Hematological parameters and antioxidant responses in olive flounder *Paralichthys olivaceus* in biofloc depend on water temperature

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A B S T R A C T

The purpose of this study was to ascertain the optimum water temperature for breeding juvenile *Paralichthys olivaceus* in biofloc. Hemoglobin and hematocrit were significantly decreased when the temperature was higher than 28 °C. Plasma calcium, glucose, cholesterol, glutamic-oxaloacetic transaminase, and glutamic pyruvic transaminase were significantly elevated at high temperatures, whereas total protein was substantially lower. Superoxide dismutase and glutathione-S-transferase activities in the liver and gills were significantly elevated at high temperatures, whereas glutathione was significantly lower. This indicates that temperatures greater than 26 °C induced hematological changes and oxidative stress in the juvenile *P. olivaceus* in biofloc. We ascribe these changes to thermal stress.

1. Introduction

Biofloc technology, a sustainable aquaculture technique, is an alternative system for reducing environmental pollution (Kim et al., 2015). The technology can regenerate the quality of the breeding water without the need for exchange, because it uses microorganisms to remove ammonia and nitrite that occur naturally in fish farms (Kim et al., 2018a, 2018b). It is more economical than standard aquaculture practices, because it reduces the cost of feed, by improving the efficiency of protein utilization by fish (Schryver et al., 2008). In addition, it is cost-effective compared with recirculating systems, with lower energy demands (Crab et al., 2012).

Water temperature is one of the most important environmental factors for fish. Fish are thermophiles, and among various environmental factors that affects them, they are most sensitive to water temperature (Cheng et al., 2017, 2018). Water temperature affects their distribution, physiology, behavior, and most reproductive processes including maturation, ovulation, spawning, hatching, embryogenesis, gamete development, and spermiation (Narum et al., 2013; Kim et al., 2017a). Temperatures that are higher than optimal can induce physiological stress in fish, which is reflected in metabolic changes (Kim et al., 2017b). The increase in water temperature reduces the amount of oxygen available to fish, thereby inducing respiratory stress, in the from of hyperventilation and increased oxygen exchange in the gill surface (Lewis et al., 2012). Therefore, thermal tolerance and biological responses to temperature are considered key parameters in determining whether fish survive temperature challenges (Perez-Casanova et al., 2008a). Considering the importance of water temperature in fish, it is essential to establish standard indicators for fish.

Hematological parameters are reliable indicators of physiological stress caused by thermal stress in fish (Lermen et al., 2004). High temperature causes a reduction in blood oxygen transport and in the oxygen utilization ratio in fish (Portner, 2002). In addition, thermal stress causes an increase in the oxygen affinity of hemoglobin molecules and in the oxygen-carrying capacity of blood, and this alters the hematological indicators (Lewis et al., 2012). Many authors have used the blood parameters of fish exposed to environmental stressors to determine the toxic effects of these stressors on fish (Cheng et al., 2017; Zaragoza et al., 2008).

Sudden changes in water temperature in a short time can induce oxidative stress in fish, by causing oxygen limitation beyond critical water temperatures, resulting in increased oxygen radical generation and metabolic rate. Under hypoxia, oxygen radical generation is increased via elevated auto-oxidation of haem groups in mitochondria or hemoglobin (Portner, 2002). Thermal stress, especially under acute exposure, worsens oxidative stress by reducing the ability of fish to detoxify reactive oxygen species (ROS) or to recover from oxidative damage (Madeira et al., 2013).

Olive flounder *Paralichthys olivaceus* is a dominant aquaculture fish species, accounting for more than 50% of South Korean fish production, because of its good taste and texture. However, the mortality rate of olive flounder has reached more than 40% per year since 2005, and is

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major impediment to sustainability in the aquaculture industry. In particular, the olive flounder immune system becomes weakened at high water temperature in the summer, making it more vulnerable to diseases, which leads to mortality. Biofloc technology is an innovative method to prevent such mass mortalities in summer. It has the advantage of reusing the breeding water, thereby facilitating water temperature control. Considering the reported steady increase in average global water temperatures, techniques that control the water temperature, such as Biofloc, are needed. Biofloc technology has been applied in commercial aquaculture since the 1980s, but it is still underdeveloped (Azim and Little, 2008). Biofloc application in fish aquaculture has been limited to some freshwater fish species with relatively high environmental tolerance such as Nile tilapia, *Oreochromis niloticus* (Martins et al., 2017), African catfish, *Clarias garienpinus* (Dauda et al., 2018), and common carp, *Cyprinus carpio* L. (Bakhshi et al., 2018). However, biofloc application studies have not been previously conducted for marine fish aquaculture. Such studies are needed to discover ways to make biofloc technology more sustainable. Therefore, our aim was to investigate the effects of thermal stress on hematological parameters and antioxidant responses in juvenile olive flounder raised in biofloc. Further we aimed to determine optimal aquaculture conditions for use in biofloc-based aquaculture facilities.

2. Materials and methods

2.1. Ethics approval and consent to participate

The participants in this study was trained in animal protection, animal welfare and animal experimentation conducted by the National Institute of Fisheries Science. All experimental animals used in this study were maintained under a protocol approved by the Institutional Animal Care and Use Committee of the National Institute of Fisheries Science.

2.2. Experimental fish

Juvenile *Paralichthys olivaceus* (mean weight 27.96 ± 3.07 g and mean length 13.4 ± 1.4 cm) were obtained from a local fish farm in Chungnam, Korea. Fish were acclimated in 500 L circular tanks with bio-floc breeding environment for 3 weeks. The *P. olivaceus* (10 fish) were stocked in each tank, and a total of 120 fish were stocked in the 12 tanks in six water temperature groups with duplicate. The composition and environment of bio-floc breeding water refer to Kim et al. (2018a, 2018b). Water temperature (20, 22, 24, 26, 28, and 30 °C) was set at a ratio of +1 °C/day (Acclimated at 20 °C, and raise water temperature until their temperature). Water temperature was constantly maintained and automatically measured using heater with temperature sensor. Fish in each treatment tank were fed a diet (Extruded pellet) at a rate of 2% body weight daily (divided into two meals per day). The chemical components of bio-floc conditions used in the experiments are demonstrated in Table 1. Water quality was measured using YSI (YSI-EXO2, YSI Inc., USA) for pH and salinity and assay kits (Merck & Co., Inc., NJ, USA) for ammonia, nitrite, and nitrate. Breeding water was static manner to maintain the water temperature. After exposure periods (1 and 2 weeks), fish blood sampling and dissection (120 fish) were

Table 1
The chemical components of bio-floc conditions used in the experiments.

Item	Value
pH	7.74 ± 0.16
Salinity (‰)	32.1 ± 0.3
Ammonia (mg/L)	0.42 ± 0.13
Nitrite (mg/L)	0.56 ± 0.21
Nitrate (mg/L)	121.7 ± 15.6

conducted to analyze hematological parameters and antioxidant responses. No mortality was observed at the respective temperature groups during the exposure periods. At the end of each period (at 1 and 2 weeks), fish were anesthetized using MS-222 (Sigma Chemical, St. Louis, MO), and the blood and tissues were collected.

2.3. Hematological parameters

Blood samples were collected through the caudal vein of the fish in 1-ml disposable heparinized syringes. The blood samples were kept at 4 °C until the blood parameters were completely studied. The hemoglobin (Hb) concentration and hematocrit (Ht) value were determined immediately. The Hb concentration was determined using Cyan-methemoglobin technique (Asan Pharm. Co., Ltd.). Blood was added into the color reagent and calculated the absorbance compared to the absorbance of the standard solution. Absorbance is measured after 5 min at 540 nm. The Ht value was determined by the micro-hematocrit centrifugation technique. Hematocrit was centrifuged at 12,000 rpm for 10 min in a micro-hematocrit centrifuge (VS-12000, Korea), and then the microtiter was measured using a micro-hematocrit reader.

2.4. Plasma components

The blood samples were centrifuged to separate plasma from blood at 3000 g for 5 min at 4 °C. The plasma samples were analyzed for inorganic substances (calcium and magnesium), organic substances (glucose, cholesterol and total protein), and enzyme activities (glutamic oxalate transaminase (GOT), glutamic pyruvate transaminase (GPT), alkaline phosphatase (ALP)) using clinical kit (Asan Pharm. Co.,Ltd.) (Kim and Kang, 2017a). Plasma calcium is measured by the principle of forming chelate compounds of OCPC (Orthocresolphthalein complexone), a chelating agent, in an alkaline solution. Plasma magnesium produces a magnesium complex in a reddish color with xylidyl blue. Plasma glucose reacts with enzymes and water by the action of glucose-oxidase to become gluconic acid and hydrogen peroxide, and this hydrogen peroxide condenses phenol and 4-aminoantipyrine by peroxidase action, forming a red color. Plasma cholesterol was measured by the principle that ester cholesterol in plasma is hydrolyzed to free cholesterol and fatty acid by cholesterol esterase. Plasma protein was measured by using alkaline copper ions to cause plasma to form complexes and to show bluish purple color. Plasma GOT and GPT were analyzed by Kind-king technique. Plasma ALP was analyzed by a method in which a certain amount of phenyl-phosphoric acid was added to the plasma with alkaline buffer.

2.5. Antioxidant responses

Liver and gill tissues were excised and homogenized with 10 vol of ice-cold homogenization buffer (0.1 M PBS buffer) using Teflon-glass homogenizer (099CK4424, Glass-Col, Germany). The homogenate was centrifuged at 4 °C 10,000 g for 30 min under refrigeration.

Superoxide dismutase (SOD) activity was measured with 50% inhibitor rate about the reduction reaction of WST-1 using SOD Assay kit (Dojindo Molecular Technologies, Inc.). One unit of SOD is defined as the amount of the enzyme in 20 μl of sample solution that inhibits the reduction reaction of WST-1 with superoxide anion by 50%. SOD activity was expressed as unit mg protein^{-1}.

*   WST-1   =   2-(4-lodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2*H* -tetrazolium, monosodium salt.

Glutathione-S-transferase (GST) activity was measured according to the method of Kim and Kang (2017b). The reaction mixture consisted of 0.2 M phosphate buffer (pH 6.5), 10 mM GSH (Sigma) and 10 mM 1-chloro-2,-dinitrobenzene, CDNB (Sigma). The change in absorbance at 25 °C was recorded at 340 nm and the enzyme activity was calculated as nmol min^{-1} mg protein^{-1}.

Reduced glutathione (GSH) was measured following the method of Kim and Kang (2017b). Briefly, 0.2 ml fresh sample supernatant was added to 1.8 ml distilled water. Three ml of the precipitating solution (1.67 g metaphosphoric acid, 0.2 g EDTA and 30 g NaCl in 100 ml distilled water) was mixed with supernatants. The mixture was centrifuged at 4500 g for 10 min. One milliliter of supernatant was added to 4.0 ml of 0.3M NaHPO4 solution and 0.5 mL 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) was then added to this solution. GSH was measured as the difference in the absorbance values of samples in the presence and the absence of DTNB at 412 nm, and the value was calculated as μmol mg protein^{-1} in the tissues.

2.6. Statistical analysis

Statistical analyses were performed using the SPSS/PC + statistical package (SPSS Inc.). Significant differences between groups were identified using one-way analysis of variance (ANOVA) and Tukey's test for multiple comparisons. The significance level was set at $P < 0.05$.

3. Results

3.1. Hematological parameters

The hematological parameters such as hemoglobin and hematocrit of *P. olivaceus* exposed to the different levels. The hemoglobin of *P. olivaceus* was significantly decreased at 30 °C after 1 week and over 28 °C after 2 weeks (Fig. 1). The hematocrit was substantially decreased over 28 °C at 1 week and over 26 °C at 2 weeks. The effect on hematological parameters of high temperature was more prominent at 2 weeks than 1 week exposure (Fig. 1).

3.2. Plasma components

In the inorganic components, the calcium of *P. olivaceus* was significantly increased over 28 °C after 1 and 2 weeks, but no change in magnesium was observed. In the organic components, the glucose of *P. olivaceus* was significantly increased over 28 °C after 1 week and over 26 °C after 2 weeks (Fig. 2). A significant increase in the cholesterol of *P. olivaceus* was observed in the concentration of 30 °C after 1 week and over 28 °C after 2 weeks (Fig. 2). The total protein of *P. olivaceus* was substantially decreased over 28 °C after 2 weeks (Fig. 2). In the enzymatic components, the GOT of *P. olivaceus* was significantly increased at 30 °C after 1 week and over 28 °C after 2 weeks (Fig. 2). The GPT of *P. olivaceus* was significantly increased over 28 °C after 1 week and over 26 °C after 2 weeks (Fig. 2). But, there was no significant change in the ALP of *P. olivaceus* by bio-floc water temperature (Fig. 2). The differences on the plasma components at 2 weeks such as glucose,

cholesterol, total protein, GOT, and GPT were more significant than those at 1 week exposure.

3.3. Antioxidant responses

Changes of antioxidant responses (SOD, GST, and GSH) in liver and gill of olive flounder, *Paralichthys olivaceus* exposed to the different levels of water temperature in bio-floc environment for 2 weeks are shown in Fig. 3. The SOD activity both in the liver and gills of *P. olivaceus* exposed to the different levels of water temperature was significantly increased in the concentration of 30 °C after 1 week and over 26 °C after 2 weeks. The GST activity in the liver of *P. olivaceus* exposed to the different levels of water temperature in bio-floc environment for 2 weeks was significantly increased over 28 °C after 1 and 2 weeks. The GST activity in the gills of *P. olivaceus* was significantly increased in the concentration of 30 °C after 1 week and over 28 °C after 2 weeks. The GSH level in the liver and gills of *P. olivaceus* exposed to the different levels of water temperature in bio-floc environment for 2 weeks was significantly decreased over 28 °C after 1 and 2 weeks. The liver and gill SOD, gill GST, and gill GSH at 2 weeks were more significant than those at 1 week exposure.

4. Discussion

Temperature affects fish physiology, biochemistry, and behavior, and high temperatures can lead to reduced growth and survival (Perez-Casanova et al., 2008b). Sudden changes in water temperature in a short time cause metabolic stress that adversely affects the health of fish (Ahmad et al., 2011). Such changes alter hematological parameters, such as the levels of erythrocytes and the various plasma components (Roche and Boge, 1996). In addition, hematological parameters can be reliable indicators of fish pathology and physiological status (Barcellos et al., 2004). Qiang et al. (2013) reported that temperatures above or below their optimal temperature affected the hematological parameters of juvenile Nile tilapia, *Oreochromis niloticus*. Erythrocytes and hemoglobin levels in fish are generally lower at temperatures above optimal, leading to reduced oxygen transport in the blood (Zarejabad et al., 2010). Here, we found that, for juvenile *P. olivaceus* raised in Biofloc, hemoglobin and hematocrit were significantly lower at high water temperatures, probably as a result of thermal stress.

Plasma calcium and magnesium are important inorganic plasma components that are essential for cell maintenance and maintaining stable functioning (Kim and Kang, 2016a). In this study, high temperature influenced the calcium ion balance of *P. olivaceus* juveniles raised in biofloc: plasma calcium was significantly elevated, whereas plasma magnesium was unchanged. In contrast, Kim and Kang (2017a, 2017c) reported significant changes in both plasma calcium and
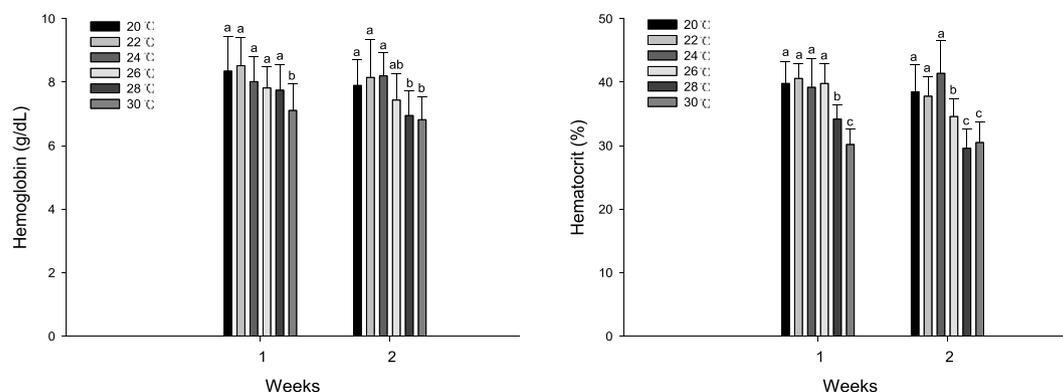

Fig. 1. Hematological parameters of olive flounder, *Paralichthys olivaceus* exposed to the different levels of water temperature in bio-floc environment for 2 weeks. Vertical bar denotes a standard error. Values with different superscript are significantly different at 1 and 2 weeks ($P < 0.05$) as determined by Tukey's multiple range test.
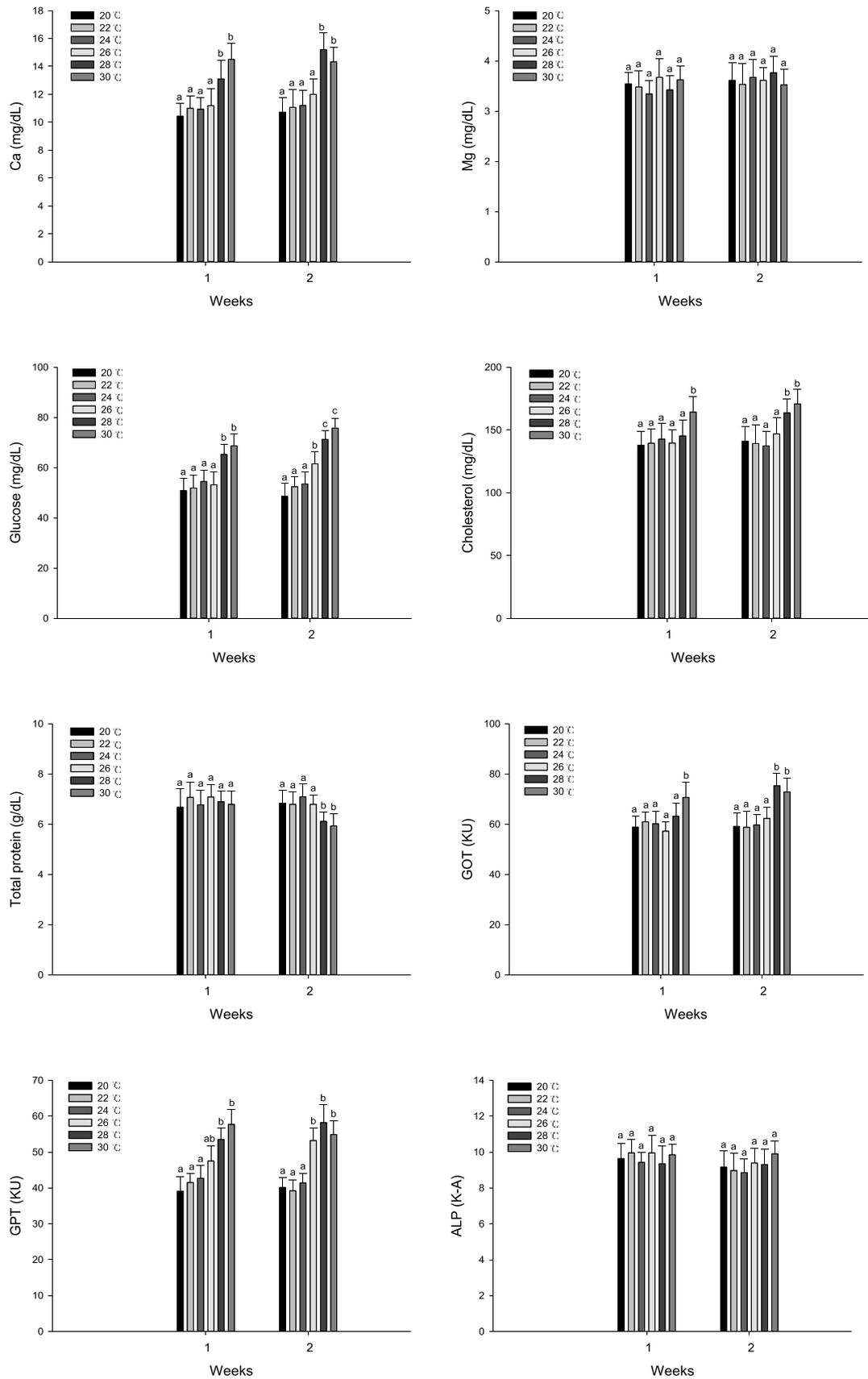
Fig. 2. Plasma components of olive flounder, *Paralichthys olivaceus* exposed to the different levels of water temperature in bio-floc environment for 2 weeks. Vertical bar denotes a standard error. Values with different superscript are significantly different at 1 and 2 weeks ($P < 0.05$) as determined by Tukey's multiple range test.

Fig. 3. Changes of antioxidant responses (SOD, GST, and GSH) in liver and gill of olive flounder, *Paralichthys olivaceus* exposed to the different levels of water temperature in bio-floc environment for 2 weeks. Vertical bar denotes a standard error. Values with different superscript are significantly different at 1 and 2 weeks ($P < 0.05$) as determined by Tukey's multiple range test.
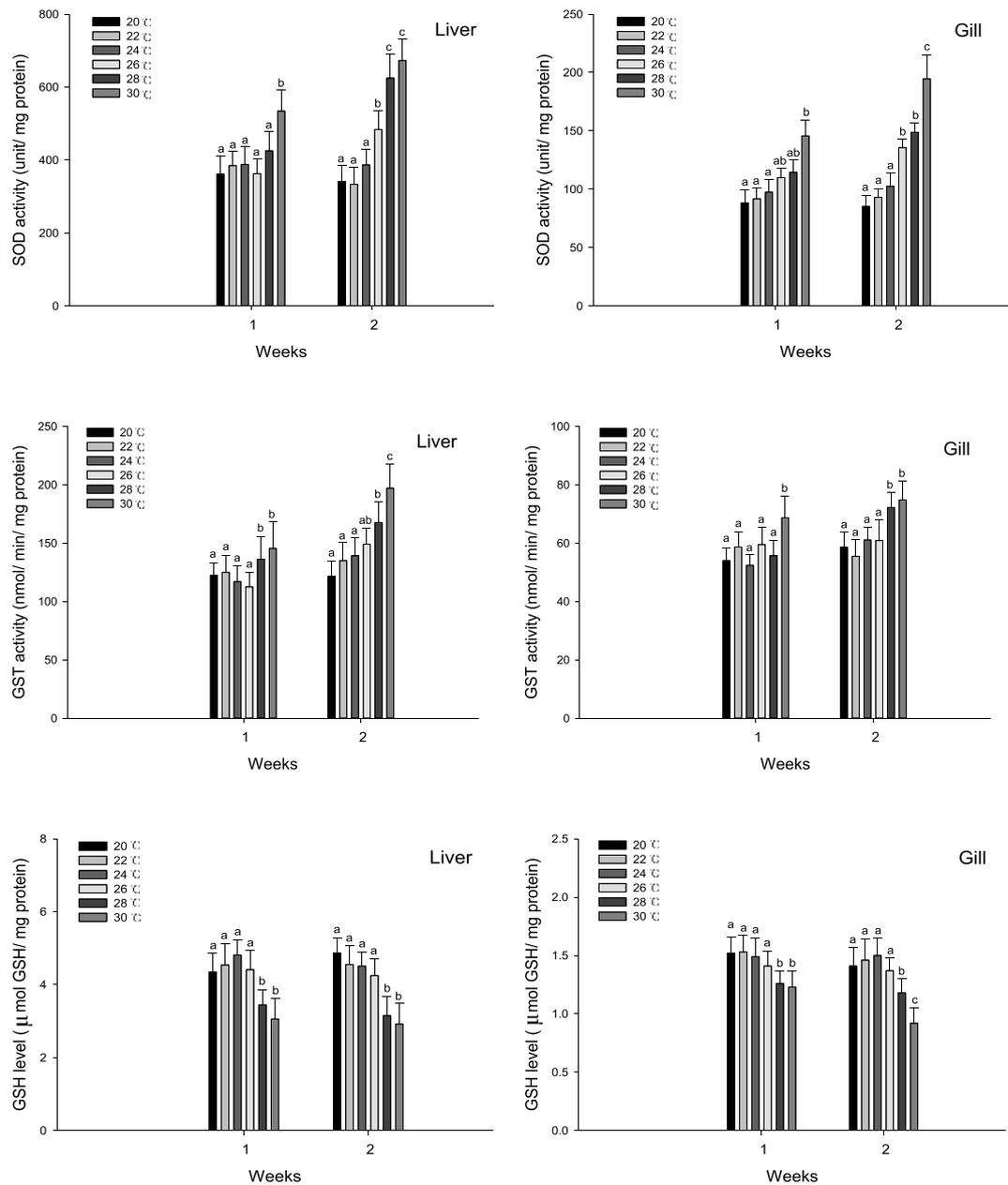
magnesium of rockfish *Sebastes schlegeli* caused by environmental stress.

Plasma glucose is a key indicator in evaluating stress in fish, because elevated glucose is a secondary response to stress (Kim and Kang, 2017c). Perez-Casanova et al. (2008b) reported a significant increase in the glucose level of Atlantic cod *Gadus morhua* L. exposed to high water temperature. The glucose level of *P. olivaceus* was significantly elevated at high temperature in a biofloc environment, in response to thermal stress. Cholesterol levels are also used as stress indicators; cholesterol is a precursor of steroid hormones, is a structural component of cells and forms the outer layer of plasma lipase proteins (Kim and Kang, 2014, 2015a). Srivastava et al. (1995) reported a substantial increase in the cholesterol of catfish *Heteropneustes fossilis* in response to an environmental stressor, malachite green; the hypercholesterolemia reflected liver damage. The cholesterol in *P. olivaceus* was significantly elevated at high temperatures in the biofloc environment, indicating that high

water temperature can induce liver damage.

Plasma protein can be altered by environmental stress, because proteins are used to maintain homeostasis, and for detoxification and tissue repair (Jenkins et al., 2003). In response to stress, fish mobilize proteins as an energy source, through amino acid oxidation. In this study, the plasma total protein *P. olivaceus* was considerably lower at high water temperatures. Additionally, Srivastava et al. (1995) suggested that environmental stressors can damage liver tissue in fish, thereby reducing total plasma protein.

Plasma GOT, GPT, and ALP are used to assess liver and kidney damage, because acid and alkaline phosphatases enter the blood after cell necrosis in tissues (Agrahari et al., 2007). Elevated enzymatic plasma components are widely considered reliable and sensitive indicators of cellular damage (Oost et al., 2003). In our study, plasma GOT, and GPT of *P. olivaceus* were significantly elevated at high water

temperatures, reflecting cellular damage by thermal stress, whereas there was no change in ALP. Similarly, Agrahari et al. (2007) reported significant increases in the GOT and GPT of *Channa punctatus* (Bloch) by environmental stress. We found that water temperatures above 26 or 28 °C altered the blood physiology of *P. olivaceus* in biofloc.

Changes in water temperature can lead to a significant increase in reactive oxygen species (ROS), because they disturb the balance between ROS generation and elimination; this induces cell injury (Lushchak, 2011). Temperature increases in aquatic environments stimulate metabolic pathways in fish in accordance with thermodynamic principles, and induce increased oxygen consumption, thereby causing oxidative stress (Bagnyukova et al., 2007). Fish have antioxidant mechanisms that protect them from cellular damage caused by ROS (Kim and Kang, 2015b). Of various antioxidant responses, fish produce superoxide dismutase (SOD), which is a critical antioxidant enzyme; SOD decomposes the intracellular oxygen free radicals to hydrogen peroxide and molecular oxygen (Kim and Kang, 2015c). In this study, we found that high water temperatures induced elevated SOD activity in the liver and gills of *P. olivaceus*, as a defense mechanism against oxidative stress. Bagnyukova et al. (2007) suggested that SOD activity functions as a critical defense mechanism against heat shock caused by water temperature increase, and reported a significant increase in the SOD activity of rotan *Perccottus glenii* in response to heat stress. Parihar et al. (1997) also reported a significant increase in SOD activity of freshwater catfish *Heteropneustes fossilis* following short-term temperature increase.

Glutathione S-transferase (GST) catalyzes the linkage between xenobiotics or ROS and GSH to produce xenobiotic metabolites for excretion (Kim et al., 2017c). Klein et al. (2017) reported a significant increase in the GST of Antarctic fish, *Notothenia rossii* caused by elevated water temperature. In this study, the GST activity in the liver and gills of *P. olivaceus* was substantially elevated by exposure to high water temperatures. Considering that GST is a critical enzymatic antioxidant in cells, we expect high water temperatures to cause oxidative stress for fish. Glutathione (GSH), a non-enzymatic antioxidant, oxidizes reduced glutathione to oxidized glutathione (glutathione disulfide, GSSG) to remove ROS directly by converting O_2^- to H_2O_2 (Kim and Kang, 2016b). The GSH levels in the liver and gills of *P. olivaceus* were significantly reduced at high water temperature, which may reflect increased GST activity due to its function to activate GST. Kim et al. (2017c) also reported a significant reduction in GSH and increase in GST in *Sebastes schlegelii* as a result of environmental stress.

In general, the antioxidant levels in *P. olivaceus* responded to oxidative stress caused by high water temperature. Temperature increases cause oxidative stress in the fish, by increasing oxygen consumption and ROS production; there is a clear connection between oxidative stress resistance and tolerance of high temperatures (Lushchak and Bagnyukova, 2006). Therefore, the responses of antioxidants should be reliable and sensitive indicators for assessing oxidative stress caused by high water temperatures. They are useful for determining the physiological responses to thermal stress, and tolerance of fish to high water temperatures. In this study, the antioxidant responses of *P. olivaceus* were significantly altered by exposure to high water temperature. This indicates that water temperatures above 28 °C resulted in oxidative stress.

We carried out an experiment to determine the water temperature tolerance limits, optimum water temperature, and stress responses in juvenile *P. olivaceus* in a biofloc breeding environment. The National Institute of Fisheries Science (NIFS), South Korea, suggests that the optimum water temperature for *P. olivaceus* is 21–24 °C, and the optimum breeding water temperature range is from 10 °C to 27 °C in a running water fish culture system (NFRDI, 2006). However, little research has been conducted on the physiological effects of thermal stress in *P. olivaceus* aquaculture. Furthermore, biofloc may have different effects on thermal stress responses, because it changes the composition of the breeding water. The results of this study showed that water temperatures of 28 °C or more altered the hematological parameters

and caused oxidative stress in *P. olivaceus* in a biofloc environment, which indicate that temperatures above 28 °C were stressful to *P. olivacues*. The temperatures we used were not significantly different from the range of optimum aquaculture temperatures used to breed *P. olivaceus* in running water fish culture systems. Our results suggest that the optimal water temperature for breeding *P. olivaceus* using biofloc technology is below 26 °C. The *P. olivaceus* aquaculture industry is threatened by mortality from thermal stress and fish disease. In this study, breeding guidelines were established by confirming the proper temperature range for breeding olive flounder in bio-floc environment, and sustainable rearing standards were established through the limit water temperature of high temperature. There is a need to stabilize the aquaculture industry, and commercialization of biofloc technology may help to do this. This will require ongoing basic research into biofloc technology, and will help to ensure the sustainable future of the *P. olivaceus* aquaculture industry. Based on the results of this study, we will carry out a positive experiment by raising olive flounder in biofloc at a water temperature of 26 °C or less. Further studies should be conducted in the near future to develop breeding guidelines for other important parameters, such as density and salinity.

Acknowledgment

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.04.013.

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