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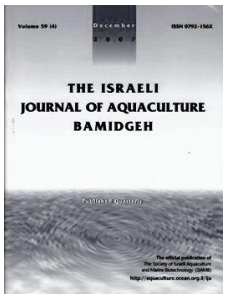

The Effect of Water Temperature on Antioxidant and Digestive Flexibility in Miiuy croaker, *Miichthys miiuy*

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Keywords: *Miichthys miiuy*; temperature; antioxidant enzyme; digestive enzyme

Abstract

Miichthys miiuy is a marine fish with high commercial value that is extensively cultured in China. Seawater temperature is an important factor that affects the cultivation of this fish. However, whether and how seawater temperature affects enzymatic activity during cultivation remains unclear. We treated *M. miiuy* with average body weight of 232.59 ± 48.79 g with four seawater temperature treatments (13.0, 20.0, 24.0, and 30.0 °C) for 12 days, and measured activities of liver antioxidant enzymes (SOD and CAT) and intestine digestive enzyme (lipases, amylase, and trypsin) at the beginning and end of the treatment. Temperature had a significant effect on the activity levels of antioxidant enzymes. SOD activity decreased from 13.0°C to 24.0°C, and increased at 30.0°C, where SOD activity was highest in samples placed in 30.0°C seawater, and lowest at 24.0°C. There were no clear trends in the CAT activity pattern with increasing temperature, and highest CAT activity levels were observed at 20.0°C and lowest levels were observed at 24.0°C. The activity rates of digestive enzymes were also significantly affected by temperature. However, there were no clear trends in activity pattern with increasing temperature, similar to CAT. Lipase activity was highest at 24.0°C, and amylase activity was highest at 24.0°C. Trypsin activity was similar across the different temperature treatments after 12 days of culturing. The present study investigated the effects of seawater temperature over time on the activities of antioxidant and digestive enzymes in *M. miiuy*, and the results will help develop better culture practices for *M. miiuy*.

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Introduction

Temperature affects metabolic functions of most organisms (Viña. 2002). This is especially important for ectothermic organism such as fish (Via et al., 1998), as it can directly affect the rate of biological processes, such as development (Qu et al., 2012, Hu et al., 2017, Argüello-Guevara et al., 2017, Guevara-Fletcher et al., 2017), immune function, and susceptibility to pathogens (Heidari et al., 2017). Temperature changes lead to stress responses in fish, altering the activity rates of antioxidant and digestive enzymes. Superoxide dismutase (SOD) serves as the primary defense against antioxidants in animals (Akhtar et al., 2012), and fish respond to temperature-induced oxidative stress by increasing SOD activity when exposed to heat stress (Lushchak and Bagnyukova, 2006), and elevated SOD activity leads to higher dismutation of O_2^- to H_2O_2, which in turn stimulates the activity of the H_2O_2 scavenger enzyme catalase (CAT) (Hermes-Lima, 2004). It has been reported that environmental stress leads to changes in SOD and CAT activities in *Paralichthys olivaceus* (Lou et al., 2011) and *Pampus argenteus* (Yin et al., 2011). Digestive enzymes are an important link between ingestion and assimilation (Bowyer et al., 2012), and different classes of enzymes catalyze reactions with different types of substrates. Main digestive enzymes include lipase, amylase, and trypsin, and all three enzymes are important for food utilization and growth of fish. Enzyme activities are influenced by temperature; testing the effects of abiotic factors on enzyme kinetics provides insights into how basal metabolic processes respond to a changing environment. Many studies focusing on enzyme activities of fish at different temperatures have been reported (Miegel et al., 2010, Zhang et al., 2016, Jiang et al., 2016).

Miichthys miiuy (Basilewsky) is a marine fish endemic to the western Japan Sea and the East China Sea (Xu et al., 2010). It is extensively cultured for consumption in China and has high commercial value (Hong et al., 2003, Lou et al., 2004). Enzyme activity in fish is affected by temperature as mentioned above, however, whether *M. miiuy* respond similarly to shifts in temperature is largely unknown. In the present study, one-year old *M. miiuy* were exposed to four different temperatures (13.0, 20.0, 24.0, and 30.0°C) for 12 days and the activity levels of liver antioxidant enzymes and intestinal digestive enzymes were measured.

Materials and Methods

Fish and experimental design.

The experiment was carried out on Xishan Island located in Zhoushan (Zhejiang, China). Healthy *M. miiuy* with an average individual weight of 232.59 ± 48.79 g were obtained from the Dengbu Aquatic Company in Zhoushan (Zhejiang, China). Fish were acclimated to laboratory conditions in 18 cylindrical fiberglass aquariums (1 m^3) for 7 days prior to the experiment under flow-through sand-filtered seawater conditions at 13°C. The stocking density of each aquarium was 20 *M. miiuy* per aquarium. Four levels of temperatures, 13.0, 20.0, 24.0, and 30.0°C, were tested in triplicate per aquarium. The following conditions were applied during the acclimatization period: pH, 7.7-8.5; salinity, 27.0-29.0 ppt; dissolved oxygen > 5.0 mg/L. During acclimation, fish were fed twice daily at 08.00 AM and 04.00 PM to satiation with a formulated diet (Table 1). About 70% of the water was exchanged after each feeding. Once animals were acclimated, three aquariums of the first group were maintained at 13.0 ± 0.5°C, and the temperatures of the other three groups were increased using electronic thermostats at a rate of 2°C/d until the test temperatures of 20.0 ± 0.5, 24.0 ± 0.5, and 30.0 ± 0.5°C were attained. Once the aquariums reached the experimental temperatures, the temperatures were maintained until the end of the experiment. The tested fish were fed according to their appetite during the tests. The culture conditions of the fish tested were similar as described above for the acclimatization stage.

Tissue sampling and preparation.

Tissue samples were collected at zero days, that is, the time when the aquarium reached the experimental temperature, and after 12 days of treatment at the attributed temperatures. Three *M. miiuy* per replicate were dissected. Prior to sampling, fish were

individually anesthetized in 2% eugenol solution, and then dissected immediately. Livers and intestines were carefully excised, the surfaces of the tissues were dried with tissue paper, thoroughly washed with saline solution (1.2 %, pH = 7.5), then placed in tubes and immediately frozen in liquid nitrogen. Samples were stored at -80°C and used to measure enzyme activities.

Table 1. Composition and nutrient levels of diets used in this study (dry matter basis).

Items	*Content %*
Fish meal	45.00
Soybean meal	20.00
Wheat gluten meal	7.00
Dextrin	9.00
Fish oil	3.67
Soybean oil	3.67
Soybean lecithin	1.00
Vitamin premix [1]	0.40
Mineral premix [2]	0.30
Choline chloride	0.30
Ca(H2PO4)2	0.50
Cellulose	9.16

[1] Vitamin Premix provided the following per kg of diets: thiamine 25mg, riboflavin 36.7mg, VA 32mg, VE 120mg, VD_3 5mg, VK 5.1mg, VC 142mg, pyridoxine hydrochloride 20mg, VB_{12} 0.1mg, biotin 1.2mg, calcium pantothenate 60mg, folic acid 20mg, niacin 200mg, inositol 792mg.
[2] Mineral premix provided the following per kg of diets: $MgSO_4 \cdot 7H_2O$ 1826mg, $FeSO_4 \cdot H_2O$ 119mg, $ZnSO_4 \cdot H_2O$ 76mg, $MnSO_4 \cdot H_2O$ 44mg, $CoCl_2 \cdot 6H_2O$ 2mg, KI 0.8mg, $CuSO_4 \cdot 5H_2O$ 1mg, NaCl 100mg, KH_2PO_4 233.2mg, NaH_2PO_4 137.0mg.

Biochemical assays

Assays were performed using substrates from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Liver antioxidant enzymes (SOD and CAT) activities were assayed following the methods described by Hu et al. (2015), and intestinal digestive enzymes (lipases, amylase, and trypsin) activities were assayed following the methods described by Gao et al. (2009). Results of all enzyme activities were expressed as unit per milligram of protein (U/mg protein).

Statistical analysis.

All analyses were performed using the statistical software SPSS 19.0. Data are presented as means ± SEM (standard error of the mean). One-way analysis of variance (ANOVA) was performed for enzyme activity to test for temperature-effects. When a significant effect of temperature was found, a multiple comparison (Tukey) test was conducted to compare significant differences among treatments. For all analyses, a probability value of $P < 0.05$ was considered significant.

Results

Changes of the Antioxidant enzyme activities.

Seawater temperature significantly affected the activity levels of liver antioxidant enzymes SOD and CAT. We found that among the four groups, SOD activity at day 0 and 12 showed similar tendency of changes when the temperature increased from 13.0 to 30.0°C. SOD activity was highest at 30.0°C and lowest at 24°C. Compared to SOD activity obtained at day zero, SOD activity was higher at 12 days of culture in each group, where a significant difference was observed at 13.0°C and 24.0°C. Therefore, longer exposure to a certain temperature would affect SOD activity (Fig. 1).

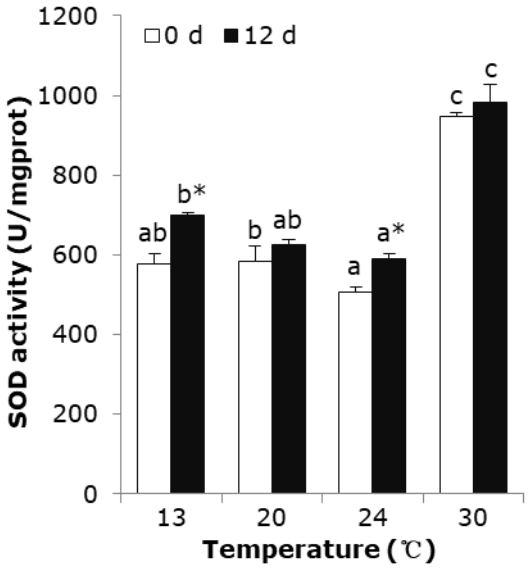

Fig. 1 SOD activity in liver of *M. miiuy* exposed to four different temperatures for 12 days. Values (mean ± SEM; n = 9) with different uppercase letters among treatment days for each temperature indicate a significant difference ($P < 0.05$). * indicates a significant difference in enzyme activities between 0 and 12 days of culture ($P < 0.05$).

CAT activity was similar across all treatments at day 0 of treatment, and activity levels changed significantly after animals were exposed to different temperatures for 12 days. Unlike SOD, changes in CAT activity did not show any obvious trends with increasing temperature. CAT activity was highest at 20.0°C and lowest at 24.0°C, and CAT activity was different between day 0 and 12 for animals exposed to 20.0°C and 24.0°C. Additionally, activity levels of CAT at 12 days of culture were significantly lower than 0 day of culture at 24.0°C and 30.0°C (Fig. 2).

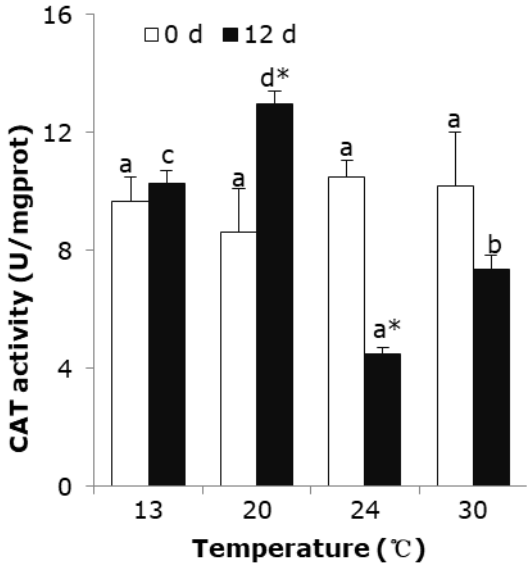

Fig. 2 CAT activity in liver of *M. miiuy* exposed to four different temperatures for 12 days. Values (mean ± SEM; n = 9) with different uppercase letters among treatment days for each temperature indicate a significant difference ($P < 0.05$). * indicates a significant difference in enzyme activities between 0 and 12 days of culture ($P < 0.05$).

Changes of the digestive enzyme activities.

Temperature had significant effects on the activity levels of digestive enzymes ($P < 0.05$). Lipase activity was highest in animals cultured at 13.0°C for 0 days and at 24.0°C for 12 days. Animals exposed to 20.0, 24.0, and 30.0°C for 12 days had higher lipase activity than those exposed to the experimental temperature for 0 days (Fig. 3).

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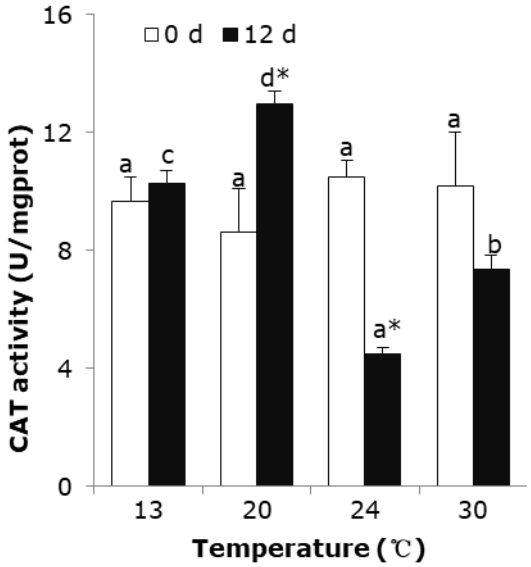

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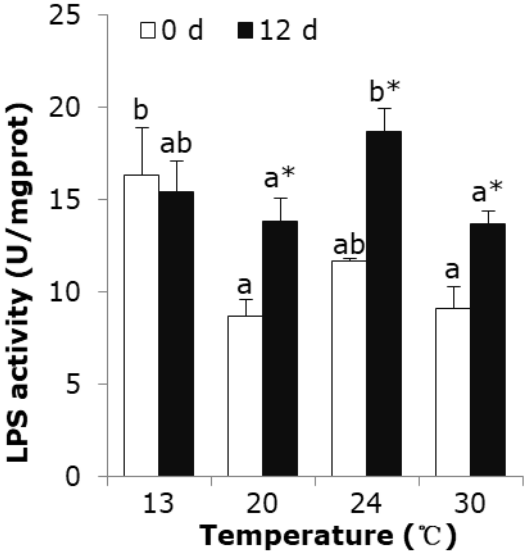

Fig. 3 Lipase activity in intestine of *M. miiuy* exposed to four different temperatures for 12 days. Values (mean ± SEM; n = 9) with different uppercase letters among treatment days for each temperature indicate a significant difference ($P < 0.05$). * indicates a significant difference in enzyme activities between 0 and 12 days of culture ($P < 0.05$).

Intestinal amylase activity was highest at 13.0°C after 0 and 12 days of culture. These time points had significantly higher amylase activity than individuals exposed to 20.0°C and 30.0°C (Fig. 4).

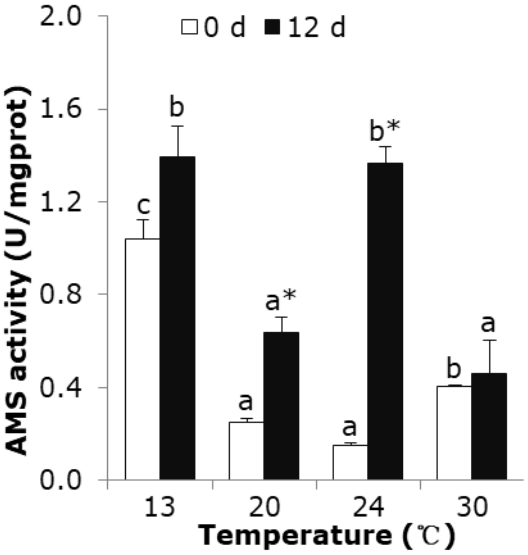

Fig. 4 Amylase activity in intestine of *M. miiuy* exposed to four different temperatures for 12 days. Values (mean ± SEM; n = 9) with different uppercase letters among treatment days for each temperature indicate a significant difference ($P < 0.05$). * indicates a significant difference in enzyme activities between 0 and 12 days of culture ($P < 0.05$).

As with the other digestive enzymes, trypsin activity was also significantly influenced by seawater temperatures (Fig. 5). Trypsin activity was highest in animals exposed to 13°C for 0 day. We did not observe differences in trypsin activity in 12 days cultured animals across the different temperatures. Otherwise, animals exposed to 13.0, 20.0, and 24.0°C for 12 days had significantly higher trypsin activity than those cultured for 0 days.

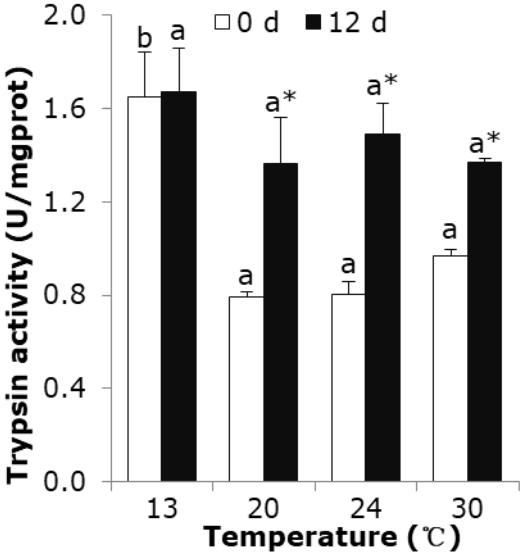

Fig. 5 Trypsin activity in intestine of *M. miiuy* exposed to four different temperatures for 12 days. Values (mean ± SEM; n = 9) with different uppercase letters among treatment days for each temperature indicate a significant difference ($P < 0.05$). * indicates a significant difference in enzyme activities between 0 and 12 days of culture ($P < 0.05$).

Discussion

The aquatic environment presents multiple stressors that can alter the antioxidant profile of organisms, one of which is elevated water temperature (Trenzado et al., 2006). Changes in temperature can either fortify or weaken antioxidant defense systems (Martínez-Álvarez et al., 2005). For example, higher temperatures can lead increased oxidative alteration, where antioxidant enzymes work to reduce damage inflicted by reactive oxygen species (Hu et al., 2015). The changes in antioxidant enzyme reflect the physiological condition of the organism in different environments, therefore, the activity levels of antioxidant enzymes can be used to evaluate the suitability of the environment for fish. Here, we found that the temperature and the duration at which fish were exposed to a particular temperature had significant effects on SOD and CAT activities. SOD activity at 24.0°C was lowest among the four temperatures that were tested. These

suggest that 24.0°C is less stressful than the other three temperatures, since less stressful environment would lower SOD activity. Therefore, 24.0°C is most likely to be the optimum temperature for culturing *M. miiuy*. SOD levels were higher at 13.0°C and 30.0°C than at 24.0°C, suggesting that extreme high and low temperature increases SOD capacity in *M. miiuy*. This observation is in agreement with the report of SOD activity levels in the digestive glands of *Mytilus coruscus* (Hu et al., 2015). CAT activity levels were affected by temperature in the present study. CAT had no obvious regular trend as SOD with increasing temperature, but CAT activity was lowest in samples cultured at 24.0°C for 12 days, further supporting the finding that 24.0°C is the optimal temperature for culturing *M. miiuy* and 13.0 and 30.0°C are not suitable temperatures for long-term culture of this species.

Digestive enzymes play a key role in absorbing nutrients, and their activities correlate with digestive capacity and fish growth rate (Ling et al., 2011). Digestive enzyme activity levels can serve as an indicator of nutrient retention ability, metabolic status, and the degree of adaptation to the environment, thus can be used as biomarkers to monitor the environment in which the organisms are placed (Lai et al., 2011). We found that temperature significantly affected digestive enzyme activities. Among the three digestive enzymes investigated in this study, the activity levels of lipases of fish cultured at 24°C were significantly higher than the other temperatures, which may have been caused by increased secretions of digestive enzymes at optimal culture temperatures. Both low and high temperatures can alter ion concentrations and pH and inhibit digestive enzyme activity and metabolism in fish (Li et al., 2011). Amylase of fish cultured at 13.0 and 24.0°C was also significantly higher than the other two temperatures. Amylase activity of fish cultured at 13.0°C was high, and future studies are needed to explore the cause of this change. Trypsin activity levels were not changed significantly in animals cultured for 12 days at different temperatures, and this differed from the findings with *Apostichopus japonicus* reported by Gao et al. (2009).

Optimum culturing temperatures can lead to improved survival, growth, and production of fish (Choa et al., 2010, Tsuji et al., 2014), which is crucial for maximizing growth and nutrient retention, and decreasing feed waste (Handeland et al., 2008; Ye et al., 2011). Based on our analysis of the activity levels of liver antioxidant enzymes and intestinal digestive enzymes, the optimum temperature for culturing *M. miiuy* was 24.0°C.

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