of Mariculture and Enhancement, Marine Fishery Institute of Zhejiang Province. The juveniles were reared in a semi-recirculation system consisting of 30 fiberglass tanks (diameter: 1.5 m, water volume: 700 L). Juveniles was maintained at 23°C and acclimatized in the laboratory for a week before exposure to experimental temperatures. The temperature shift was controlled within 1°C.

Experimental design

Juveniles exposed to 25°C were treated as control groups. Juveniles reared at 28, 30, and 32°C were treated as high temperature stress groups. The fish were maintained in their tanks (n = 40 per tank) separately at test temperatures of 25, 28, 30, and 32°C. The water temperatures were controlled using submersible heaters and temperature controllers (Xinlian, China) and the temperature shift of each treatment was held within 0.5°C. Temperatures were also manually checked twice a day. There were five replicates per treatment, i.e., 20 experimental units. Of the 5 replicates in a treatment, 3 were used to measure survival and growth performance, while the other 2 were used for sampling to measure SOD and CAT activities. For the experiments, the water temperature was increased at a rate of 1°C per day to reach treatment temperatures. After achieving the desired test temperatures, fish were maintained in their respective temperatures for 18 days, and then, the temperature of all treatments was recovered to 25°C.

During the experimental period, approximate two thirds of the water in the tanks was changed with preheated water every time, and the water exchange was carried out twice a day. Tanks were cleaned daily, and any dead fish were removed and weighed. The dissolved oxygen content was maintained above 6.0 mg L^{-1} by continuous aeration using 2HP centralized air blower. Other conditions were as follows: pH 7.8 to 8.2, and salinity 28 to 30 gL^{-1}.

At the beginning of the experiment, fish were starved for 24 h. Total length and body weight measurements were performed on all fish in each tank. Fish averaged 46.21 ± 10.77 g in body weight and 16.53 ± 1.34 mm in total length, and there was no difference in mean initial body weight and initial total length among the fish in the various tanks (Body weight: $F_{3,8} = 3.21$, $P > 0.05$; Total length: $F_{3,8} = 2.67$, $P > 0.05$).

Feeding procedure

The fish were fed a commercial pelleted diet to apparent satiation twice a day (at 0900 and 1500). Uneaten food was collected after 40 min and dried to determine feed intake.

Sampling

Sampling was performed on the 7, 13, 19, and 23rd days, that is four days after water temperature had returned to 25°C. Three individuals from each tank were randomly selected for tissue sampling. Hepatic tissue were removed immediately and frozen at -80°C prior to analysis.

Enzyme assay

Hepatic tissue samples were thawed, washed with physiological saline solution (0.9% NaCl), and weighed. Then, they were homogenized in 10 mM Tris–HCl buffer (pH 7.4) to give a 10% homogenate by using double glass homogenizer immersed in an ice water bath. The remaining homogenate was centrifuged at 16,000 g for 10 min at 4°C. The supernatant fluid was used for determination of enzymatic activity.

All the enzyme activities were determined according to the instruction on the assay kits (Nanjing Jiancheng, China). SOD activity was measured as previously described by Bagnyukova et al. (2007). One unit of enzyme activity (U) was defined as the amount of enzyme exhibiting 50% inhibition of the auto-oxidation rate of 0.1 mM pyrogallol in 1 ml solution. CAT activity was measured as previously described by Cao et al. (2010). One unit of CAT activity is defined as the amount of enzyme that catalyzes the degradation of 1 µmol of H_2O_2 per min and specific activity corresponding to 1 µmol transformation of substrate (H_2O_2) per minute per milligram protein. SOD and CAT activities were expressed in U mg^{-1} protein.

Calculations

Growth performance was evaluated as %WG, %LG, the specific growth rate (SGR, $\%day^{-1}$), feeding rate (FR, $\%day^{-1}$) and food conversion efficiency, which were defined as:

$\%WG = 100(\text{final weight} - \text{initial weight})/\text{initial weight}$,

$\%LG = 100(\text{final total length} - \text{initial total length})/\text{initial total length}$,

$SGR\ (\%\ day^{-1}) = (\ln W_t - \ln W_0) \times 100t^{-1}$

$FR\ (\%\ day^{-1}) = I_d\ (t \times (W_t + W_0)/2)^{-1}$

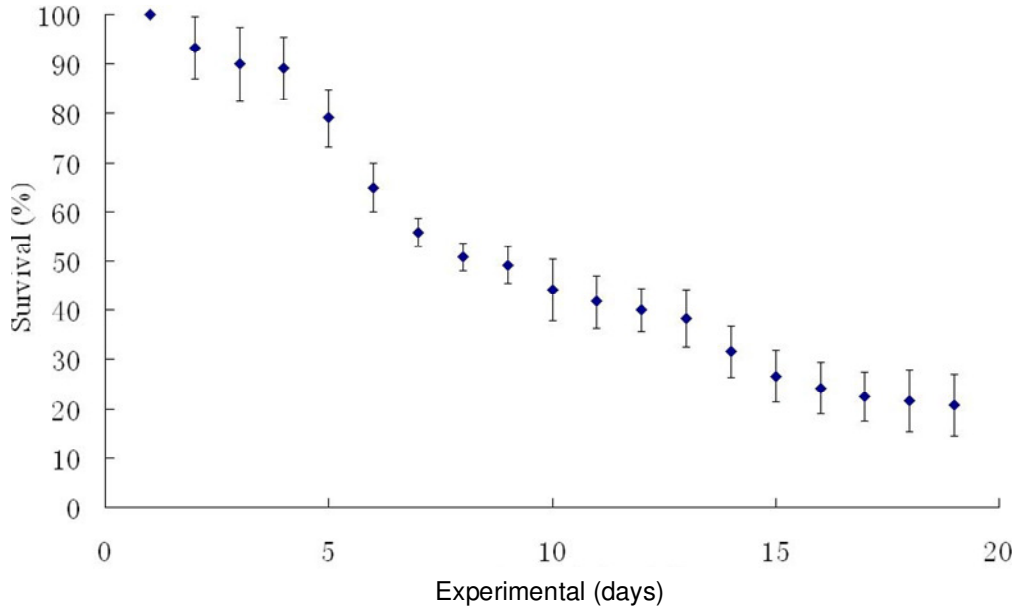

Figure 1. The survival of Japanese flounder rearing at 32℃ during the experimental period.

Table 1. Comparisons of survival and growth performance in Japanese flounder at different treatments.

	25℃	28℃	30℃	32℃
Survival (%)	100[a]	100[a]	100[a]	20.83 ± 2.20[b]
Final body weight (g)	83.17±18.33[a]	72.94±20.07[b]	61.24±12.22[c]	46.29±12.83[d]
Final total length (cm)	19.26±1.39[a]	18.58±1.51[b]	17.73±1.40[c]	16.27±1.66[d]
Percentage weight gain (WG, %)	82.22±7.26[a]	47.34±2.27[b]	39.04±5.25[c]	-7.00±1.77[d]
Percentage length gain (LG, %)	15.80±0.46[a]	9.78±0.24[b]	6.92±0.71[c]	-4.73±0.45[d]
Specific growth rate (SGR, %day^{-1})	3.15±0.09[a]	2.16±0.04[b]	1.56±0.06[c]	-0.47±0.01[d]
Feeding rate (FR, %day^{-1})	2.09±0.05[a]	1.73±0.08[b]	1.57±0.03[b]	0.47±0.05[c]
Feed conversion efficiency (FCE, %)	154.61±9.44[a]	122.95±1.29[b]	115.04±11.72[b]	

Different letters in the same row indicate significant difference among treatments ($P < 0.05$).

$FCE\ (\%) = 100 \times (W_t - W_0)\ I_d^{-1}$

where W_0 and W_t are the initial wet weight and final wet weight, respectively. “t” (d) is the experimental duration (days), and I_d is food intake (g?).

Data analysis

Data were expressed as mean ± SE, and subjected to a one-way ANOVA test implemented with SPSS 14.0 software. Significant differences were determined using Tukey’s post hoc tests at the 0.05 level of significance.

RESULTS

Survival

At the end of the 18 day experimental period, there were no mortalities at temperatures of 25, 28, and 30℃. At 32℃, mortality was first observed on the second day and continued until the end of the experiment (Figure 1). The average survival rate was 20.83 ± 2.20% at the end of the experimental period, significantly lower than those in other temperatures ($P < 0.05$).

Growth performance

Compared with initial body weight and total length, an increase in body weight and total length was observed at 25, 28, and 30℃, while a decrease in body weight and total length was observed at 32℃ (Table 1). Significant differences in body weight and total length were found among different temperature treatments (Body weight: $F_{3,8} = 155.41$, $P < 0.01$; Total length: $F_{3,8} = 117.45$, $P < 0.01$). The highest values were observed at 25℃, and

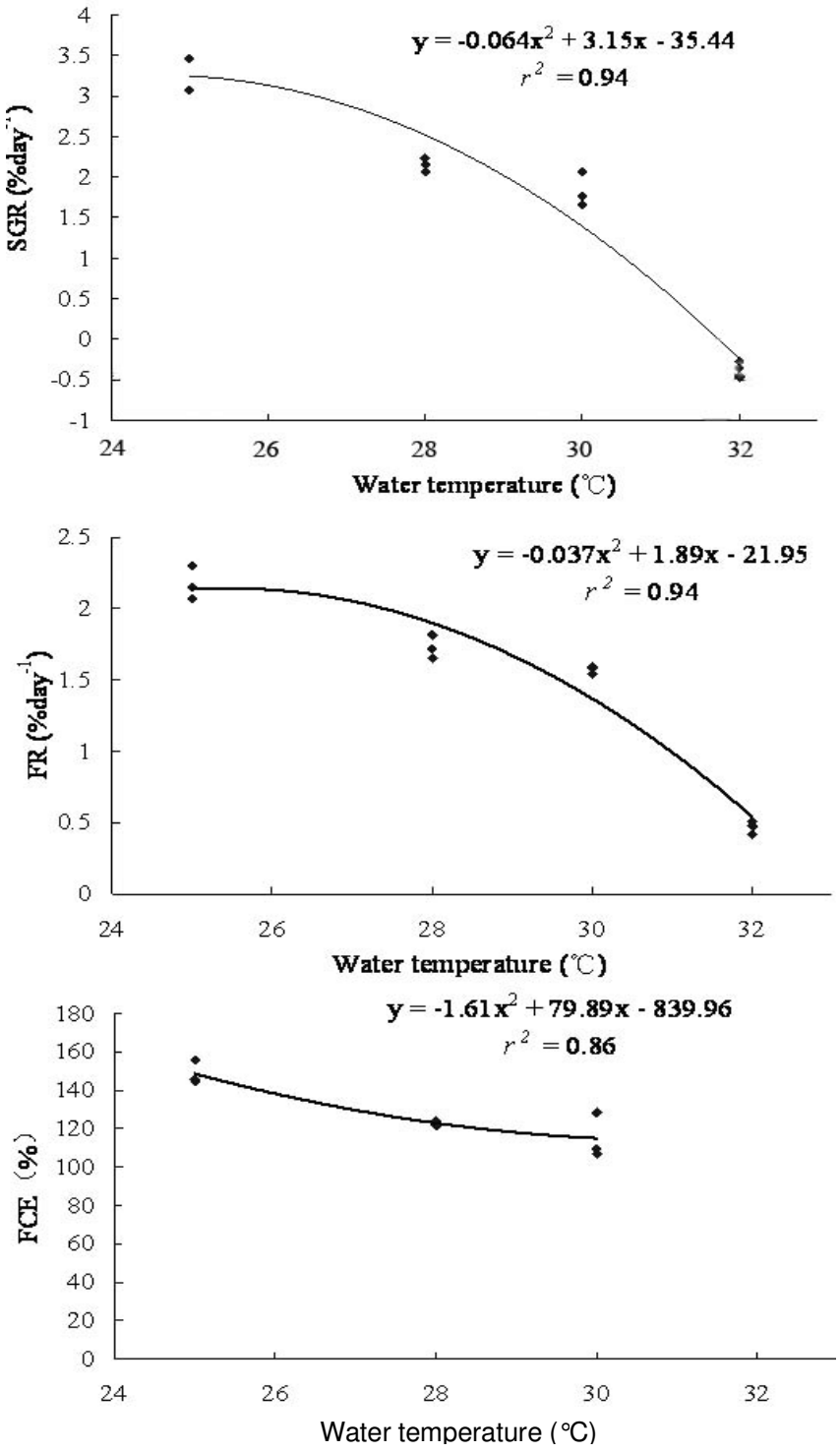

Figure 2. Relationship between the specific growth rate (SGR), feeding rate (FR), food conversion efficiency (FCE), and water temperatures in juvenile Japanese flounder.

the lowest were observed at 32℃, thus, growth of juveniles was significantly influenced by the water temperature. Regression models were fitted to describe the relationships between SGR and water temperature as $SGR = -0.064t^2 + 3.15t - 35.44$ ($n = 12$, $r^2 = 0.94$, $P < 0.01$). The SGR decreased with increasing water temperature in the temperature range of 25 to 32℃ (Figure 2).

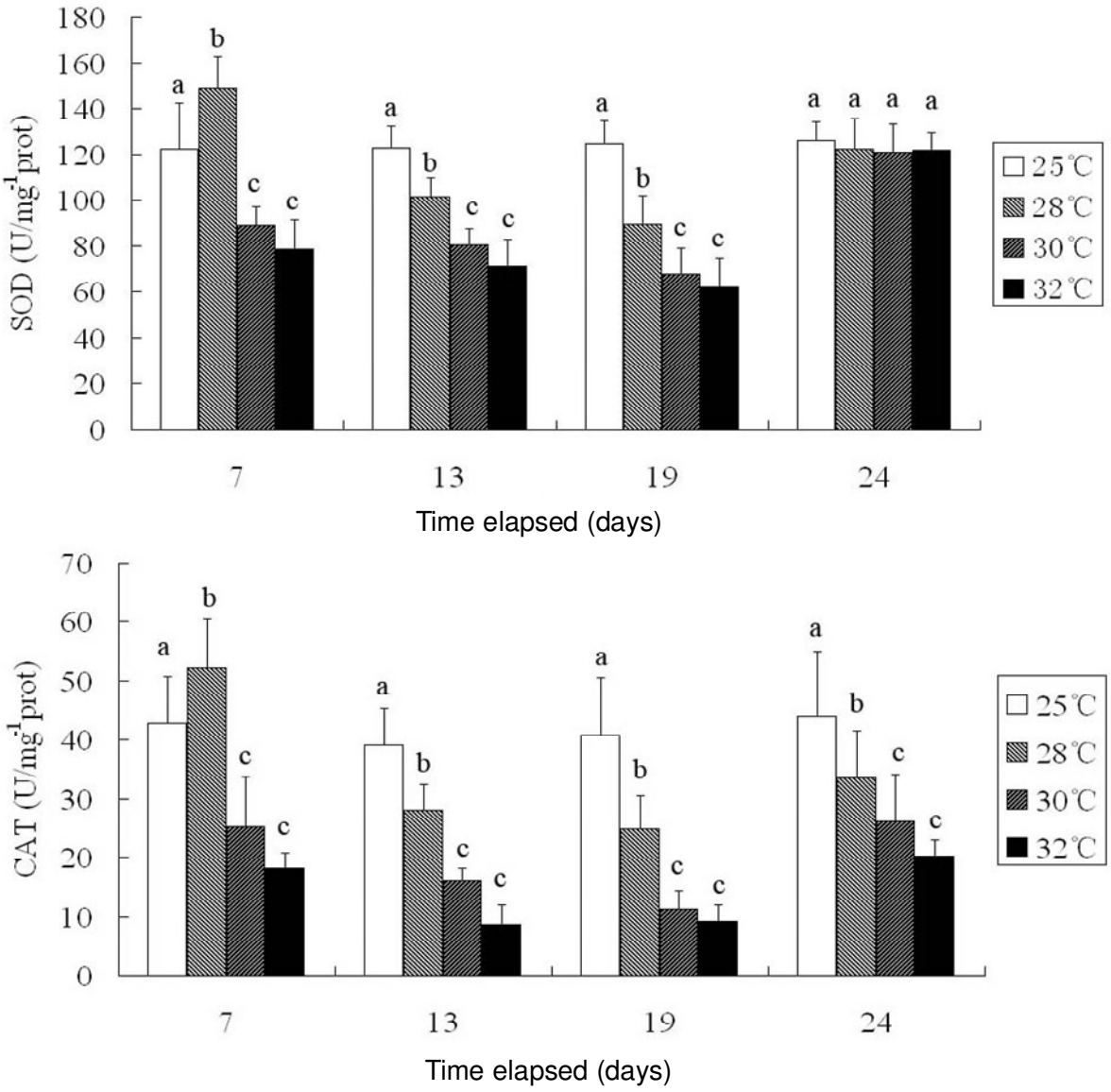

Figure 3. Effects of water temperature on superoxide dismutase (SOD) and catalase (CAT) activities in the hepatic tissue of juvenile Japanese flounder. Different letters indicate significant difference among treatments ($P < 0.05$).

Feeding rate and food conversion efficiency

Feeding rates and food conversion efficiency followed a pattern similar to that of growth. Regression models were fitted to describe relationships between FR, FCE, and water temperature as $FR = -0.037t^2 + 1.89t - 21.95$ ($n = 12$, $r^2 = 0.94$, $P < 0.01$); $FCE = -1.61t^2 + 79.89t - 839.96$ ($n = 9$, $r^2 = 0.86$, $P < 0.01$). The FR and FCE decreased with increasing water temperature within the temperature range of 25 to 32℃ (Figure 2).

SOD and CAT activities

SOD and CAT activities from the hepatic tissue were measured (Figure 3). At 28℃, SOD and CAT activities were significantly higher than those of control on the 7th day ($P < 0.05$). However, SOD and CAT activities decreased on day 13 and 19. At 30 and 32℃, SOD and CAT activities were significantly lower than those of the control through the entire period of the experiment. After the 4 day recovery period, SOD activities of the Japanese flounder in the high temperature treatments increased, and no significant differences were found among the high temperature treatments and controls ($F_{3,20} = 0.15$, $P > 0.05$). The CAT activities in high temperature treatments also increased after the 4 day recovery period. However, CAT activities in the high temperature treatments remained significantly lower than those of the controls ($F_{3,20} = 15.38$, $P < 0.05$).

Table 2. The correlation coefficients between SGR, FR, FCE and SOD activities.

	SGR			FR			FCE	
Day	n	Pearson correlations	P-value	n	Pearson correlations	P-value	n	Pearson correlations	P-value
7	12	0.67	0.02	12	0.69	0.02	9	0.27	0.59
13	12	0.90	0.001	12	0.87	0.001	9	0.88	0.02
19	12	0.84	0.001	12	0.81	0.001	9	0.90	0.001

Table 3. The correlation coefficients between SGR, FR, FCE and CAT activities.

	SGR			FR			FCE	
Day	n	Person correlations	P-value	n	Person correlations	P-value	n	Person correlations	P-value
7	12	0.73	0.01	12	0.74	0.006	9	0.34	0.38
13	12	0.92	0.001	12	0.90	0.001	9	0.87	0.002
19	12	0.84	0.001	12	0.81	0.002	9	0.89	0.001

The data from SGR, FR, FCE and antioxidant enzyme activities at different temperatures were subjected to bivariate analysis using Pearson's correlation coefficient (Tables 2 and 3). The SGR and FR were significantly associated with SOD and CAT activities on the 7, 13 and 19th day ($P < 0.05$). The FCE was also significantly associated with SOD and CAT activities on 13th and 19th day ($P < 0.05$), while not significantly associated on the 7th day ($P > 0.05$).

DISCUSSION

Effect of high water temperature on growth and survival of the Japanese flounder

As described in previous reports, the optimal temperature for the growth of the Japanese flounder ranged from 20 to 25°C (Iwata et al., 1994; Fonds et al., 1995; Kikuchi and Takeda, 2001). The growth rate is expected to decline as temperature exceeds the optimum temperature for growth. Although the experimental period is short, the present study evidently examined the effect of high temperature on growth. The SGR of juvenile Japanese flounder decreased with an increasing water temperature range between 25 and 32°C, thus, water temperature significantly affected the growth of juveniles with the fish kept at the highest temperature experiencing mortality. A higher water temperature imposes two antagonistic effects on growth: increasing temperature has a negative effect due to higher energy cost for maintenance of metabolism, but also a positive effect due to higher efficiency of transforming food energy into net energy. Within the temperature range tolerated by a fish species, growth rate increases with rising temperature, but when the experimental temperature reaches the upper extreme of the tolerated range, growth decreases (Van Weerd and Komen, 1998). A loss of body weight for the Japanese flounder was observed at 32°C. There may be a marked increase in the energy requirements for maintenance above the optimal temperature range and a lower conversion efficiency, which resulted in slower growth.

The present study suggested that juvenile Japanese flounder can survive water temperature as high as 30°C for 18 days, and some can survive a temperature as high as 32°C. The water temperature of 32°C might be close to the upper lethal temperature for the Japanese flounder. This was consistent with the observations of Fonds et al. (1995), who presumed that the temperature of 32°C was the critical temperature for the Japanese flounder. Sakamoto et al. (2005) also suggested that the whole body thermal tolerance for the Japanese flounder was 32°C. Upper temperature tolerances for other species in the order Pleuronectiformes have been reported, for example, 28°C for turbot (*Scophthalmus maximus*) (Gao et al., 2006; Arnason et al., 2009), 31°C for *Paralichthys californicus* (Mendez et al., 2009), and 38°C for southern flounder (*Paralichthys lethostigma*) (Toro-Silva et al., 2008). This information is valuable for fish culture.

Effect of high water temperature on antioxidant enzyme activities

SOD and CAT play important roles in detoxifying harmful reactive oxygen species (ROS). SOD catalyzes the conversion of O_{2^-} and H^+ into less reactive species, molecular oxygen and H_2O_2. H_2O_2 produced by SOD is sequentially reduced to water and oxygen to prevent oxidative stress. Many species increase activities of antioxidant enzymes to prevent oxidative damage in response to high temperature stress (Martinez-Alvarez et al., 2005). For instance, goldfish responded to temperature-induced oxidative stress by increasing

activities of SOD when exposed to heat stresses (Lushchak and Bagnyukova, 2006). In the present study, SOD and CAT activities at 28°C increased in the early stage as well, which were probably induced by the high water temperature to deal with a high presence of ROS. However, activities of antioxidant enzymes in juveniles decreased at a later stage. Similar findings have been reported by An and Choi (2009) in the ark shell, in which exposure to heat stress increased activities of antioxidant enzymes by inducing mRNA expression compared to controls in the early stage. However, continued stress damaged tissue and led to a decrease in antioxidant enzyme activities (An and Choi, 2009). We suggested that juveniles were unable to suppress ROS induced by continued stress, followed by a decrease in the antioxidant enzyme activities (Chen et al., 2007). At the temperature treatments of 30 and 32°C, activities of antioxidant enzymes decreased throughout the high-temperature period. The loss of enzymatic antioxidant activity might be related to disturbances in protein synthesis or heat-induced antioxidant enzyme denaturation (Heise et al., 2006; An and Choi, 2009). When the high temperature stress was released, the fish was able to repair its antioxidant defense-system (Martinez-Alvarez et al., 2005; Lesser, 2006; Lushchak and Bagnyukova, 2006). In the present study, the SOD activities recovered completely after a 4 day recovery while the CAT activities of juveniles did not completely recover. SOD is the first and the most important defense line among the antioxidant systems and Downs et al. (2001) suggested that SOD was more sensitive to environmental stresses.

Possible implications of high water temperature for summer mortalities of the Japanese flounder

The results of present study revealed that the high water temperature significantly affected the growth and antioxidant enzyme activities of the Japanese flounder. Juveniles grew slowly and mortality was high under high temperature stress, which was consistent with poor antioxidant enzymatic activities. Activities of antioxidant enzymatic such as SOD and CAT correlate well with immune competence and are appropriate biomarkers to evaluate environmental effects on fish (Martinez-Alvarez et al., 2005; Cao et al., 2010). In summer, it is suspected that the overall health of the Japanese flounder would be compromised, and the likelihood of being affected by pathogens might increase during this period. Although several causes, such as environmental stress, infectious disease and genetic inbreeding, are hypothesized to account for summer mortality (Zheng et al., 2004; Lin et al., 2005; Xiao et al., 2005), the high seawater temperatures are likely to be amongst most important factors responsible for the death of the cultured Japanese flounder in summer.

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