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A comparison of the physiological responses to heat stress of juvenile and adult starry flounder (*Platichthys stellatus*)

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Abstract

Water temperature is the most important factor in fish farming as changes in water temperature cause physiological stress in fish. There have been few studies on physiological responses to high temperatures, which vary with aging. This study investigated the responses of juvenile and adult *Platichthys stellatus* to heat stress. Plasma cortisol, glucose, lactate, and lysozyme levels, antioxidant enzyme activities, and expression of heat shock proteins 70 and 90 (HSP70 and HSP90) of *P. stellatus* were determined at water temperatures of 16, 20, 24, 28, and 30°C. As a result, it was confirmed that several plasma parameters of adult were significantly higher than that of juvenile under heat stress. Plasma cortisol and glucose levels of adult were increased than juvenile at 24 and 28°C. Plasma lactate level of adult were higher than that of juvenile at 28°C. Comparisons of survival and physiological changes showed that juveniles have better thermal tolerance, resulting in a higher cumulative survival rate. Moreover, the relationship between thermal tolerance and HSP gene expression revealed that expression of HSP70 and HSP90 was significantly upregulated at 28°C in both juvenile and adult fish, and HSP70 expression was significantly higher in juvenile fish than in adult fish. It is judged that the adult's HSP70 activity was lower than juvenile, so the demand for plasma parameters for heat response was relatively high, whereas juvenile's HSP70 activity increased at 24 and 28°C, indicating a relatively stable value of plasma parameters. These results indicate that the thermal tolerance of juvenile fish is greater than that of adult fish, based on the differences in plasma parameters and HSP expression. These findings improve our understanding of age-related changes in *P. stellatus* during thermal stress and may help guide the management of fish farms.

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Introduction

Water temperature influences ectotherm physiology (Paaajmans et al., 2013; Hamdan et al., 2015; Payne et al., 2016). Recent increases in water temperature due to climate change have had a profound effect on aquatic ectotherms, including teleosts (Lee et al., 2014; Alfonso et al., 2021). Fish adapt to changes in water temperature by controlling the rate of metabolic activities, although the fish must maintain their biological activity within tolerable thermal limits (Radoslav et al., 2013; Kim et al., 2019). Temperatures above tolerance thresholds can lead to changes in growth rate, enzymatic activities, and metabolic rate, leading to homeostatic overload or failure. Thermal stress exerts adverse effects on immune function and the cellular response of fish; these can be measured using known indicators (Liu et al., 2010; Lu et al., 2016). Thermal stress initially stimulates the hypothalamic–pituitary–internal axis, secretes cortisol into the blood, and initiates primary responses to stress (Wu et al., 2017). Cortisol is the predominant circulating corticosteroid in fish, and stress results in elevated plasma cortisol levels (Barton and Iwama, 1991; Pottinger et al., 1994). As cortisol increases the metabolic rate of fish, it may be involved in an adaptive mechanism to prevent excess energy mobilization during recovery from stress, thereby assisting in regaining metabolic homeostasis (Sathiyaa et al., 2001). Several studies have described plasma cortisol profiles in response to various stressors and the physiologic consequences of elevated cortisol levels in fish (Fevolden et al., 1991; Pottinger and Carrick, 1999; Ruane et al., 2001; Fast et al., 2008; Di Marco et al., 2008; Ramsay et al., 2009). Plasma glucose levels in fish also increase following exposure to stressful stimuli (Pottinger and Carrick, 1999; Barton, 2000; Begg and Pankhurst, 2004; Lowe and Davison, 2005; Mirghaed et al., 2017; Jiang et al., 2017). Induction of glucose production increases the energy demand under high water temperature by regulating carbohydrate metabolic pathways (Hemre et al., 2002; Guillen et al., 2019). Increased levels of lactate in plasma have been observed in flatfish exposed to different stressors such as air exposure, handling, high density, and thermal shock (López-Patiño et al., 2013; Gesto et al., 2016). Lactate is significantly increased under stress conditions and can be used as a stress response marker in Senegalese sole, *Solea senegalensis* (Conde-Sieira et al., 2018). Some of the effects of thermal stress on innate immunity in fish include alterations in lysozyme activity (Fletcher and White, 1976; Muona and Soivio, 1992; Ndong et al., 2007; Cheng et al., 2009). For example, in an experiment with rainbow trout, *Oncorhynchus mykiss* (Möck and Peters, 1990), chronic 2-h transport stress reduced lysozyme activity significantly. Thermal stress can lead to oxidative stress via the production of reactive oxygen species (ROS) and in organisms unable to detoxify the ROS or repair injury (Paital and Chainy, 2016; Paital et al., 2016). ROS damage cells, and to cope with these injuries, cells express antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Yu et al., 2017).

High levels of heat-shock proteins (HSPs) are induced in response to environmental changes such as heat stress to protect both prokaryotic and eukaryotic cells from environmental stresses by acting as a molecular chaperone (Feder and Hofmann, 1999; Voellmy and Boellmann, 2007; Jacob et al., 2017). HSP70 is involved in chronic temperature acclimation and the acute response of organisms to temperature challenge (Pérez-Casanova et al., 2008; Yamashita et al., 2010). HSP90 supports various components of the cytoskeleton and is involved in a variety of cellular processes, including control of apoptosis in the stress response (Csermely et al., 1998; Fu et al., 2011; Wei et al., 2013; Xu et al., 2014). Heat stress upregulates HSP70 and HSP90 expression in teleosts (Iwama et al., 1998; Basu et al., 2002; Kayhan and Duman, 2010; Kelly et al., 2018); therefore, both HSP70 and HSP90 can be useful biomarkers for assessing heat stress in fish (Tedeschi et al., 2015). Many of the functional roles of HSPs are known, but the underlying mechanisms are still unclear. HSPs may also be useful as markers of cellular injury and for diagnostic and therapeutic purposes (Kregel, 2002).

The starry flounder, *Platichthys stellatus*, is an important commercial flatfish in Korea, North China, and Japan. Olive flounder, *Paralichthys olivaceus*, Korea's main aquaculture fish species, has been suffering from a decline in price competitiveness due to mass

production, and *P. stellatus* is emerging as a high-value-added aquaculture target species to replace *P. olivaceus*. The optimum temperature for growth of *P. stellatus* ranges from 15°C to 19°C (Oh et al., 2009; Kim, 2012); feeding and growth are maintained at under 18°C (Byun et al., 2008). Above 24°C, the fish are stressed, with osmoregulatory disturbances (Min et al., 2015). Since 2016, hot summers have repeatedly affected *P. stellatus* aquaculture farms close to the shore on the east coast of Korea, with temperatures climbing to 28°C or higher, inducing long-term stress responses, lowered immunity and mass mortality. As a result, the farms have suffered heavy financial losses. However, during recent high summer temperatures, only adult fish died; juvenile fish survived. Thus, it was hypothesized that there is an age-related difference in the heat resistance of *P. stellatus* at relatively high temperatures. The thermal tolerance of fish may depend on intrinsic factors such as age (Fowler et al., 2009; Turko et al., 2020). Several studies have revealed that juvenile and subadult fishes are often more tolerant of warm conditions compared with larvae (Drost et al., 2016; Moyano et al., 2017) and mature adults (Troia et al., 2015). These studies highlighted the need to evaluate the thermal tolerance of fish species at different stages, and that it is important to understand how heat resistance varies with age to predict the effects of thermal stress in fish populations.

In this study, differences in the physiological responses to heat stress of juvenile and adult *P. stellatus* were investigated. We compared their survival rates and physiological changes using plasma analysis and analyzed the relationship between thermal tolerance and HSP gene-expression patterns.

Materials and Methods

Ethical statement

All procedures were performed in accordance with the guidelines for the ethical treatment of animals and were approved by the institutional animal care and use committee of Mokpo National University (No. 1183; December 17, 2013).

Experimental fish

The experiments were conducted at the Marine Seed fish farm located in Yeosu, Korea. Two experimental groups were examined: 90 *P. stellatus* juveniles with a total length of 122.1 ± 6.47 mm and weight of 28.08 ± 4.8 g, and 90 *P. stellatus* adult fish with a total length of 313.0 ± 21.64 mm and weight of 524.45 ± 64.03 g. The experimental fish were first acclimated to a water temperature of 16°C and salinity of 33 psu for 7 days and were fed twice a day.

Heat stress and sample collection

The fish were housed in three tanks containing 30 juvenile and three tanks containing 30 adult *P. stellatus*. First, the temperature of each tank was elevated at a rate of 1°C per 12 h until reaching the target temperature, 34°C. This temperature was then maintained using thermostatic heaters. Dissolved oxygen was maintained at above 7.0 mg/L by supplying liquefied oxygen during the experiment. The control temperature was 16°C. **Figure 1** shows details of the increase in temperature. Fish survival was checked every 12 h; juvenile and adult fish without opercular movement and with no response to mechanical stimuli were considered dead and were removed from the tanks. Lethal temperature (LT50), defined as the temperature at which 50% of a population dies, was measured based on the method of Stirling (1982) with some modifications (Kilgour and McCauley, 1986). Blood and tissue sampling were conducted at 20, 24, 28, and 30°C. Fish were anesthetized with 100 mg/L tricaine methane sulfonate (MS-222, Sigma, Burlington, MA, USA) before blood samples and tissues were collected. Blood samples were obtained from the caudal vein of six juvenile and six adult fish from each tank using 18G and 23G syringes, respectively. Blood samples were centrifuged at $1000 \times g$ for 10 min. Plasma was collected and stored at -80°C until analysis. After blood collection, the

fish were dissected and kidney tissue was sampled. The specimens were then frozen in liquid nitrogen until RNA extraction.

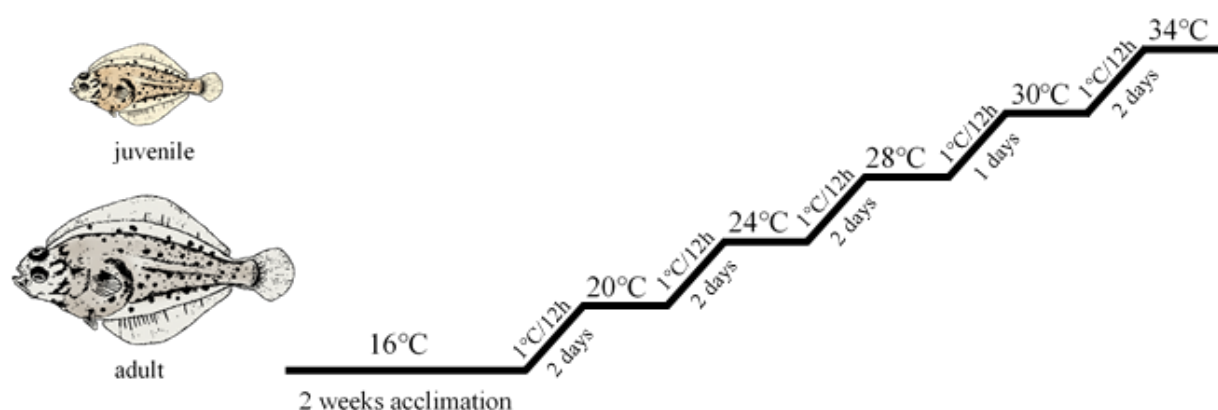


Figure 1 Scheme of the increasing water temperature protocol from 16°C to 30°C in juvenile and adult *Platichthys stellatus*.

Plasma analysis

Plasma levels of cortisol were measured using a Fish Cortisol ELISA kit (MyBiosource, San Diego, CA, USA), and all plasma samples were analyzed in duplicate. For the assay, 50µl of fish plasma sample and 50µl of standard solution were added to per well. 50µl of antibody-HRP-conjugate solution was added to each well except blank, and mixed well, incubated for 1 hours at 37°C. Then, the solution of each well removed and washed three times with 250µl of 1X wash buffer. 50µl of substrate A and 50µl of substrate B solution were added to each well, and incubated for 15 min at 37°C. After added 50µl of stop solution, determined the optical density (OD) of each well within 5 min, using a SpectraMax 190 microplate reader (Molecular Devices, San Jose, CA, USA) set to 450nm. Standard curve constructed and the concentration of samples can be determined by regression analysis.

Plasma levels of glucose were measured using a Glucose Assay Kit (MyBioSource). 50µl of each glucose standard and plasma samples were added into wells, and 50µl of reaction mix solution was added to each well. Then, incubated for 40 min at 37°C protected from light. After incubation, using a microplate reader in the 540-570nm range. The concentration of glucose within samples calculated by comparing the sample OD to the standard curve.

Plasma lactate levels were measured using an L-Lactate Assay Kit (Abcam, Cambridge, UK). 50µl of standard and plasma samples were added to per well, and 50µl of reaction mix including assay buffer, substrate mix, and enzyme mix was added to wells. Then, 50µl of background reaction mix to control wells, and incubated at room temperature for 30 min. The plate measured at OD 450nm in a microplate reader. Average of each standard and samples were subtracted the sample background control, and absorbance values were plotted for as a function of the final concentrations of lactate.

Plasma lysozyme activity was measured using a Fish Lysozyme ELISA kit (MyBiosource). 50µl of standard and samples were added to wells, and 100µl of HRP-conjugate reagent was added to every well except blank wells. The plate was covered and incubated for 60 min at 37°C, and all wells were washed 4 times. 50µl of chromogen solution A and 50µl of chromogen solution B were added to every well, and incubated for 15 min at 37°C. Then, 50µl of stop solution was added to all wells, the OD of the plate was read at 450nm using a microplate reader within 15 min. Average of all standard and samples were subtracted average OD of the blank, and standard curve was made and calculate the level of the analyses.

Antioxidant enzyme activities were analyzed using a Fish Superoxide Dismutase ELISA kit (MyBiosource) and a Fish Catalase ELISA kit (MyBiosource). According to protocol of

SOD assay kit, 50µl of standard and samples were added to per well. And 50µl of antibody-HRP-conjugate solution was added to each well, incubated for 60 min at 37°C. Each well was aspirated and washed using 250µl of wash buffer three times. 50µl of substrate A and B were added to all wells, and incubated for 15 min at 37°C. After added 50µl of stop solution, the OD of each well was determined using a microplate reader set to 450nm. The levels of SOD within samples calculated by comparing the sample OD to the standard curve. Plasma levels of CAT were performed in the same way as the SOD assay kit protocol.

HSP gene-expression analysis

Total RNA extraction and cDNA synthesis

Total RNA was extracted from kidney using the TRIzol® protocol (Invitrogen, Waltham, MA, USA). DNase I (Qiagen, Hilden, Germany) was added to prevent DNA contamination. RNA degradation and contamination were assessed on a 1% agarose gel. cDNA was synthesized from purified total RNA using a Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The synthesized cDNA was quantified using Nanodrop One C (Thermo) and stored at –80°C.

Quantitative real-time PCR

The primers used for quantitative real-time PCR (qRT-PCR) are listed in **Table 1**. qRT-PCR was performed using a Pikoreal 96 RealTime PCR System (Thermo Fisher Scientific) to examine the expression of HSP70 and HSP90 with increasing water temperature. The concentration of cDNA was adjusted to 100 ng/µL. The triplicate 20-µL reaction volumes contained 11 µL SYBR Green PCR Master Mix (Promega, WI, USA), 1 µL each primer (10 pM), 1 µL cDNA, and 7 µL sterile water. The qRT-PCR cycling conditions were pre-incubation at 50°C for 2 min, pre-denaturation at 95°C for 7 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 20 sec. Melting curves were also plotted (60–90°C) to ensure that a single PCR product was obtained for each pair of primers. The expression levels of HSP70 and HSP90 in kidney were calculated relative to those of the housekeeping gene glyceraldehyde3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta C_t}$ method with mean C_t values.

Table 1 Primer sequences used in this study.

Primers	Primer sequence 5' - 3'	Primer efficiency
Hsp70_FW	CGATTGTCTACGGCTTGGAT	97.50%
Hsp70_RV	TCTTCGCTCTCTCACAAGCA	97.50%
Hsp90_FW	GACCAAGGCCGATCTGATAA	95.04%
Hsp90_RV	CTGCTCGTCATCATTGTGCT	95.04%
GAPDH_FW	CCAGAACATCATCCCAGCTT	98.78%
GAPDH_RV	GGCCTTCACAACCTTCTTGA	98.78%

Statistical analyses

For each analysis, the mean and standard error were calculated, and one-way ANOVA, Duncan's multiple range test, and Student's *t*-test were performed using the SPSS statistics program (version 24) to test for significant differences between measurements ($P < 0.05$).

Results

LT₅₀ test

Until the water temperature reached 24°C, there were no significant differences between juvenile and adult *P. stellatus*, but from 28°C to 34°C, the mortality rate of adult was significantly higher than that of juvenile fish. The results for upper LT₅₀ are shown in **Figure 2**. The upper LT₅₀ values for adult and juvenile *P. stellatus* were 28.5°C and 33.5°C.

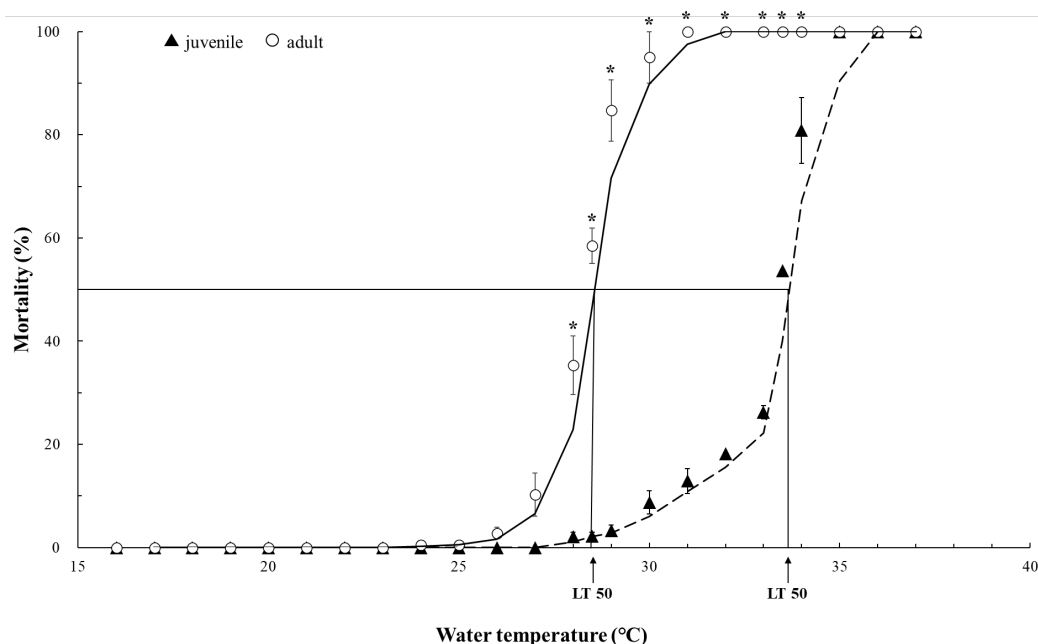


Figure 2 Determination of the 50% lethal temperature (LT₅₀) of juvenile and adult *Platichthys stellatus*. The arrow indicates the LT₅₀. Each mark corresponds to the mean \pm SE of three determinations. Asterisks indicate significant differences between juveniles and adults at that sampling time ($P < 0.05$).

Plasma levels

The biochemical activities following exposure to increasing water temperatures are presented in **Figure 3**. During the experiment, the plasma cortisol levels of adult *P. stellatus* increased significantly at water temperatures of 24°C and 28°C (7.09 ± 0.88 ng/mL and 8.55 ± 0.19 ng/mL, respectively). Those of juvenile fish increased significantly at 28°C (4.98 ± 0.69 ng/mL). In addition, levels in adult fish were significantly higher than in juveniles at 24°C and 28°C ($P < 0.05$).

Plasma glucose levels differed significantly between juvenile and adult fish at water temperatures $\geq 24^\circ\text{C}$, similar to cortisol levels ($P < 0.05$). At 24°C, the glucose level in juvenile fish was 131.77 ± 5.48 $\mu\text{g/mL}$, compared with 298.85 ± 60.15 $\mu\text{g/mL}$ in adult fish. At 28°C, the glucose level was significantly higher in adult fish than in juvenile fish (361.93 ± 98.62 vs. 149.5 ± 14.36 $\mu\text{g/mL}$). At 30°C, the glucose level in adult fish decreased, and there was no longer a significant difference from that in juveniles (130.49 ± 3.69 vs. 115.16 ± 14.87 $\mu\text{g/mL}$).

Plasma lactate levels also differed significantly between juvenile and adult fish at water temperatures $\geq 24^\circ\text{C}$ ($P < 0.05$). At 24°C, the lactate level was 1.08 ± 0.16 nmol/L in juvenile fish and 8.03 ± 0.83 nmol/L in adult fish. The lactate levels of juvenile and adult fish increased rapidly to 7.81 ± 1.1 and 23.22 ± 0.61 nmol/L at 28°C and decreased to 6.15 ± 0.62 and 18.76 ± 1.72 nmol/L at 30°C, respectively.

Plasma lysozyme levels were significantly higher in adults than in juveniles at 20 and 28°C, but significantly higher in juveniles than in adults at 30°C ($P < 0.05$). The lysozyme

concentrations in juvenile and adult fish were 128.22 ± 4.06 and 168.01 ± 5.74 $\mu\text{g/mL}$ at 20°C , 129.81 ± 7.66 and 179.06 ± 10.41 $\mu\text{g/mL}$ at 28°C , and 129.13 ± 4.64 and 100.39 ± 9.45 $\mu\text{g/mL}$ at 30°C , respectively.

Plasma SOD levels were significantly higher in adult fish than in juvenile fish at all temperatures except 20°C ($P < 0.05$). The SOD level in juvenile fish was highest at 35.81 ± 1.38 IU/mL at 20°C and decreased to 2.05 ± 0.7 IU/mL as the water temperature increased to 30°C . The SOD level in adult fish was highest at 61.02 ± 0.95 IU/mL at 24°C and decreased significantly to 29.56 ± 6.37 IU/mL at 30°C .

Plasma CAT concentrations differed significantly between juvenile and adult fish at 16°C (5.1 ± 1.26 vs. 15.0 ± 1.62 U/mL, $P < 0.05$). The CAT concentrations in juvenile and adult fish were highest at 28°C (15.59 ± 1.29 and 18.18 ± 1.14 U/mL, respectively).

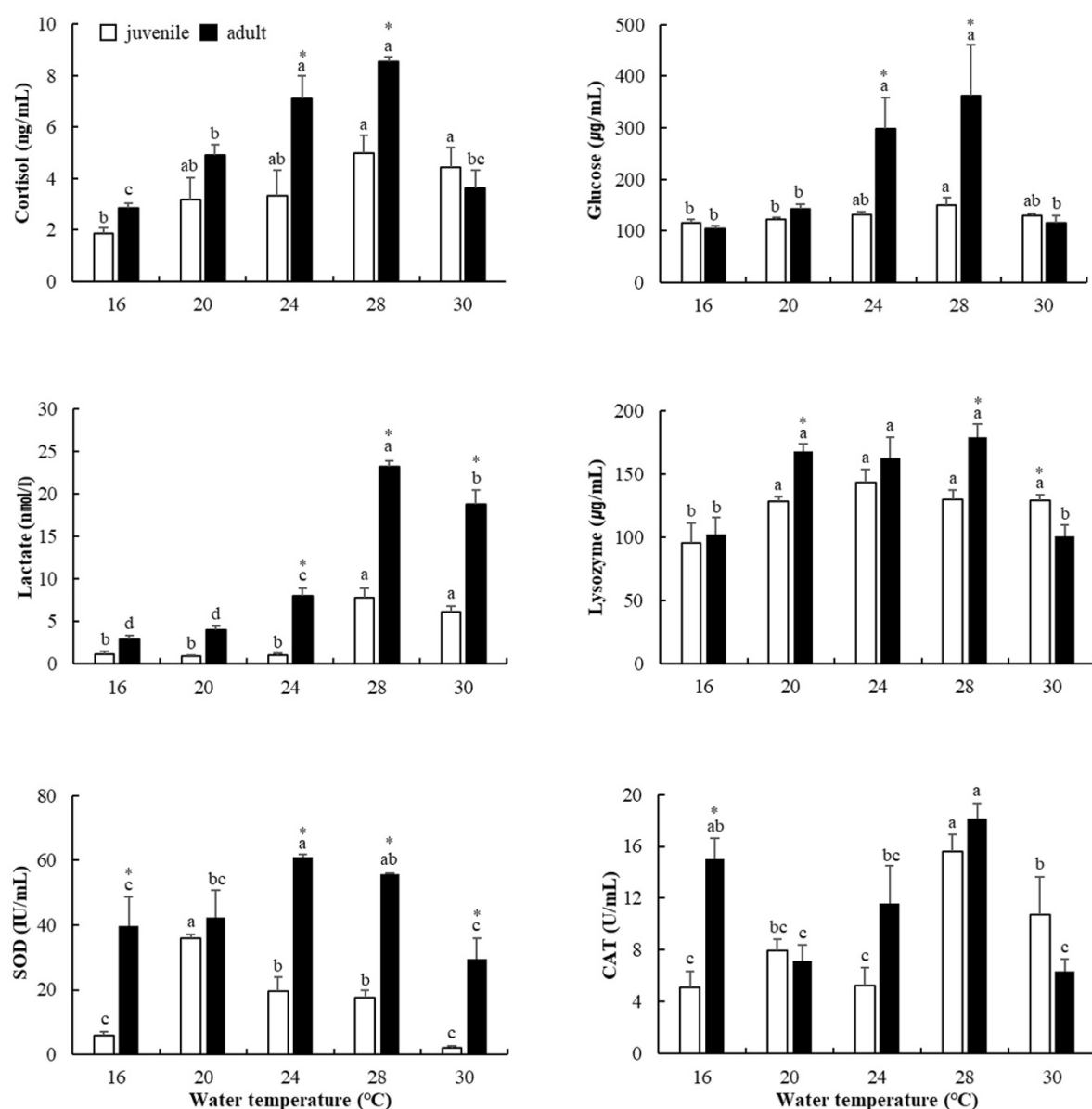


Figure 3 Levels of plasma parameters of *Platichthys stellatus* with increasing water temperatures. Data are presented as means \pm SEM. Bars with different letters indicate significant differences among the water-temperature groups ($P < 0.05$, $n = 6$). Asterisks indicate significant differences between juveniles and adults at that sampling time ($P < 0.05$).

HSP gene expression

The relative gene expression levels of HSP70 and HSP90 according to water temperature are shown in **Figure 4**. Expression of HSP70 in kidney was significantly higher in juvenile *P. stellatus* than in adults at 24, 28, and 30°C ($P < 0.05$). Expression of HSP70 at 28°C, when expression was highest, was 43.5-fold and 74.9-fold higher in adults than in juveniles, respectively. At 30°C, expression of HSP70 was 33.8- and 21.8-fold lower in juvenile and adult fish than that at 16°C, respectively. Expression of HSP90 did not differ significantly between juvenile and adult fish ($P < 0.05$) at any tested temperature. The levels in both juvenile and adult fish increased sharply at 28°C, by 53.1- and 50.7-fold, respectively, relative to levels at 16°C. Expression levels of HSP90 were 8.2- and 13.6-fold lower in juvenile and adult fish at 30°C than at 16°C, respectively.

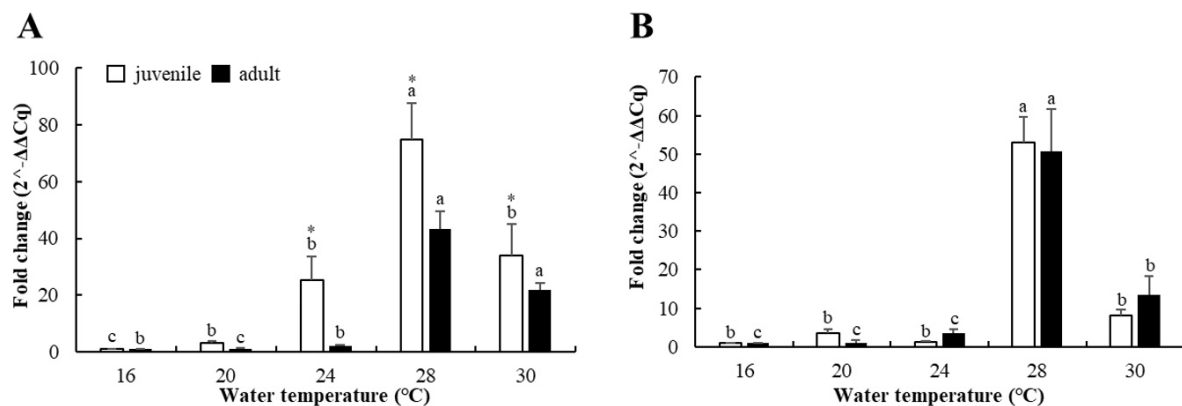


Figure 4 Relative mRNA expression of HSP70 (A) and HSP90 (B) of *Platichtys stellatus* during thermal stress. Data are presented as means \pm SEM. Bars with different letters indicate significant differences among the water-temperature groups ($P < 0.05$, $n = 6$). Asterisks indicate significant differences between juveniles and adults at that sampling time ($P < 0.05$).

Discussion

P. stellatus is an economically important flounder species in East Asia (Min et al., 2015). However, mass mortality frequently occurs during hot summer months, resulting in very heavy losses and hindering the development of the *P. stellatus* aquaculture industry. During periods of high temperature in recent summers, juvenile fish in Korean fish farms survived, whereas large numbers of adult fish died. In this study, a comparative analysis of the physiological responses of juvenile and adult fish underlying thermal tolerance and summer mortality syndrome in *P. stellatus* was conducted. Cultivated organisms have the ability to overcome stress resulting from changes in their external environment to some extent, but above a certain threshold level stress can result in reduced physiological activity (Park et al., 2011). In general, the levels of hepatic markers rise dramatically under stress and are considered indicative of a key response to stress in teleosts (Jia et al., 2020). When fish are exposed to stress, corticotrophin-releasing hormone is secreted from the hypothalamus of the brain to the pituitary gland, and adrenocorticotrophic hormone is secreted from the pituitary gland, resulting in increased cortisol secretion from the adrenal glands (Cockrem et al., 2019). Secreted cortisol acts as a glucocorticoid hormone, which releases glucose by allowing glycogen secretion from the liver (Vijayan et al., 2003). Therefore, blood sugar levels increase and a secondary reaction occurs that uses the released glucose as an energy source to cope with the stress (Faught and Vijayan, 2019). In the current study, the plasma cortisol levels of adult fish began to increase at 24°C, whereas those of juvenile fish did not. Levels in both juvenile and adult fish increased significantly at 28°C, reaching their highest values, followed by a decrease at 30°C. Similar results under heat stress have been found in rainbow trout (Basu et al., 2001; Lewis et al., 2010), milkfish, *Chanos chanos* (Hanke et al., 2019), and

olive flounder (Kim et al., 2019). Patterns of plasma glucose levels were similar pattern to those of cortisol, with adult levels starting to increase significantly at 24°C and peaking at 28°C. Glucose levels were also highest at 28°C in juvenile fish, but were significantly lower than in adults. A recent study in Antarctic fish showed that thermal stress stimulates glycogenolysis to enable acclimatization (Guillen et al., 2019).

Lactate levels increase when blood flow decreases due to illness or shock, resulting in a decreased oxygen supply (Dando, 1969; Milligan and Girard, 1993). Plasma lactate levels were evaluated as primary and secondary responses to stress in Senegalese sole (Conde-Sieira et al., 2018); lactate levels increased significantly in response to thermal stress in olive flounder (Lu et al., 2016) and black rockfish (Song et al., 2019). In the present study, plasma lactate levels increased significantly at 28°C in juvenile and adult *P. stellatus* before decreasing at 30°C. Lactate levels at 24, 28, and 30°C were significantly lower in juvenile fish than in adults. This implies that stress levels were higher in adult fish than in juveniles, at the same temperatures.

Defense against pathogenic infective organisms is first provided by the non-specific immune system, and includes lysozyme activity (Möck and Peters, 1990). Several studies have investigated plasma lysozyme activity to determine the effects of heat stress on the immune systems of fish (Subramanian et al., 2007; Simide et al., 2016; Dawood et al., 2020; Vakili et al., 2021), as lysozyme content is an indicator of non-specific immune function (Jung et al., 2016). Mahmoud et al. (2020) found that in Nile tilapia, *Oreochromis niloticus*, lysozyme activity increased significantly at 32°C but then decreased at 37°C. By contrast, plasma lysozyme activity in goldfish, *Carassius auratus*, decreased significantly following exposure to 30°C (Jung et al., 2016). In the present study, lysozyme activity in both juvenile and adult *P. stellatus* increased significantly from 20°C to 28°C, and was significantly higher in adult than in juvenile fish; the opposite was true at 30°C.

In general, thermal stress can stimulate the production of endogenous ROS, thereby inducing cell injury and antioxidant responses in aquatic organisms (Heise et al., 2003; Kaur et al., 2005; Banh et al., 2016). The antioxidant enzymes SOD and CAT can remove excessive free radicals, thereby minimizing damage to cells (Shin et al., 2010). SOD activity influences the concentrations of molecular oxygen and hydrogen peroxide, the two substrates of the Haber–Weiss reaction, and is therefore likely to be critical for defense mechanisms (Bowler et al., 1992). Hydrogen peroxide is scavenged by CAT and peroxidases, which convert hydrogen peroxide to water and molecular oxygen (Bowler et al., 1992). Recent studies have reported that thermal stress can induce oxidative stress and damage to fish, including sea bream (*Diplodus vulgaris*) (Madeira et al., 2013), puffer fish (*Takifugu obscurus*) (Cheng et al., 2018), olive flounder (Lu et al., 2016), and other fish species (Mueller et al., 2012; Rossi et al., 2017; Klein et al., 2017).

Yang and Yeo (2004) reported that SOD and CAT activities significantly increase in olive flounder as the temperature rises. On the other hand, significant differences in SOD and CAT activities were not observed at high temperatures during chronic heat stress (Liu et al., 2014). In the present study, SOD activity in juvenile *P. stellatus* increased significantly at 20°C and then decreased at 30°C. In adult fish, SOD activity was significantly higher at 24°C, peaked at 28°C, and then decreased. In adult fish, CAT activity differed significantly between 16°C and 28°C, while in juvenile fish activity was significantly higher at 28°C. Considering that the CAT activity in adult *P. stellatus* was high at 16°C, handling during blood collection may have induced stress. Thus, SOD activity was significantly higher in adult *P. stellatus* than in juveniles, and there was no significant difference in CAT activity between adult and juvenile fish. These results indicate that thermal stress elevated cortisol levels in *P. stellatus*, thereby increasing glucose to provide additional energy for metabolism and to maintain homeostasis. In addition, levels of lactate, involved in anaerobic metabolism, increased with increasing water temperature and oxygen consumption. As ROS increased due to environmental stress, the antioxidant enzymes SOD and CAT also gradually increased, with the exception of SOD activity in juvenile *P. stellatus*.

In animals, thermal stress increases levels of HSPs and stress hormones (Iwama et al., 1999; Sharma et al., 2013). HSP family members play a critical role in unstressed and stressed cells as molecular chaperones responsible for the repair of damaged proteins and for the folding of new proteins (Jia et al., 2020). HSP expression increases following cellular stress resulting from exposure to high temperatures and oxidative damage, and has been detected in every organism evaluated (Feder and Hofmann, 1999; Kregel, 2002). Environmental stressors can affect expression of HSP70 and HSP90, which have been used as biomarkers to assess organisms' response to environmental stress (Iwama et al., 2004; Akbarzadeh et al., 2018). In the present study, expression of HSP70 in juvenile *P. stellatus* was significantly higher at 28°C, and then decreased at 30°C. In adult fish, HSP70 expression was significantly higher at both 28 and 30°C. In addition, expression differed significantly between juvenile and adult *P. stellatus* from 24 to 30°C. In both juvenile and adult fish, HSP90 expression was significantly higher only at 28°C, with no significant difference between the two age groups.

Several studies have shown that the response to high temperature varies depending on the age or developmental stage of the fish. Fowler et al. (2009) compared thermal resistance between juvenile and adult rainbow trout and showed that an enhanced heat shock response may contribute to greater thermal resistance in juvenile fish. Meakin et al. (2014) reported that juveniles can cope with high temperatures better than adults, which offers a partial explanation for the movement patterns of fish in nature, in which younger fish inhabit near-shore waters and then migrate to deep water as they mature. The reason for the difference in thermal tolerance with age is deterioration of the HSP system (Wheeler et al., 1995; Verbeke et al., 2001; Tower, 2009), which can react to thermal stress; once sexual maturity is reached, the HSP system may be evolutionarily dispensable, as increased fitness may be attained by spending less energy on protection and more on reproduction (Sørensen and Loeschcke, 2002). In the present study, the physiological response changed, and expression of HSP genes decreased as fish aged, indicating that juvenile fish were more resistant to high temperatures than were adult fish.

Overall, this study demonstrated that the levels of several parameters, including plasma cortisol, glucose, lactate, SOD, CAT, and HSP70/90 expression, increased with thermal stress. In addition, by comparing the physiological response to high temperatures of juvenile and adult *P. stellatus*, we confirmed stronger thermal tolerance in juvenile than in adult fish. These results indicate that adult *P. stellatus* initiate a positive physiological response to cope with thermal-stress-induced damage at 28°C, while low mortality rates were observed in juveniles. As HSP70 expression is more active in juvenile, it is judged that the requirements of plasma parameters related stress were higher due to the slow HSP70 activity of adult *P. stellatus*. In the case of juvenile, HSP70 activity was high, and heat response could appear earlier, so it is thought that the levels of plasma parameters could remain relatively stable. In addition, these data strengthen our understanding of the age-related physiological mechanism of *P. stellatus* during thermal stress and may help guide the management of *P. stellatus* in fish farms. Finally, to further elucidate the differences in heat tolerance between juvenile and adult *P. stellatus*, additional studies are needed, not only to investigate hematological parameters and HSP genes but also the expression of other genes.

Acknowledgements

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Conflict of interests

The authors have declared that no competing interests exist.

Author contributions

HB Lee designed the experiments. HB Lee and IY Lee performed the experiments. JH Yoon and JY Park assisted with the experiments and analyzed the data. HB Lee interpreted the data and drafted the manuscript. HK Lim revised the manuscript and supervised the study design and data analysis. All authors contributed intellectual content to the revisions and approved the final version for publication.

Data availability statement

The data supporting the findings of this study are available upon request from the corresponding author.

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