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Effects of changes in water temperatures on physiological stress responses of bay scallops *Argopecten irradians*

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ABSTRACT

Changes in seawater temperature that occur during the changing of the seasons are a serious challenge faced by scallop aquaculture farms. In this study, we investigated the physiological stress responses of bay scallop *Argopecten irradians*, to increases and decreases in water temperature. We measured the changes in the levels of expression of copper- and zinc-containing superoxide dismutase (Cu/ZnSOD), and heat shock protein (HSP) 90, as well as the concentrations of lipid peroxide (LPO) in tissues of bay scallops exposed to thermal stress conditions (low-temperature condition: 17°C → 11°C; high-temperature condition: 17°C → 25°C). The expression levels of Cu/ZnSOD and HSP90 mRNA were temperature-dependent following thermal changes. LPO concentrations increased significantly in response to temperature changes. Additionally, greater Cu/ZnSOD and HSP90 expression levels and LPO concentrations were observed under the high-temperature condition than under the low-temperature condition. The results indicate that low and high water temperature conditions induced oxidative stress in bay scallops, and that this effect was greater at high water temperatures than at low water temperatures. This study provides basic knowledge about thermal stress in bay scallops, and our results may contribute to the management of scallop aquaculture farms.

Keywords: Antioxidant, *Argopecten irradiance*, Bay scallop, Stress response, Thermal stress

Introduction

Scallops are widely cultivated worldwide, and are known to have very high economic value (Hannam *et al*., 2009). More than 20 species of scallops occur in South Korea, including bay scallop *Argopecten irradians*, yesso scallop *Patinopecten yessoensis*, and Zhikong scallop *Chlamys farreri* (Lee and Min, 2002). Among these, the bay scallop is a species that is frequently exposed to rapid water temperature changes because it inhabits waters that are less than 10 m deep (Barber and Davis, 1997).

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When selecting scallops for aquaculture farms, water temperature is one of the most important external factors that can determine whether farming is possible. Due to increases in seawater temperatures during the summer, thermocline layers are formed. Accordingly, the temperature of each water layer may vary rapidly by up to 15℃, depending on the region (Côté *et al*., 1993; Pearce *et al*., 2004). Due to such a rapid water temperature changes, bay scallops are frequently exposed to both low and high water temperatures, as well as sudden water temperature changes (Lafrance *et al*., 2002).

When shellfish are exposed to environmental stressors, such as rapid temperature changes, this leads to an excess of reactive oxygen species (ROS) in the tissues, which damage unsaturated lipids, leading to the production of lipid peroxide (LPO) (Hannam *et al*., 2009). The LPO produced in the tissues is highly reactive, and induces the degeneration of hormones, reduces cell function, and causes cell death (Matoo *et*

al., 2013; Jiang *et al*., 2016). Therefore, to protect themselves from ROS, organisms release antioxidant enzymes such as superoxide dismutase (SOD), which activate antioxidant systems that convert toxic substances such as ROS into less- or non-toxic substances (Kashiwagi *et al*., 1997). These antioxidant enzymes are also used as indicators of stress levels in various species, including scallops (Klumpp *et al*., 2002).

In addition, heat shock protein (HSP) is generated by bivalves and other aquatic organisms in response to thermal stress, and HSP protects organisms from cell damage caused by water temperature stress (Brown *et al*., 2004; Lin *et al*., 2018). On the other hand, when HSP is over-expressed, it adversely affects the immunity of the organism, for example through disturbing antigen recognition by adversely affecting the activity of immune substances (Song *et al*., 2015; Giri *et al*., 2016). Data related to changes in antioxidant enzymes and stress indicators caused by thermal stress can be used to understand the physiological stress response of various aquatic organisms, including scallops, exposed to changes in the water temperature (Heise *et al*., 2003).

Therefore, this study was conducted to understand the stress responses of bay scallops, an important aquaculture species in South Korea, to low and high temperature environments. To achieve this aim, bay scallops were exposed to water temperature changes, and the mRNA expression levels of an antioxidant enzyme (SOD) and HSP90, as well as the concentrations of LPO in digestive diverticula were evaluated.

Materials and methods

1. Experimental animals

Bay scallops (shell length: 65 ± 10 mm; weight: 48.81 ± 4.01 g) were purchased from a commercial market (Tongyoung, Korea), and kept in four 400 L circulation filter tanks containing filtered and aerated seawater, prior to experiments in the laboratory. Two experimental conditions were established in duplicate, with 40 bay scallops per tank. Half of the total volume of seawater in each tank was changed daily. The bay scallops were reared with automatic temperature regulation systems (JS-WBP-170RP; Johnsam Co., Buchoen, Korea), and the water temperature and salinity were maintained at 17°C and 30 ‰, respectively. Bay scallops were allowed to acclimate to the experimental conditions for one week. All scallops were initially exposed to water temperatures of 17°C. The water temperature was then increased from 17°C to 25°C, or reduced from 17°C to 11°C in daily increments of 1°C. Sampling was performed at 11, 13, 15, 17, 19, 21, 23, and 25°C by randomly collecting five scallops from each experimental treatment. The digestive diverticula tissues were collected from each scallop, and were stored at −80°C until analysis.

2. Total RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from the digestive diverticula using the TRI reagent® (Molecular Research Center, Inc., Ohio, USA), according to the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed in a total volume of 20 μL, using an oligo-d$(T)_{15}$ anchor and M-MLV reverse transcriptase (Promega, Madison, WI, USA), according to the manufacturer's protocol. Synthesized cDNA was stored at −20°C until use.

3. quantitative PCR (qPCR)

qPCR was conducted to determine the relative expression levels of copper- and zinc-containing superoxide dismutase (Cu/ZnSOD, referred to hereafter as 'SOD', for brevity) and HSP90 mRNA using cDNA reverse-transcribed from the total RNA extracted from the digestive diverticula. Ribosomal protein S18 (RPS18) mRNA was used as an internal control. The primers used for qPCR are shown in Table 1. PCR amplification was conducted using a Bio-Rad CFX96™ Real-time PCR Detection System (Bio-Rad, California, USA) and iQ™ SYBR Green Supermix (Bio-Rad, California, USA), according to the manufacturer's instructions. The qPCR program was as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 20 s and 55°C for 20 s. RPS18 was used as an internal control,

Table 1. Primers used for QPCR amplification

Genes	Primer	DNA sequences
Cu/ZnSOD (EU683812)	Forward	5′- TCT GAC GAG GCA TTT GAC AG-3′
	Reverse	5′- GCA TGC ATT GTC TGC TCA CT-3′
HSP90 (EF532406)	Forward	5′- ATG ACG GAA AGA CCC TTG TG-3′
	Reverse	5′- CAA GGC GGT TGG ATA CAG TT-3′
RPS18 (AF526232)	Forward	5′- GTC TGC AAG AAG GCT GAT GT-3′
	Reverse	5′- GGG TTG GAC ATG ATT GTG AT-3′

and all data are expressed relative to the corresponding RPS18 threshold cycle (ΔCt) levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal controls (RPS18) were calculated using the 2-ΔΔCt method: [Δ ΔCt = 2^-($\Delta Ct_{sample} - \Delta Ct_{internal\ control}$)].

4. LPO assay

LPO concentrations were quantified by measuring malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which are degradation products of polyunsaturated fatty acid (PUFA) hydroperoxides (Esterbauer *et al*., 1991). LPO concentrations were quantified using a Lipid Hydroperoxide Assay Kit (Cayman Chemical, Michigan, USA), according to the manufacturer's instructions. Tissue (0.1 g) was homogenized in 1 mL of HPLC-grade water. 50 μL of the cytosolic fraction of the homogenate was added to a glass tube. 50 μL of chloroform, 45 μL of chloroform methanol, and 5 μL of ferric thiocyanide solution (FTS) reagent 1 and FTS reagent 2 mixtures (Cayman Chemical, Michigan, USA) were added to the glass tube and mixed. The samples were incubated for 5 min at room temperature. Samples (30 μL per well) were added to flat bottom 96 well plates. The absorbance was read at 500 nm using a plate reader. LPO is expressed as nM of MDA and 4-HNE/g protein.

5. Statistical analysis

All data were analyzed using the SPSS statistical package (version 19.0; SPSS Inc., USA). A one-way ANOVA followed by Tukey's post-hoc test was used to compare differences in the data ($P < 0.05$). The values are expressed as the means ± standard error (SE).

RESULTS

1. Expression of SOD mRNA in the digestive diverticula

SOD mRNA expression levels in all experimental groups were significantly increased by changes in temperature (Fig. 1). mRNA expression levels were significantly higher at 25°C, the highest temperature in this study (4.57 ± 0.23), and at 23°C (4.26 ± 0.32), than at 11°C, the lowest temperature in this study (4.22 ± 0.31).

2. LPO concentrations

LPO concentrations in tissues of bay scallops exposed to temperature changes were quantified using a plate reader (Fig. 2). LPO concentrations were significantly increased by temperature changes. The LPO concentrations at the control temperature (17°C) were 17.11 ± 0.88 nM/g. The LPO concentrations were significantly higher at 25°C, the highest temperature in this study (28.51 ± 1.02 nM/g), and at 23°C (26.18 ± 1.12 nM/g), than at 1°C, the lowest temperature in this study (25.55 ± 0.84 nM/g).

3. Expression of HSP90 mRNA in the digestive diverticula

HSP90 mRNA expression levels were temperature-dependent following changes in temperatures (Fig. 3). As the change in water temperature increased, the mRNA expression gradually increased. Bay scallops in the high-temperature treatment (17°C: 1.00 ± 0.13; 19°C: 1.27 ± 0.36; 21°C: 2.38 ± 0.36; 23°C: 4.74 ± 0.56; 25°C: 6.43 ± 0.84) showed significant up-regulation more rapidly, compared to bay scallops in the low-temperature treatment (17°C: 1.00 ± 0.13; 15°C: 1.40 ± 0.31; 13°C: 2.82 ± 0.12; 11°C: 4.09 ± 0.31).

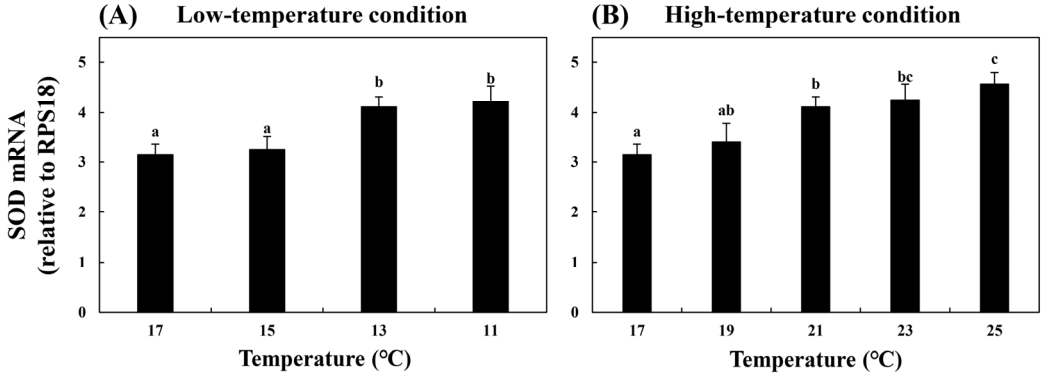

Fig. 1. Changes in superoxide dismutase (SOD) mRNA expression levels in the digestive diverticula of bay scallops, *Argopecten irradians*, during temperature changes (**A**: low water temperature conditions; **B**: high water temperature conditions), as measured by quantitative real-time PCR. The lowercase letters indicate significant differences ($P < 0.05$) between different temperatures within the same temperature treatment (i.e. 17°C → 11°C and 17°C → 25°C). All values are shown as means ± SE (n = 5).

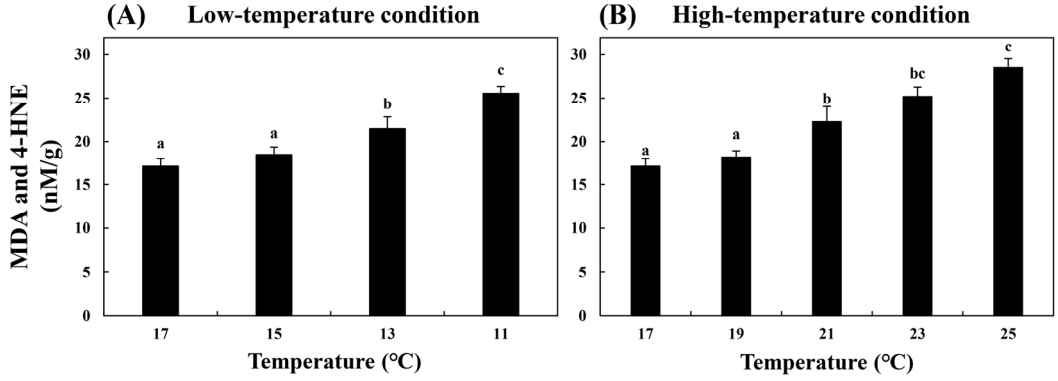

Fig. 2. Changes in lipid peroxide (LPO) concentrations in bay scallops, *Argopecten irradians*, exposed to changes in water temperature (**A**: low water temperature conditions; **B**: high water temperature conditions). The lowercase letters indicate significant differences ($P < 0.05$) between different temperatures within the same temperature treatment (i.e. 17°C → 11°C and 17°C → 25°C). All values are shown as means ± SE (n = 5).

Discussion

Changes in water temperature experienced by organisms living in aquatic environments are known to be one of the main factors affecting the physiological stress response of individuals, and cause oxidative stress (Zhang *et al*., 2019).

Among the physiological reactions of organisms, oxidative stress occurs when excessive ROS are produced in response to external factors. Of all the external environmental factors, aquatic organisms are particularly affected by water temperatures. In response to environmental stressors, such as changes in water temperatures, organisms secrete antioxidant enzymes from cells to reduce ROS (Kashiwagi *et al*., 1997). In a previous study, golden and brown noble scallops *Chlamys nobilis* were exposed to rapidly lowered temperatures (Tan *et al*., 2019). As a result, SOD activity and LPO concentrations in the tissues increased, and in particular, LPO concentrations increased more as the time during which the scallops were exposed to the changes in water temperature increased (Tan *et al*., 2019). In addition, another study reported that SOD activity increased when clams

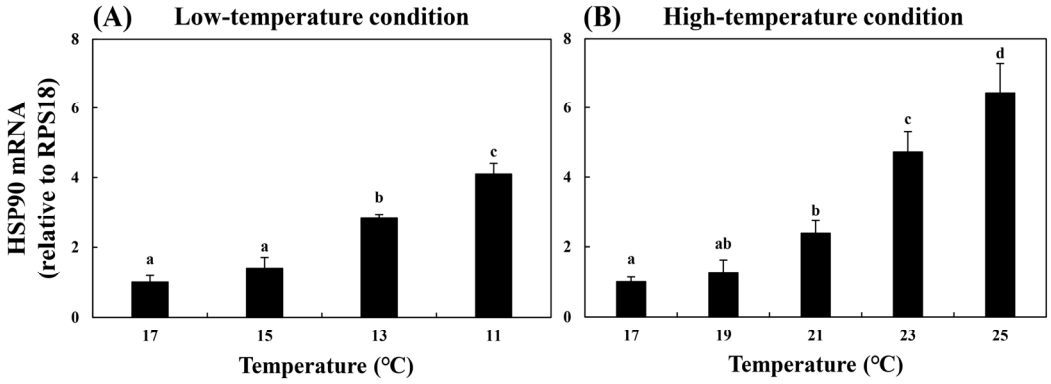

Fig. 3. Changes in the HSP90 mRNA expression in the digestive diverticula of bay scallops, *Argopecten irradians*, exposed to changes in water temperature (**A**: low water temperature conditions; **B**: high water temperature conditions), as measured by quantitative real-time PCR. The lowercase letters indicate significant differences ($P < 0.05$) between different temperatures within same the same temperature treatment (i.e. 17°C → 11°C and 17°C → 25°C). All values are shown as means ± SE (n = 5).

Chamelea gallina which have an optimal habitat temperature of 20°C, were exposed to high-temperature environments of 25 and 30°C (Monari *et al*., 2007).

Since the results of this study are similar to those of the previous studies described above, it is likely that the occurrence of oxidative stress in aquatic species increases as the water temperature increases or decreases from the optimum. For this reason, it is likely that the bay scallops used in this study increased the expression of the antioxidant enzyme SOD to suppress the accumulation of free radicals produced in the tissues.

When the ROS generated by water temperature stress are not sufficiently decomposed and accumulate in the tissues despite increases in the expression levels of antioxidant enzymes, LPO concentrations increase. A study by Rahman *et al*. (2019) reported that ROS activities were increased in all three species of bivalve shellfish (Pacific oysters *Crassostrea gigas*, Mediterranean mussels *Mytilus galloprovincialis*, and mud cockles *Katelysia rhytiphora*) exposed to high-temperature conditions. Matoo *et al*. (2013) reported that the exposure of hard-shell clams *Mercenaria mercenaria* to high temperatures resulted in an increase in LPO concentrations in tissues.

The results of these previous studies are consistent with the results of this study, in which the LPO concentrations in bay scallop tissues increased with increasing water temperatures. As the changes in water temperatures increase, oxidative stress in bay scallops exposed to this environmental stressor increased. This seems to lead to the accumulation of LPO in the tissues, due to the formation of a large amount of ROS that cannot be degraded by antioxidant enzymes, suggesting the possibility of cell dysfunction and death.

In addition, HSP is a molecular chaperone that plays an important role in the response of cells to various external stressors. In a study by Lin *et al*. (2018), the clams *Paphia undulata* were exposed to high temperatures, and the expression of HSP90 was found to increase rapidly. Jiang *et al*. (2017) reported that the expression of HSP mRNA increased with increasing exposure time when Zhikong scallop were exposed to high- or low-temperature conditions. In addition to the results of this study, the results of previous studies indicate that the expression levels of HSP mRNA increase with changes in water temperature. In bay scallops, HSP90 is also believed to be involved in adaptation to environmental water temperatures and thermal stress. This suggests that HSP plays an important role as the scale of the changes in water temperatures increases.

In conclusion, our results confirm that water temperatures affected the physiological stress

responses of bay scallops. In addition, we confirmed that rapid changes in water temperature, including both increases and decreases in temperature, negatively affected the physiological stress responses of bay scallops. Moreover, since the expression levels of the stress indexes were higher in the high temperature treatment than in the low temperature treatment, it is likely that exposure to high temperatures may be more stressful to bay scallops than exposure to lower temperatures. The results of this study provide basic data on the physiological stress responses of bay scallops to changes in temperature, and can be used to evaluate the impact of environmental changes on bay scallops, such as rising sea water temperatures, which may occur irregularly due to climate change.

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