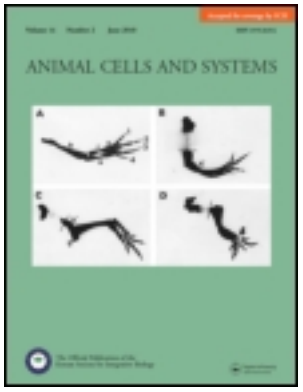

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Effect of hypoosmotic and thermal stress on gene expression and the activity of antioxidant enzymes in the cinnamon clownfish, *Amphiprion melanopus*

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Effect of hypoosmotic and thermal stress on gene expression and the activity of antioxidant enzymes in the cinnamon clownfish, *Amphiprion melanopus*

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We studied oxidative stress in cinnamon clownfish exposed to hypoosmotic (35 psu → 17.5 psu and 17.5 psu with prolactin (PRL)) and low temperature (28°C → 24°C and 20°C) conditions by measuring the expression and activity of Cu/Zn-superoxide dismutase (Cu/Zn-SOD), catalase (CAT), and glutathione peroxidase (GPX). The expression and activity of the antioxidant enzymes were significantly higher after the fish were exposed to 24°C, 20°C, and 17.5 psu, and expression was repressed by PRL treatment. Furthermore, we measured H_2O_2 and lipid peroxidation levels and found that they were significantly higher after exposure to the hypoosmotic and low-temperature environments. Additionally, we investigated changes in plasma AST and ALT levels after exposure to low temperature and hypoosmotic stress. These levels increased upon exposure of the clownfish to 24°C, 20°C, and 17.5 psu, but the levels of these parameters decreased in the 17.5 psu with PRL treatment during a salinity change. The results indicate that hypoosmotic and low-temperature conditions induce oxidative stress in cinnamon clownfish and that the parameters tested in this study may be indices of oxidative stress in the cinnamon clownfish.

Keywords: cinnamon clownfish; hydrogen peroxide; lipid peroxidation; lysozyme; oxidative stress

Introduction

Salinity and temperature are important environmental factors that affect the growth, survival, reproduction, and immune functions of teleost fishes (Schreck et al. 1989; Bly and Clem 1992). Acute changes in salinity and water temperature can cause stress (Barton and Iwama 1991; Abele et al. 1998). Stress induced by changes in salinity is associated with enhanced generation of reactive oxygen species (ROS), which may seriously affect immune function and lead to oxidative stress (Fisher and Newell 1986; Shin et al. 2010a). Furthermore, overproduction of ROS in response to thermal stress can lead to oxidative damage (Halliwell and Gutteridge 1989) and increased lipid peroxidation, and may affect cell viability by causing membrane damage and enzyme inactivity (Kim and Phyllis 1998; Pandey et al. 2003).

Complex antioxidant defense systems maintain homeostasis in changing environments and protect aerobic organisms against ROS and subsequent oxidative stress-induced damage (Bagnyukova et al. 2007). Antioxidants are enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione *S*-transferase (GST) or compounds such as metallothionein, vitamin C, and vitamin E (α-tocopherol) (McFarland et al. 1999). Antioxidant defense systems are found in the liver and kidneys of marine organisms (Basha and Rani 2003) and have the following functions. SOD, CAT, and GPX directly scavenge ROS. SOD breaks down O_2^- through the process of dismutation to O_2 and H_2O_2 ($2O_2^- + H^+ \rightarrow H_2O_2 + O_2$; Kashiwagi et al. 1997). H_2O_2 produced by SOD is sequentially reduced to H_2O and O_2 by CAT (Kashiwagi et al. 1997). CAT and GPX are oxidoreductases that break down two molecules of H_2O_2 into two molecules of H_2O and O_2 ($2H_2O_2 \rightarrow 2H_2O + O_2$), thereby counteracting the toxicity of H_2O_2 (Kashiwagi et al. 1997).

These ROS are the most powerful oxidants formed in biological systems and can readily attack any biological molecule and also attack polyunsaturated fatty acids to initiate lipid peroxidation (Gutteridge 1995).

Furthermore, oxidative stress may seriously affect immune function (Fisher and Newell 1986; Shin et al. 2010a). Indeed, immune biomarkers may be useful in evaluating the effects of exposure to environmental

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stress (Reynaud and Deschaux 2006). Lysozyme is part of the nonspecific immune system (Jollès and Jollès 1984) and its activity level is affected by stress, although the response following acute or prolonged stress is ambiguous (Fevolden and Røed 1993; Demers and Bayne 1997). Enzymatic, nonenzymatic antioxidant, and innate immune systems contribute to important biological defenses against environmental stress (Lopes et al. 2001). The extent of liver injury is usually assessed by increases in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels (Rahman and Sultana 2006).

The cinnamon clownfish is a popular seawater ornamental fish. Clownfish aquaculture in Korea is conducted completely in tank culture. However, rainfall, which is highly concentrated in the rainy season of the summer, causes the water temperature and/or salinity to change, which stresses the cinnamon clownfish.

Therefore, to examine the oxidative stress resulting from temperature and salinity changes, we investigated the mRNA expression and activity of antioxidant enzymes (Cu/Zn-SOD, CAT, and GPX), and the levels of H_2O_2, lipid peroxidation (LPO), and lysozyme as potential biomarkers of oxidative stress attributable to hypoosmotic and/or low-temperature stress in the cinnamon clownfish. Additionally, we investigated the expression and activity of the antioxidant enzymes H_2O_2 and LPO after prolactin (PRL) treatment to study the role of PRL when fish were exposed to a hypoosmotic environment. PRL is a freshwater-adapting hormone whose synthesis and release increases as teleosts adapt to fresh or brackish water (Olivereau et al. 1981). Furthermore, we analyzed the changes in AST and ALT levels to measure liver damage induced by the hypoosmotic and low-temperature conditions.

Materials and methods

Fish and experimental conditions

Immature cinnamon clownfish ($n = 100$, 8.1 ± 1.8 g) were purchased from the Center of Ornamental Reef & Aquarium (CCORA; Jeju, Korea), and were allowed to acclimate to the experimental conditions for one month in 300 L circulation filter tanks in the laboratory. Before the experiment, the water temperature and photoperiod were 28 ± 0.5°C and 12L:12D, respectively. The fish were fed commercial marine aquarium fish feed (Jeilfeed; Kyoungnam, Korea) twice a day (09:00 and 17:00).

Salinity changes

The cinnamon clownfish were transferred from seawater (35 psu) to a hypoosmotic environment (17.5 psu) as follows: groundwater was poured into two 50 L circulating filter tanks, the salinity was adjusted to 17.5 psu, and the fish were exposed to this water for 48 h. The livers were removed from five fish at each of the following time periods after transfer: 0, 12, 24 and 48 h. The water temperature and photoperiod were maintained at 28 ± 0.5°C and 12L:12D, respectively.

PRL treatment

To investigate the role of PRL in the effects of salinity changes on cinnamon clownfish, the fish were treated with PRL (Sigma; St. Louis, MO), and the expression of Cu/Zn-SOD, CAT and GPX mRNA was measured. The fish were adapted in 50 L circulation filter tanks in the laboratory, and they were then anesthetized with tricaine methane sulfonate (MS-222; Sigma) prior to PRL injection. PRL was dissolved in saline and each fish was given an intraperitoneal injection of PRL (5 μg/g body mass (BM)) and a sham group of fish was injected with a dissolved equal volume of saline (10 μL/g BM). After intraperitoneal injection, the fish were transferred from seawater to the hypoosmotic environment. Livers were removed from five fish at each of the following time periods after transfer: 0, 12, 24 and 48 h. During the experimental period, the water temperature and photoperiod were maintained at 28 ± 0.5°C and 12L:12D, respectively.

Water temperature changes

The cinnamon clownfish were placed in seawater in two circulating filter tanks (50 L) with cooler systems (SH-1000; Samhwa Co.; Korea), and they were allowed to acclimatize to the conditions for 24 h. The water temperature was then decreased from 28°C to 20°C in daily decrements of 1°C. During the experimental period, the salinity and photoperiod were maintained at 35 psu and 12L:12D, respectively.

Cu/Zn-SOD, CAT and GPX activity analysis

The liver tissues (50 mg) were homogenized in 0.1 M ice-cold phosphate-buffered saline (PBS; pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA). The homogenates were centrifuged at $10{,}000 \times g$ for 15 min at 4°C; the supernatant was removed, and the remaining sample was used for analysis. Cu/Zn-SOD, CAT, and GPX activities were determined using commercial kit (Cayman Chemical; Ann Arbor, MI) according to the manufacturer's instructions.

Cu/Zn-SOD activity was assessed using a tetrazolium salt for detecting the superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit

of Cu/Zn-SOD is defined as the amount of enzyme required to catalyze 50% dismutation of the superoxide radical. Absorbance was read at 450 nm. Each assay was performed in duplicate, and the enzyme activity was recorded in units per milliliter. The assay for the assessment of CAT activity is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced was measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald®) as the chromogen (Wheeler et al. 1990). Purpald® specifically forms a bicyclic heterocycle with aldehydes; this bicyclic heterocycle changes from colorless to purple upon oxidation. Absorbance was read at 540 nm. Each assay was performed in duplicate, and the CAT activity was expressed in nanomoles per minute per milliliter. GPX activity was measured indirectly using a coupled reaction with glutathione reductase. Oxidized glutathione, which is produced upon reduction of H_2O_2 by GPX, is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in the absorbance at 340 nm, and GPX activity was expressed in nanomoles per minute per milliliter.

H_2O_2 assay

The H_2O_2 concentration was measured using the modified methods of Nouroozzadeh et al. (1994) and a PeroxiDetect Kit (Sigma). For this, 10 μL of cinnamon clownfish plasma was added to each well of flat-bottomed 96-well plates. The plates were left at room temperature for 20 min to allow the plasma to settle and adhere to the plate. A working solution of a color reagent was prepared by mixing 12.5 mL distilled water containing 100 mM sorbitol and 125 μM xylenol orange (Sigma) with 125 μL of 25 mM ferrous ammonium sulfate prepared in 2.5 M sulfuric acid (Sigma). Next, 100 μL of this reagent was added to each well and incubated at room temperature for 1 h. The absorbance was read at 560 nm, and the concentration of H_2O_2 was interpolated from a standard curve and expressed in nanomoles per milliliter.

LPO assay

LPO was quantified by measuring the amounts of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which are the degradation products of polyunsaturated fatty acid (PUFA) hydroperoxides (Esterbauer et al. 1991), with a Lipid Hydroperoxide Assay Kit (Cayman Chemical) according to the manufacturer's instructions. Tissue (50 mg) was homogenized in 10 mL of HPLC grade water. Then, 250 μL of the cytosolic fraction of the homogenate was added to a glass tube, and 250 μL of chloroform, 225 μL of chloroform–methanol, and 25 μL of a mixture of ferric thiocyanide solution (FTS) reagent 1 and FTS reagent 2 (Cayman Chemical) were added to the glass tube and mixed. This sample was incubated for 5 min at room temperature and then added at 300 μL/well to flat-bottomed 96-well plates. The absorbance was read at 500 nm by using a plate reader, and LPO was expressed in terms of nanomoles of MDA and 4-HNE per gram protein.

Lysozyme activity

Plasma (10 μL) was added to 190 μL of a suspension of *Micrococcus lysodeikticus* (0.2 mg/mL) in 0.05 M sodium phosphate buffer (pH 6.2).The reactions were carried out at 25°C, and the absorbance at 530 nm was measured between 0.5 and 4.5 min by using a spectrophotometer. A lysozyme activity unit was defined as the amount of enzyme catalyzing a 0.001/min decrease in absorbance.

Results

Effect of salinity and temperature changes on antioxidant enzyme expression

The expression of Cu/Zn-SOD and CAT mRNA in the liver increased gradually in the hypoosmotic environment and was highest at 48 h after transfer to 17.5 psu (approximately 2.5- and 4.2-fold greater than that at 35 psu), and GPX mRNA increased at 12 h and was maintained until 48 h (approximately 3.2-fold greater than that at 35 psu). Cu/Zn-SOD and CAT mRNA expression in the PRL-treated group was lower than that in the untreated group (Figure S-1 in supplementary data[1]). Furthermore, hepatic mRNA expression of these genes was the highest at 24°C (approximately 8.5-, 5.5-, and 20-fold higher than that at 28°C) (Figure S-2 in supplementary data[1]).

Effect of salinity and temperature changes on antioxidant enzyme activity

Cu/Zn-SOD and GPX activity in the liver was highest at 24 h after transfer to 17.5 psu (approximately 3.1-, 1.45-, and 2.7-fold higher than that at 35 psu), and CAT increased at 6 h and was maintained until 48 h (approximately 1.45-fold higher than that at 35 psu). These activities were lower in the PRL-treated group than in the untreated group (Figure 1). Activity gradually increased with the change in temperature (Figure 2).

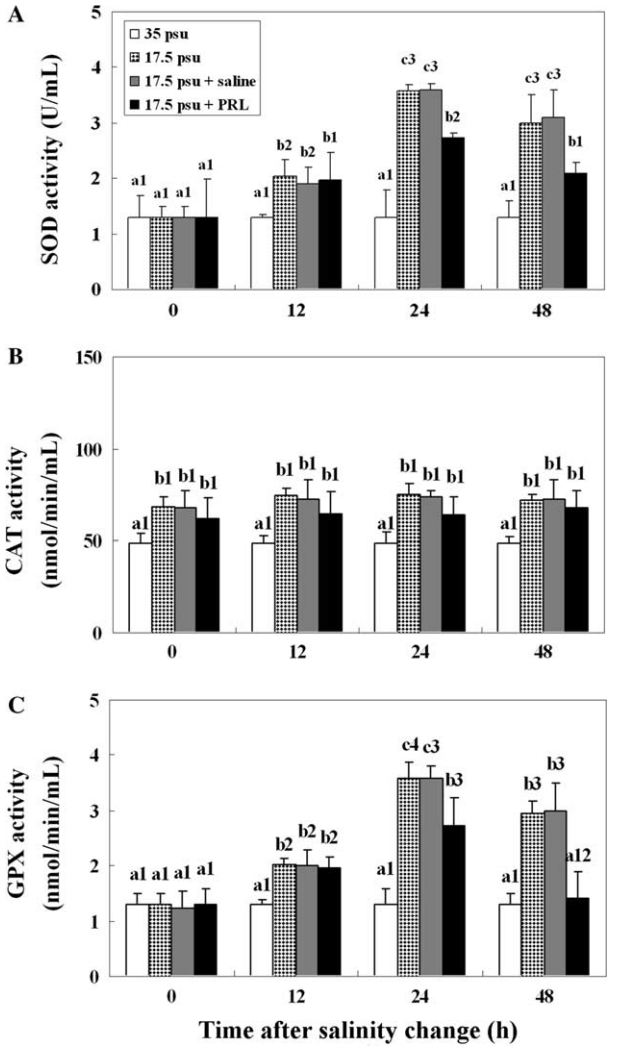

Figure 1. Activity of Cu/Zn-superoxide dismutase (Cu/Zn-SOD) (A), catalase (CAT) (B), and glutathione peroxidase (GPX) (C) in the liver of cinnamon clownfish during a salinity change. Values are means ± SD ($n = 5$).

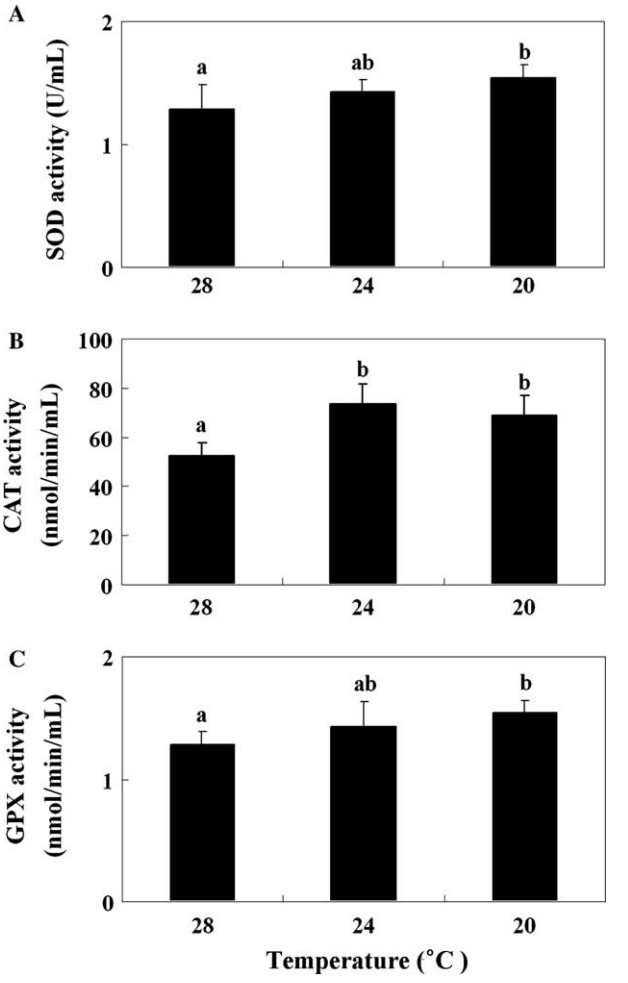

Figure 2. Activity of Cu/Zn-superoxide dismutase (Cu/Zn-SOD) (A), catalase (CAT) (B), and glutathione peroxidase (GPX) (C) in the liver of cinnamon clownfish during a temperature change. Values are means ± SD (n=5).

H_2O_2 levels

The plasma H_2O_2 level was 14.7 ± 8.1 nmol peroxide/mL in the control group (35 psu), which increased significantly to 56.5 ± 8.7 nmol peroxide/mL at 12 h after transfer to 17.5 psu. Furthermore, during the change in temperature, the H_2O_2 levels were high at 24°C (30.6 ± 5.4 nmol peroxide/mL) and 20°C (28.4 ± 4.8 nmol peroxide/mL) (Figure 3).

LPO levels

The LPO levels (expressed in terms of the amounts of malondialdehyde (MDA) and 4-hydroxy nonenal) were 42.5 ± 9.8 nM/g in the controls (35 psu), and gradually increased to 125.6 ± 10.5 nM/g at 48 h after transfer to 17.5 psu. In addition, the level increased to 115.1 ± 12.3 nM/g at 20°C (Figure 4).

Lysozyme activity

The lysozyme activity in the control was 1.4 ± 0.2 μg/mg protein (35 psu), which gradually decreased to 0.7 ± 0.15 μg/mg protein at 48 h after transfer to 17.5 psu. The level then decreased to 0.9 ± 0.14 μg/mg protein at 20°C (Figure 5).

AST and ALT levels

Plasma AST levels were 50 ± 23 IU/L in the control group, and increased significantly to 966.1 ± 80 IU/L at 24 h after transfer to 17.5 psu (Figure S-3A in supplementary data[1]). AST increased to 415.4 ± 54 IU/L at 20°C (Figure S-4A in supplementary data[1]). Plasma ALT levels were 1.3 ± 0.2 IU/L in the control

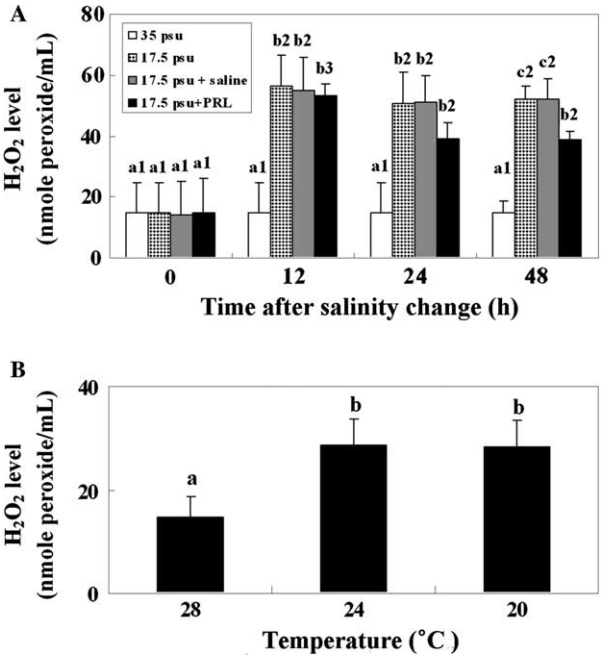

Figure 3. Plasma H_2O_2 levels during a salinity change (A) and during a temperature change (B) in cinnamon clownfish. Values are means $\pm$ SD ($n = 5$).

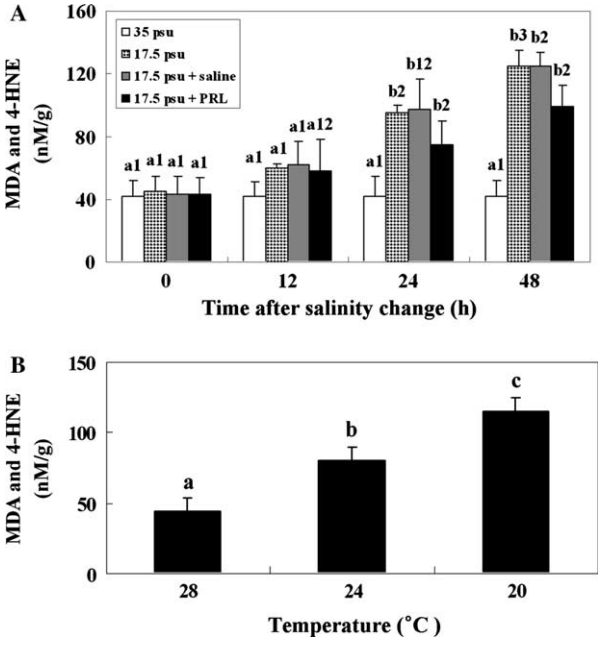

Figure 4. Plasma malondialdehyde (MDA) and 4-hydroxy nonenal (4-HNE) levels during a salinity change (A) and during a temperature change (B) in cinnamon clownfish. Values are means $\pm$ SD ($n = 5$).

group; they increased significantly to 8.3 ± 0.6 IU/L at 24 h after transfer to 17.5 psu (Figure S-3B in supplementary data[1]) and increased to 9.6 ± 0.3 IU/L at 20°C (Figure S-4B in supplementary data[1]).

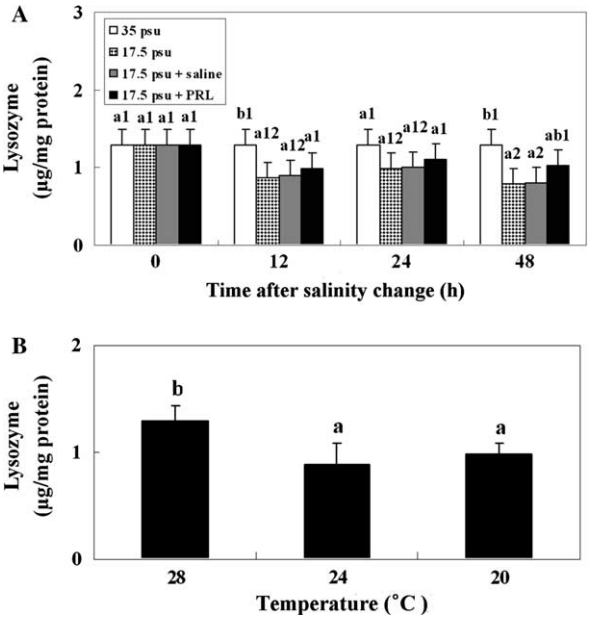

Figure 5. Plasma lysozyme levels during a salinity change (A) and during a temperature change (B) in cinnamon clownfish. Values are means $\pm$ SD ($n = 5$).

Discussion

We examined LPO, plasma H_2O_2 concentration, and the expression and activity of antioxidant enzymes (Cu/Zn-SOD, CAT, and GPX) in cinnamon clownfish exposed to a hypoosmotic low-temperature environment to examine the effects of oxidative stress. We also examined lysozyme-related immune function and plasma ALT and AST levels to investigate the physiological changes induced by oxidative stress.

First, we found that the expression and activation of antioxidant enzyme genes in the liver of cinnamon clownfish exposed to a hypoosmotic (17.5 psu) environment increased (Figures 1 and 2). Generally, marine organisms, including fish, experience oxidative stress due to changes in water salinity (Halliwell and Gutteridge 1989; Liu et al. 2007). The results of the present study were in particular agreement with those of recent studies in which the expression and activation of antioxidant enzyme genes increased in olive flounder (Choi et al. 2008; Shin et al. 2010b), black porgy (An et al. 2010), and crab (Paital and Chainy 2010) exposed to hypoosmotic environments. Our results indicate that the expression and activation of antioxidant enzymes in the cinnamon clownfish increased to reduce the oxidative stress induced by osmotic stress. Furthermore, the H_2O_2 concentration increased in the hypoosmotic environment (17.5 psu), indicating that the cinnamon clownfish experienced oxidative stress upon exposure to a hypoosmotic environment (Figure 5A). In contrast, expression and activation of the antioxidant enzyme genes decreased in fish exposed to a hypoos-

motic environment after PRL treatment. PRL, which is involved in freshwater adaptation, appeared to help fish adjust to hypoosmotic environments and reduce stress, consequently reducing oxidative stress. The basis of this conclusion lies in the finding that the levels of H_2O_2 and LPO decreased following PRL treatment (Figure 3A and 4A).

Similar to the results in the hypoosmotic environment, low temperature resulted in an increase in the expression and activation of antioxidant enzyme genes and H_2O_2 concentration in the livers of cinnamon clownfish (Figure S-2 in supplementary data[1] and Figure 2). The reason for this is that cinnamon clownfish, which live in subtropical environments, experience stress when exposed to low temperatures, whereby oxidative stress also increases. Although previous studies have examined antioxidant enzyme gene expression under different water temperatures, they included fish such as goldfish and olive flounder that were exposed to high-temperature environments (Bagnyukova et al. 2007; An et al. 2010; Shin et al. 2010a). To date, no study has been conducted on the intracorporeal oxidative stress due to low temperature. ROS or oxygen free radicals seem to be produced in the body because of oxidative stress when marine organisms are exposed to temperatures other than their normal habitat temperature, and the expression and activation of antioxidant enzyme genes subsequently increase to eliminate these oxygen free radicals (Abele et al. 1998; An et al. 2010). Although no deaths were recorded during the experiment, the results led to the speculation that cinnamon clownfish continuously experience oxidative stress in hypoosmotic or low-temperature environments.

The ROS produced due to hypoosmotic and low-temperature stress damage lipids and promotes LPO (Bagnyukova et al. 2007). The current study also showed that LPO tended to increase in cinnamon clownfish exposed to hypoosmotic and low-temperature environments, so LPO may be induced by oxidative stress caused by salinity and temperature changes. This result was in agreement with previous results in black porgy; i.e. LPO increased due to oxidative stress induced by salinity and water temperature changes (An et al. 2010). Furthermore, the current results are also in agreement with those of Lushchak and Bagnyukova (2006a, b), who found that the amounts of LPO products, namely, lipid peroxides and thiobarbituric acid-reactive substances, increased quickly in goldfish (*Carassius auratus*) tissues because of oxidative stress induced by thermal stress (35°C). Additionally, Chien and Hwang (2001) reported that the amount of MDA increased in thornfish (*Terapon jarbua*) exposed to 36°C water.

In contrast, plasma lysozyme activation tended to decrease in cinnamon clownfish exposed to hypoosmotic and low-temperature environments. Lysozyme is used as an immunity index. When the external environment changes rapidly, the stress occurring in the body suppresses immune function, which activates lysozyme (Wang et al. 2008). The current results are in agreement with those of Cheng et al. (2009) and Shin et al. (2010b), who found that lysozyme activity decreased in olive flounder and orange-spotted grouper, *Epinephelus coioides*, exposed to hypoosmotic and low-temperature conditions, respectively. Thus, one can speculate that a sudden change in environment induces intracorporeal stress, resulting in a decrease in immune function.

In this study, we examined the immune function changes due to salinity and water temperature changes by analyzing oxidative stress, and we measured physiological changes, such as changes in plasma ALT and AST concentrations, to measure the stress levels in cinnamon clownfish. ALT and AST are amino transferase enzymes, and their concentration in blood is a general index of liver function in vertebrates. These enzymes can also be used to evaluate the stress response to temperature change, low oxygen, pH, ammonia, or heavy metals (Pan et al. 2003).

In this study, plasma AST and ALT concentrations increased in cinnamon clownfish exposed to both hypoosmotic stress and low-temperature environments. Therefore, we can conclude that oxidative stress occurs in cinnamon clownfish because of salinity and temperature changes, and that liver cells are damaged in consequence. Furthermore, considering the continuous increase in antioxidant enzyme gene activation upon exposure to hypoosmotic and low-temperature environments in relation to the increase in the concentrations of AST and ALT, continuous oxidative stress due to salinity and temperature changes seems to cause liver damage, which in turn affects normal antioxidant actions.

Acknowledgements

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Note

1. Supplementary material can be found by clicking on the Supplementary Content tab at http://dx.doi.org/10.1080/19768354.2011.604941.

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