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Effects of Thermal Stress on the mRNA Expression of SOD, HSP90, and HSP70 in the Spotted Sea Bass (*Lateolabrax maculatus*)

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Abstract – The aim of this study was to elucidate the molecular mechanisms underlying the thermal stress response in the spotted sea bass (*Lateolabrax maculatus*). Spotted sea basses were exposed to 4 different water temperatures (20, 22, 24, and 28°C) in increasing increments of 2°C/h from 18°C (control) for different time periods (0, 6, 12, 24, 48, 72, and 96 h). Subsequently, 3 tissues (liver, muscle, and gill) were isolated, and the levels of SOD, HSP90, and HSP70 mRNA were assessed. SOD mRNA expression was maintained at baseline levels of control fish at all water temperatures in the liver, while muscle and gill tissue showed an increase followed by a decrease over each certain time with higher water temperature. HSP90 mRNA expression increased in the liver at ≤ 24°C over time, but maintained baseline expression at 28°C. In muscle, HSP90 mRNA expression gradually increased at all water temperatures, but increased and then decreased at ≥ 24°C in gill tissue. HSP70 mRNA expression exhibited an increase and then a decrease in liver tissue at 28°C, but mainly showed similar expression patterns to HSP90 in all tissues. These results suggest the activity of a defense mechanism using SOD, HSP90, and HSP70 in the spotted sea bass upon rapid increases in water temperature, where the expression of these genes indicated differences between tissues in the extent of the defense mechanisms. Also, these results indicate that high water temperature and long-term thermal stress exposure can inhibit physiological defense mechanisms.

Keywords – thermal stress, SOD, HSP90, HSP70, *Lateolabrax maculatus*

1. Introduction

Rapid increases in water temperature beyond normal tolerance ranges can cause a suite of physiological changes in fish (Stacey 1984; Hochachka and Somero 2002; Johnston 2006). The global average water temperature has increased by 0.6°C since the late 19th century, and is expected to increase 2~4°C further by 2100 (Metz and Davidson 2007; Pachauri and Reisinger 2007). This has necessitated various studies on the effects of water temperature on a number of fish species (Lushchak and Bagnyukova 2006; Healy and Schulte 2012).

When fish undergo stress as a result of environmental changes, reactive oxygen species (ROS) are generated inside the body (Madeira et al. 2013), and the excessive production of these causes structural changes in nucleic acids and proteins, sometimes resulting in a loss of function and deleterious physiological effects (Pandey et al. 2003). To eliminate these ROS, antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are activated to compensate (Martínez-Álvarez et al. 2005). SOD primarily hydrolyzes ROS into O_2 and H_2O_2, and its presence indicates oxidative damage caused by stress (Kashiwagi et al. 1997; Lu et al. 2015).

When water temperature rises past the normally tolerable range, heat shock proteins (HSPs) are synthesized to maintain protein homeostasis (Sanders 1993). The various types of HSPs are differentiated by molecular weight (Lindquist and Craig 1988). Of these, HSP90—which is expressed in most tissues—is involved in various physiological functions and plays a protective role for proteins (Minami et al. 1991; Jakob and Buchner 1994). HSP70 translocates proteins across cellular membranes and protects neurons from apoptosis (Mailhos et al. 1994; Feder and Hofmann 1999). Both of these HSPs have been used as indicators for various stressors (Cui et al. 2014).

Although numerous studies have shown the effects of water temperature on fish growth (Bermudes et al. 2010),

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maturation (Sudo et al. 2011), and food intake (Bogevik et al. 2010), studies analyzing thermal stress and the physiological mechanisms underlying the response in fish are still lacking. Therefore, our study aimed to examine the thermal stress response of spotted sea bass (*Lateolabrax maculatus*) to increased water temperature by analyzing the mRNA expression of the antioxidative enzyme SOD, as well as the HSPs (HSP90 and HSP70) in liver, muscle, and gill tissues. This study provides basic data for understanding the temporal characteristics of thermally induced defense mechanisms in fish by analyzing the effects of thermal stress over time on the expression of these genes.

2. Materials and Methods

Maintenance of fish and experimental conditions

Spotted sea bass individuals of 13.2 ± 0.5 cm in length and 22.9 ± 1.6 g in weight were maintained in tanks at 18°C for an acclimation period of 30 days. Seawater was filtered using a 10 μl-diameter filter and was ventilated once a day via a peristaltic pump. Fish feed corresponding to 5% of fish weight was provided twice per day.

To precisely maintain water temperature, chillers (SinSung-1100, Sinsungcold Co., Pocheon, Korea) and heaters (OKE-6422H, SEWON OKE Co., Busan, Korea) were installed in all fish tanks used in the experiment. Temperature and dissolved oxygen levels were measured every 30 s using an Oxygen Optode 4531 (AADI, Bergen, Norway). Water temperature deviated from the set temperature of 18°C by no more than ± 0.3°C. Four 300-l fish tanks were used for thermal stress experiments, with each tank containing 20 fish. Fish were starved for one day prior to experiments. The seawater in each tank was ventilated once per day during the experimental period, and salinity was maintained at 31.0 ± 0.5 and pH 7.7 ± 0.1. Dissolved oxygen levels were maintained at ≥ 6 mg/l for all water temperatures to exclude the effects of hypoxia.

Thermal stress experiments

Prior to the experiment, two fish from each fish tank were collected as a control. Then, the water temperature of all tanks was gradually increased from the initial temperature of 18°C by 2°C/h, to reach the experimental temperatures of 20, 22, 24, or 28°C. The 0 h of the experiment was designated as the time at which each tank reached the experimental temperature. Fish mortality was measured at 0, 6, 12, 24, 48, 72, and 96 h, and 2 surviving fish were randomly collected from each tank. The collected individuals were anesthetized using 100 mg/l of MS-222 (Ethyl 3-aminobenzoate methanesulfonate, Sigma Aldrich Co., St. Louis, USA), and liver, muscle, and gill tissues were excised. Isolated tissues were placed in 1.5 ml tubes, fixed using DNA/RNA shield (Zymo Research, Irvine, USA), and stored at −80°C prior to subsequent analysis. This experimental process was repeated three times, yielding a total of 60 fish per each temperature treatment and 240 experimental fish in total.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from the aforementioned tissues using RNAiso Plus (TaKaRa Bio.; Tokyo, Japan) according to the manufacturer's instructions, and was stored at −80°C prior to further analysis. cDNA was synthesized using the PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa Bio., Tokyo, Japan), and was stored at −20°C prior to further analysis.

Sequencing

To analyze the DNA sequence of SOD, HSP90, HSP70, and β-actin, degenerate primers were designed and PCR amplification was performed. Degenerate primers were designed for each gene using the DNA sequences obtained from GenBank (Table 1). After aligning the sequences of each gene, primers were generated using regions with the highest homology. PCR products resulting from degenerate PCR were used to verify the sequences of SOD, HSP90, HSP70, and β-actin.

Real-time PCR analysis of mRNA expression

Real-time RT-PCR was conducted to quantify mRNA expression in each sampled tissue in response to thermal stress. Primers for SOD, HSP90, HSP70, and β-actin were generated using partial sequences of the genes generated by sequencing (Table 2).

To amplify specific products, SYBR® Premix Ex Taq™ (TaKaRa) was used in the following thermal cycling program: 95°C for 30 s; 40 cycles of 98°C for 5 s, 60°C for 30 s; 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. A TaKaRa Thermal Cycler Dice® TP815 (TaKaRa) was used for the amplification and detection of SYBR® Green. Real time RT-PCR measurements were displayed as threshold cycle (CT) values and used to calculate ΔCT. Gene expression levels were measured as a relative value in comparison to the expression levels of β-actin, a housekeeping gene in spotted sea bass, and $2^{-\Delta\Delta CT}$ was used to determine the relative level of gene expression

Table 1. List of degenerate PCR primers used in this study

Primer name	Sequence (5'–3')	Accession number for degenerate primer design
SOD_D_F	GTG GGT TGA AAC TAC TGC AA	AF329278 AJ000249
SOD_D_R	TGG CAA CAT TAT CTG CTC CT	AY613390 KJ558392
HSP90_D_F	CCC TCA TCG ACA CTG GAA TC	AB598553 EU099575
HSP90_D_R	TCA GGT GCA GGA TGA TCT TT	JQ929760 KJ683738
HSP70_D_F	ATC ATC GCC AAC GAC CAG GG	AF053059 FJ429326
HSP70_D_R	GTT GTT GTC CTT GGT CAT GG	KM102660 KT334554
β-actin_D_F	CGA GCT GAG AGT TGC AC	AY148350 AY491380
β-actin_D_R	CAA CGG AAC CTC TCA TTG C	HE57767 JN226150

Table 2. List of real-time PCR primers used in this study

Gene name	Primer name	Sequence (5'–3')
SOD	SOD_R_F	GGT TTC CAT GTC CAT GCT TT
	SOD _R_R	CAT CAT TAG GAC CGG CAT GA
HSP90	HSP90_R_F	ACA CAA CGA TGA TGA GCA GT
	HSP90_R_R	TCA GGT GCA GGA TGA TCT TT
HSP70	HSP70_R_F	CCC AAG GTC CAA GTT GAG T
	HSP70_R_R	AGG TAG GCT TCA GCA ATC TC
β-actin	β-actin _R_F	AGG AGA AGC TGT GCT ATG TC
	β-actin _R_R	AAT GGT GAT GAC CTG TCC G

(Livak and Schmittgen 2001).

Statistical analysis

One-way ANOVA was used to examine the differences amongst the values obtained from the thermal stress experiments, followed by post hoc analysis using Tukey's multiple range test in SPSS version 18.0 (IBM Corp., Armonk, NY, USA) with $p < 0.05$ regarded as significant.

3. Result

Mortality

Mortality at each water temperature is shown in Table 3. All fish survived at 20°C, but mortality increased as the water temperature rapidly increased over a short period of time, and all fish died at 28°C within 24 h. Based on these results, samples were collected from each water temperature before mortality reached 100 %, and mRNA levels of SOD, HSP90, and HSP70 were analyzed.

Table 3. Mortality in response to thermal stress over time in the spotted sea bass

Temp. (°C) / Time (h)	Mortality (%)			
	20	22	24	28
0	0	0	0	0
6				5
12		5	7	50
24		13	33	100
48		28	100	
72		40		
96		100		

SOD mRNA expression

SOD mRNA expression levels in each tissue are shown in Figure 1. In the liver, SOD mRNA expression decreased approximately 0.25~0.5-fold over time at all water temperatures in comparison to the control, but generally exhibited similar expression levels to the baseline of control fish ($p < 0.05$; Fig. 1A).

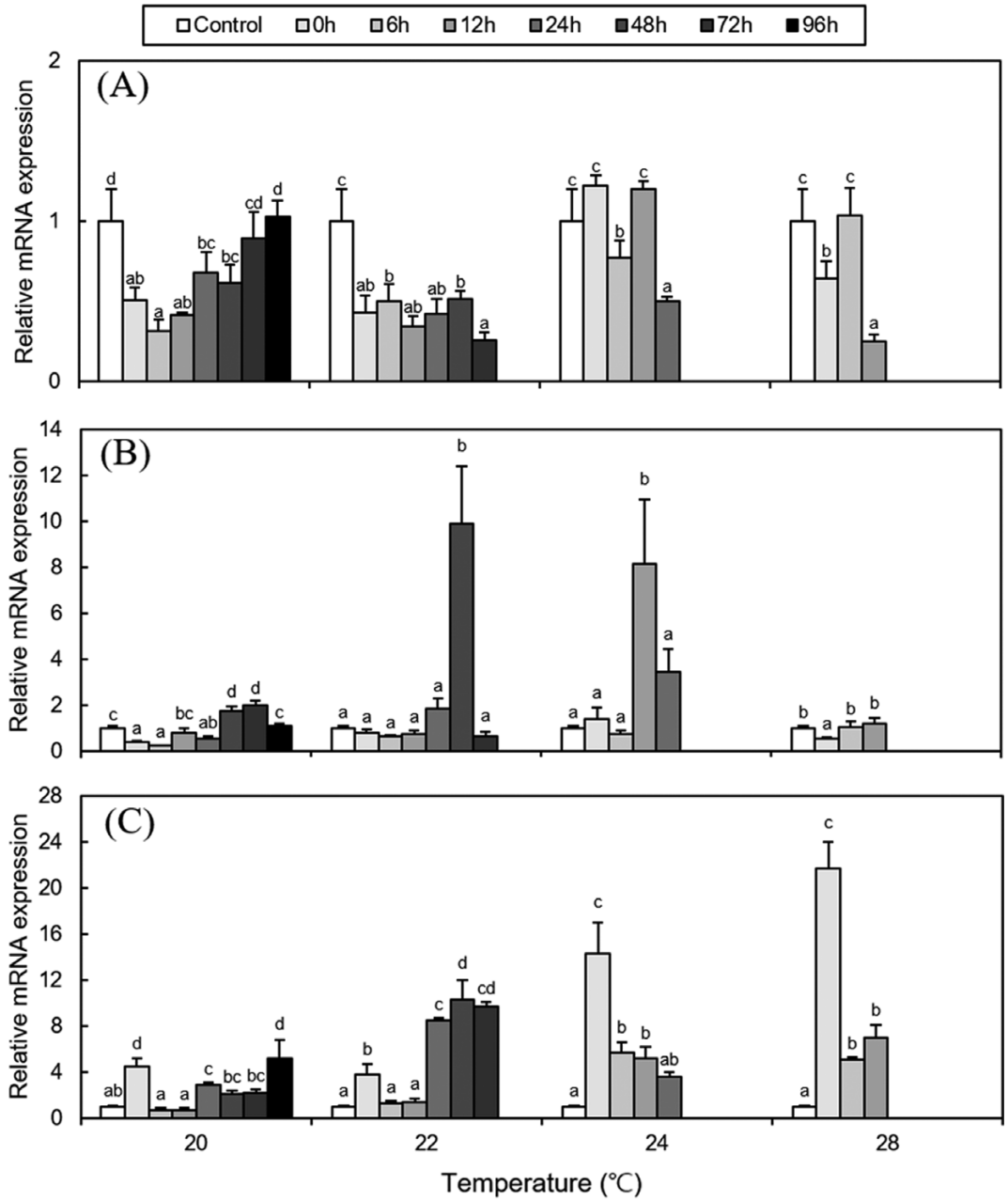

Fig. 1. Expression patterns of SOD mRNA in liver (A), muscle (B), and gill (C) in spotted sea bass, *Lateolabrax maculatus*, after exposure to 20, 22, 24, 28°C for 0, 6, 12, 24, 48, 72, or 96 h. The values were calibrated using β-actin as an internal control. The values shown are the mean ± SE. Values with dissimilar letters indicate significant differences ($p < 0.05$) at the indicated time points, but at the same temperature

In muscle tissues, SOD mRNA expression increased 9.9-fold 48 h after water temperature increased to 22°C, then rapidly decreased. At 24°C, expression increased 8.2-fold at 12 h and then decreased. In contrast, expression was maintained at baseline levels at 28°C ($p < 0.05$; Fig. 1B).

In gill tissues, SOD mRNA expression rapidly increased beginning at 0 h of all experimental threshold temperatures. At 20°C and 22°C, SOD expression respectively significantly increased 5.2~10.3-fold over time. In contrast, at 24 and 28°C, SOD expression increased 14.3-fold and 21.7-fold, respectively, and subsequently decreased ($p < 0.05$; Fig. 1C).

HSP90 mRNA expression

HSP90 mRNA expression levels in each tissue are shown

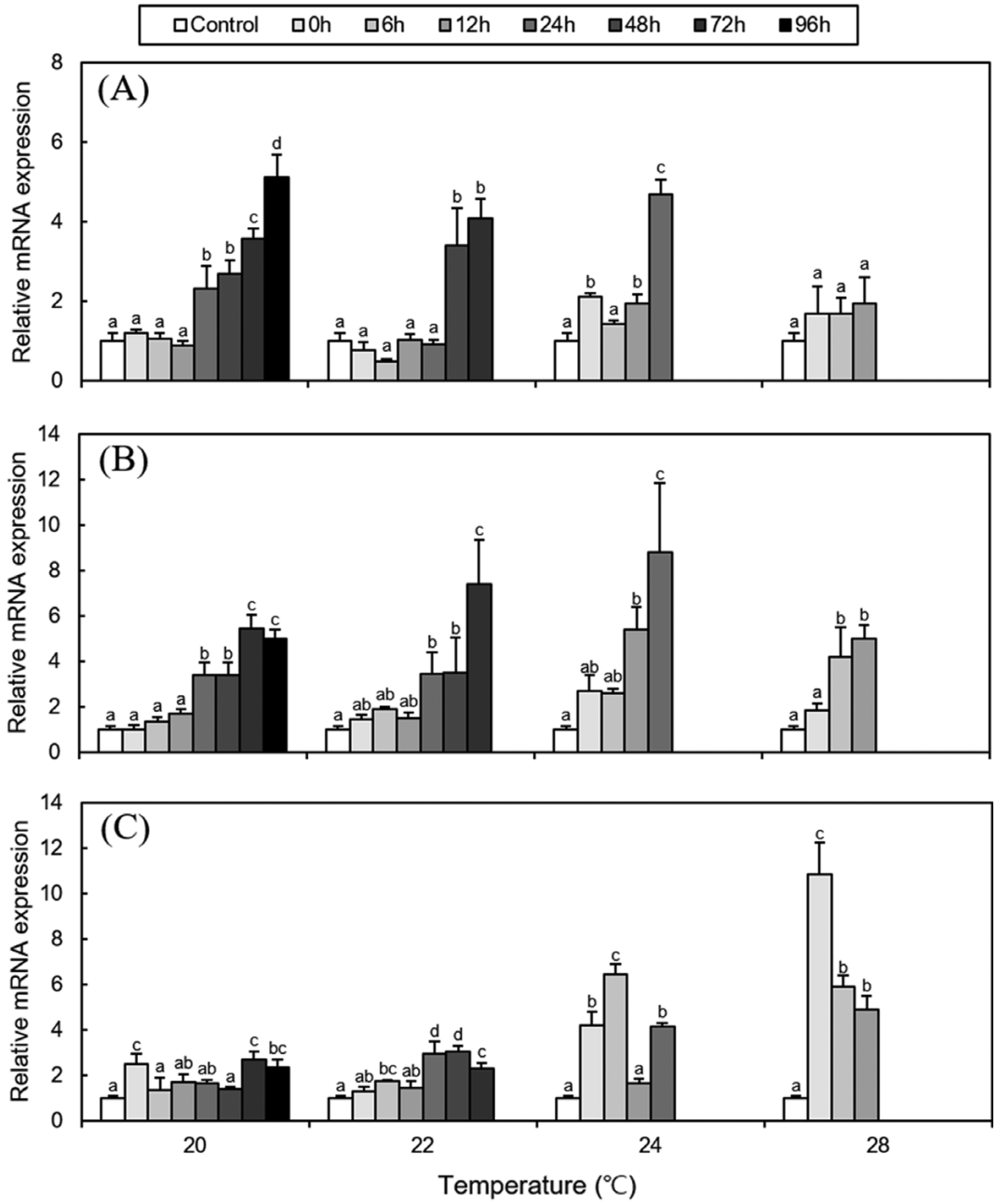

Fig. 2. Expression patterns of HSP90 mRNA in liver (A), muscle (B), and gill (C) in spotted sea bass, *Lateolabrax maculatus*, after exposure to 20, 22, 24, 28°C for 0, 6, 12, 24, 48, 72, or 96 h. The values were calibrated using β-actin as an internal control. The values shown are the mean ± SE. Values with dissimilar letters indicate significant differences ($p < 0.05$) at the indicated time points, but at the same temperature

in Figure 2. In liver tissue, expression gradually increased 4.1~5.1-fold over time at water temperatures of ≤ 24°C in comparison to the control. However, expression increased 1.9-fold at 0 h at 28°C, but there were no significant differences over time ($p < 0.05$; Fig. 2A).

In muscle tissue, HSP90 expression gradually increased 5.0~8.8-fold, with all increases statistically significant at all water temperatures and time points ($p < 0.05$; Fig. 2B).

In gill tissues, overall expression levels increased approximately 2.7-fold and 3.0-fold at 20 and 22°C, respectively. In contrast, HSP90 expression increased approximately 6.4-fold and 10.9-fold at 24 and 28°C, respectively, and subsequently decreased ($p < 0.05$; Fig. 2C).

HSP70 mRNA expression

HSP70 mRNA expression in each of the three tissues is shown in Figure 3. In the liver, expression gradually increased 4.7~5.2-fold over time at water temperatures of ≤ 24°C in comparison to the control. However, HSP70 expression increased 3.2-fold at 0 h at 28°C, and subsequently decreased ($p < 0.05$; Fig. 3A).

In muscle, HSP70 expression gradually increased significantly 2.2~3.4-fold over time at all water temperatures ($p < 0.05$; Fig. 3B).

In gill tissue, HSP70 expression displayed an overall increase of approximately 3.0-fold and 7.1-fold at 20 and 22°C, respectively. In contrast, HSP70 expression increased approximately 6.4-fold and 10.9-fold at 24 and 28°C,

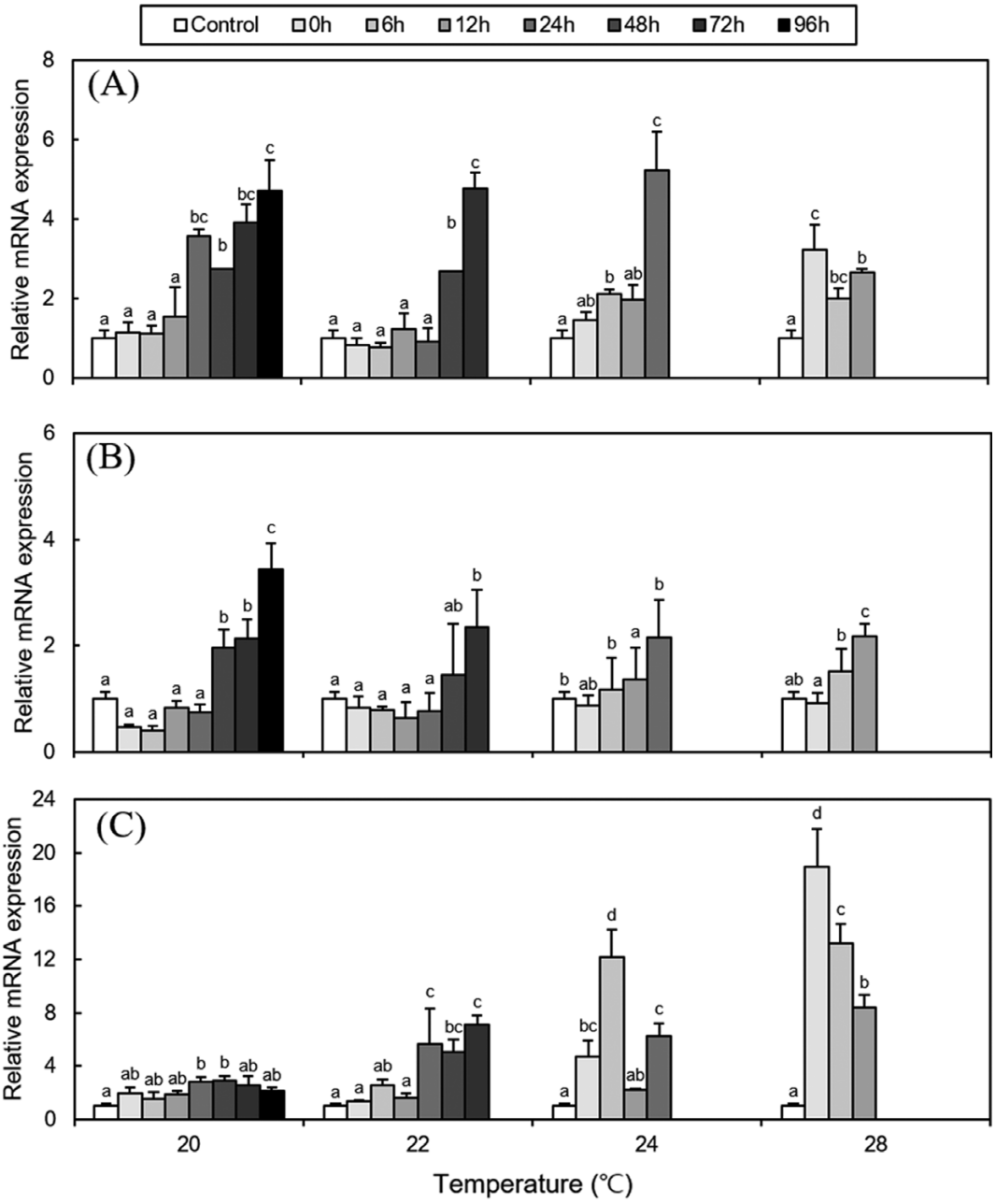

Fig. 3. Expression patterns of HSP70 mRNA in liver (A), muscle (B), and gill (C) in spotted sea bass, *Lateolabrax maculatus*, after exposure to 20, 22, 24, 28°C for 0, 6, 12, 24, 48, 72, or 96 h. The values were calibrated using β-actin as an internal control. The values shown are the mean ± SE. Values with dissimilar letters indicate significant differences ($p < 0.05$) at the indicated time points, but at the same temperature

respectively, and subsequently decreased ($p < 0.05$; Fig. 3C).

4. Discussion

In this study, we examined SOD, HSP90, and HSP70 mRNA expression in sea bass exposed to thermal stress. Thermal stress in fish induces membrane damage and enzyme inactivity, and generates ROS that result in DNA damage (Nordberg and Arnér 2001; Meng et al. 2014). SOD is an oxygen-scavenging enzyme that plays a role in eliminating ROS formed in the body, and is an indicator of oxidative damage caused by environmental changes (Kashiwagi et al. 1997; Lu et al. 2015). Our results show that SOD expression increases over time at high water temperatures in muscle and gill tissues, indicating activation of the ROS eliminating mechanism. These results are similar to those obtained in a previous study reporting SOD activity increase in black porgy exposed to high water temperature (An et al. 2010). However, SOD expression in liver tissue was maintained at baseline levels, suggesting that ROS did not accumulate in the liver of the spotted sea bass in response to thermal stress.

Weyts et al. (1999) reported that resistance to a given stressor increases as an initial response, but weakens as the stress persists. In the present study, mRNA expression of SOD increased initially in muscle and gill tissues, then decreased under sustained exposure to increased water temperatures. Such prolonged heat stress is likely to accelerate DNA damage due to ROS because the SOD defense mechanism has reached its maximum capacity. In addition, SOD expression in gills increased from 5.2 to 21.7-fold as water temperature increased, exhibiting the highest expression of all the tissues analyzed in this study. In general, oxygen consumption rates increase in response to increased water temperature in fish (Wuenschel et al. 2005). In the present study, it is likely that the increased metabolism of gill tissues, which is responsible for the direct exchange of sea water and oxygen, affected the production of ROS.

When fish undergo stress as a result of environmental changes, protective proteins are synthesized within the body (Iwama et al. 1999) to guard cells against secondary shock caused by stress (Ciavarra and Simeone 1990; Hightower 1991). HSP90 is a stress protein that accounts for approximately 1~2% of all cellular proteins (Pratt 1997), and assists cortisol—which provides energy to cells—by transmitting signals from glucocorticoid receptors (Pratt 1997). Our results showed that HSP90 expression increased with increased water temperature and exposure time in both liver and gill tissues. This is likely because the secretion of cortisol increased in order to maintain cellular homeostasis (Holloway and Leatherland 1997; Ackerman et al. 2000), thereby leading to the increased expression of HSP90 mRNA to assist with this function. On the other hand, HSP90 mRNA expression did not increase over time at the maximum water temperature (28°C) in the liver, and decreased after 6 h or 12 h at ≥ 24°C in gill tissue. This pattern is similar to changes in expression of HSP90 observed in pufferfish exposed to high temperature (Cheng et al. 2015). This result suggests that the sustained exposure to elevated water temperature reduces the supply of energy to cells. In muscle, HSP90 mRNA expression increased over time at all water temperatures, which is consistent with the results of a study by Liu and Steinbacker (2001) indicating that exposing muscle cells to heat results in upregulation of HSPs. Because *L. maculatus* is a fast-swimming fish, the energy supply to muscle cells in response to stress is likely to increase to a greater extent than in other tissues. HSP70 plays an important role in the maintenance of intracellular homeostasis via protein transport across the cellular membrane and protein synthesis (Moseley 1998). HSP70 is also known to be sensitive to environmental stressors and is associated with fluctuations in water temperature, and therefore protects cells from stress-induced apoptosis (Mosser et al. 1997; Feder and Hofmann 1999; Mallouk et al. 1999). Moreover, HSP70 protein content in any given tissue may differ from its mRNA level (Poltronieri et al. 2007), but since mRNA is a more sensitive indicator for thermal stress, the analysis in the present study was based on mRNA level (Lund et al. 2003).

Our study results show that HSP70 expression increased concomitantly with increased water temperature and exposure time in both liver and gill tissues. This is likely a result of the apoptotic defense mechanism triggered by increased water temperature. In contrast, the extent of the increase in HSP70 expression was relatively low in the liver at 28°C in comparison to lower water temperatures, while its expression decreased after 6 h or 12 h at ≥ 24°C in the gill. This is consistent with previously-published results showing that as the water temperature increased, the expression of HSP70 in Florida pompano initially increased, then decreased (Cardoso et al. 2015). Hochachka and Somero (2002) reported that proteins are destroyed and their levels decline concomitantly in environments of elevated water temperature, which might explain the low levels of HSP70 expression observed in our

study. In muscle tissue, HSP70 mRNA expression increased at all water temperatures, suggesting that the apoptotic response was active in muscle tissues in response to thermal stress.

In the present study, HSP90 and HSP70 exhibited similar expression patterns. This is likely because HSP90 and HSP70 simultaneously bind to HOP (the HSP70/HSP90 organizing protein) and are both essential proteins for the activation of hormone receptors in the nucleus (Arbeitman et al. 2000; Wegele et al. 2004). Overall, maximum gene expression levels were higher in gills than in other tissues at high water temperatures. A study by Mallatt (1985) found that the gill was more sensitive than other tissues to environmental changes, which is consistent with our results. Additionally, because the gill is in direct contact with sea water, it is likely to be acutely damaged by water temperature to a greater extent than other tissues. SOD showed a particularly high increase in expression in the gill in comparison to HSP90 and HSP70. Therefore, we suggest that SOD expression in the gills may be used as baseline data for analyzing acute thermal stress.

In summary, increased water temperature affects the expression of SOD, HSP90, and HSP70 mRNA expression in *L. maculatus*. There were tissue-specific differences in SOD, HSP90, and HSP70 expression, indicating that the thermal defense mechanism of the spotted sea bass differs between tissues.

Climate change has resulted in water temperature increase throughout much of the world (Metz and Davidson 2007), and some local areas are projected to experience rapid increases in the near future (Bamber and Seaby 2004). As increased water temperatures trigger physiological changes in fish, molecular approaches are invaluable for elucidating the underlying mechanisms of such phenomena (Clotfelter et al. 2013; Cheng et al. 2015). Hence, the results obtained from this study can be used as valuable basic data for understanding not only the effects of increased water temperature on fish, but also the defense mechanisms employed by fish to combat thermal stress.

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