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Combined Effects of Copper and Temperature on Antioxidant Enzymes in the Black Rockfish *Sebastes schlegeli*

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Abstract

Copper has been widely used to control algae and pathogens in fish culture ponds. However, its toxic effects on fish depend not only on its concentration in the water but also on the water quality. A laboratory experiment was conducted to assess copper toxicity in the black rockfish *Sebastes schlegeli* using a panel of antioxidant enzymes, including glutathione (GSH), glutathione S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR) and superoxide dismutase (SOD), at different levels of copper at three water temperatures (WT, 18, 23, 28°C) for 4 days. After exposure to two copper concentrations (100 and 200 μg/L), GSH levels and GST activities increased significantly, depending on WT ($P < 0.05$) in the liver, gill, and kidney of the black rockfish. GPx and SOD activities decreased significantly with both increasing WT and copper treatment in the organs of black rockfish ($P < 0.05$). These changes can be seen as initial responses to temperature stress and as a sustained response to copper exposure. This also indicates that GSH and related enzymes activities were sensitive indexes to stress by toxicants such as copper. The present findings suggest that simultaneous stress due to temperature change and copper exposure can accelerate changes in enzymes activities in the black rockfish. This provides another example of synergism between environmental temperature and pollutants, which may have important implications for the survival of fish in polluted environments during seasonal warming and/or global climate change.

Key words: *Sebastes schlegeli*, Copper, Water temperature, Antioxidant enzymes

Introduction

Water temperature is an environmental factor in aquaculture that affects the growth and survival of aquatic animals (Wang et al., 2006). Recently, the results of an analysis of observed temperature data by Seong et al. (2010) showed that sea surface temperatures in the East, West, and South seas of Korea were increasing. In fish, changes in temperature can affect their ability to obtain oxygen from the environment and may promote changes at the physiological level that alter the rate of oxygen consumption and its delivery to tissues (Powell and Watts, 2006). For example, from July to early September 2012, there was mass mortality in fishes, particularly the black rockfish *Sebastes schlegeli* being raised in floating fish cages along the coast of Gyeongsangnam-do, Korea. The amount of damage was 1,802,000 fishes and the cause was confirmed to be a rapidly rising water temperature (Lee et al., 2013). Previous studies showed that rising temperature resulted in higher metabolism, increasing oxygen consumption and, consequently, the production of reactive oxygen species (ROS) (Lushchak and Bagnyukova, 2006a; An and Choi, 2010). The study of the influence of elevated temperature as one of the environmental stressors on the antioxidant profile of aquatic organisms becomes important because they are often exposed

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to multiple stressors in the aquatic environment (Bocchetti et al., 2004).

Accumulation of heavy metals in fish can also lead to redox reactions, generating free radicals, and especially ROS. Among them, copper is a pollutant widespread in surface waters at concentrations of up to 100 μg/L (Roy, 1997). Copper sulfate ($CuSO_4$) has been used widely to control algae and some pathogens in fish culture ponds, further increasing copper concentrations in water (Carvalho and Fernandes, 2006). Copper is highly toxic to fish, so the concentrations used to control algae or pathogens must be below the toxicity threshold for fish. Copper toxicity varies with water chemistry, temperature, and fish species, and induces various forms of damage that can lead to the death of the fish (Sanchez et al., 2005). The effects of copper on the aquatic environment are complex and depend on the physiochemical properties of the water (Takasusuki et al., 2004). High temperatures tend to increase the diffusion rate, accelerating chemical reactions and changing the rates of various processes that may become uncoordinated, leading to imbalances (Reynolds and Casterlin, 1980). Thus, a combination of temperature and copper stress is an environmentally relevant situation for populations of intertidal poikilotherms in polluted environments.

At the cellular level, copper has been described as a promoter of oxidative stress, catalyzing the formation of ROS, such as the OH· radical, through the Haber-Weiss reaction, and causing damage to DNA, proteins, and lipids (Matés, 2000; Linde et al., 2005). Cells have a well-developed antioxidant defense system to protect against oxidative stress. Glutathione (GSH)-associated metabolism is a major protective mechanism against agents that cause oxidative stress. GSH participates in detoxification at several levels, and may scavenge free radicals, reduce peroxides, or become conjugated to electrophilic compounds. Several antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), convert ROS into less-noxious compounds. Collectively, these enzymes represent the first line of defense against superoxide and H_2O_2. However, such enzymes are not 100% effective under normal conditions. Because some of the chemicals generated following reactions between ROS and macromolecules are themselves highly reactive, these secondary oxidation products must also be detoxified to prevent them damaging DNA, proteins, and lipids. This second line of defense against ROS is provided by enzymes such as glutathione-*S*-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), aldo-keto reductase, and aldehyde dehydrogenase (Yu, 1994). Copper also binds directly to thiol-containing molecules, such as glutathione (GSH) and metallothionein (MT), and becomes trapped (Roesijadi, 1996).

Thus, in the present study, we sought to evaluate the combined effects of elevated temperature and copper on antioxidant profiles (GSH, GST, GPx, GR, and SOD) in the liver, gill, and kidney of the black rockfish.

Materials and Methods

Experimental animals and water conditions

Black rockfish *Sebastes schlegeli* were sampled from a fish farm in Gyeongsangnam-do, Korea. After being transported to the laboratory, healthy fishes without visible damage were selected for 4 weeks of adaptation. Fishes averaging 13.29 ± 1.03 cm in body length and 29.76 ± 1.04 g in body weight were grouped randomly into a glass tank (39 × 54 × 30 cm) after adaptation. The water quality parameters used for the bioassays conducted are provided in Table 1.

Exposure protocol

Prior to the temperature acclimation, fish were held for 4 weeks in aerated tanks containing 18°C seawater to ensure a common thermal history between the acclimation groups. Temperatures were then adjusted from 18°C in 1°C steps on alternate days to final temperatures of 23 or 28°C using a water heater (Electronic thermostat, MS701-H, Mink, Korea).

Toxicity tests with two replicates (12 fishes at each copper concentration and controls) were carried out in static systems with continuous aeration, at constant temperatures (18, 23, and 28°C) for 96 h in a constant-temperature room (18°C). Experimental fish were exposed to waterborne treatment of 0, 100, and 200 μg/L $CuSO_4$ (copper (II) sulfate, minimum 99%, Sigma, USA) concentrations for 96 h at each temperature. Following the toxicity test, fish were deeply anesthetized using benzocaine, and the liver, gills, and kidney were removed and stored at -80°C until analysis.

Biochemical analysis

Liver, gill, and kidney tissues for determination of enzyme activity were rinsed in 0.1 M KCl (pH 7.4) and homogenized (099CK4424, Glass-Col, Germany) in homogenization buffer (0.1 M K_2HPO_4, 0.15 M KCl, 1 mM DTT, 1 mM EDTA, 1 mM

Table 1. The chemical components of seawater and experimental condition used in the experiments

Test parameters	Value
Culture type	Renewal 24h toxicity test
Temperature (℃)	18.0 ± 0.5
pH	8.1 ± 0.5
Salinity (‰)	33.5 ± 0.6
Dissolved oxygen (mg/L)	7.1 ± 0.3
Chemical oxygen demand (mg/L)	1.13 ± 0.1
Ammonia (mg/L)	12.5 ± 0.7
Nitrite (mg/L)	1.3 ± 0.3
Nitrate (mg/L)	11.48 ± 1.0
Copper (μg/L)	≤ 0.1

PMSF). The homogenate was centrifuged (12,000 *g*, 25 min, 4°C), and the supernatant was stored at -75°C until use.

Total glutathione

The total glutathione (GSH) contents of tissues were quantified using the method of Richardson and Murphy (1975). Briefly, the working solution containing 0.01 M 5,5'-dithiobis, 2-nitrobenzoic acid (DTNB, Sigma, USA) and 0.1 M PBS buffer (pH 8.0) was added to the sample. The GSH level was evaluated at 412 nm using a spectrophotometer (Zenyth 200, Anthos Labtec Instruments GmbH, Austria) and determined using a reduced glutathione standard curve. The GSH level was expressed as nmol GSH/mg protein.

Glutathione *S*-transferase

Glutathione *S*-transferase (GST) activity was measured using a modification of the method of Habig et al. (1974). The sample was mixed with 0.2 M potassium phosphate (pH 6.5) and distilled water. The reaction was initiated by adding 10 mM GSH (Sigma, USA) and 10 mM CDNB. The reaction mixture was stored at room temperature for 1 min. GST activity was determined by the absorbance increase at 340 nm after 5 min, expressed as nmol/min/mg protein.

Glutathione peroxidase

Glutathione peroxidase (GPx) activity was measured by determining the decrease in NADPD (Sigma, USA) during the formation of reduced glutathione via glutathione reductase at 340 nm by a modified method of Bell et al. (1985). In this procedure, 2.5 mM H_2O_2 in 50 mM potassium phosphate buffer (1 mM GSH, 0.1 M NADPH, 0.5 U/mL glutathione reductase, 1 mM EDTA, 2 mM sodium azide, pH 7.4) was used as the substrate.

Glutathione reductase

Glutathione reductase (GR) activity was determined using NADPH and oxidized glutathione (GSSG) as substrate, as described by Beutler (1984). One unit of GR was defined as the NADPH consumed per min, which catalyzed the reduction of 1 mmol GSSG.

Superoxide dismutase

Superoxide dismutase (SOD) activity was measured using a SOD assay kit (Dojindo Co., Japan) to determine the 50% inhibition rate of the reduction of 2-(4-lodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-*2H*-tetrazolium, monosodium salt ('WST-1'). Supernatants were evaluated at an absorbance of 450 nm after dilution in 0.1 mM PBS. SOD activity at the 50% inhibition rate was expressed as units/mg protein.

Protein

Protein concentrations were determined using the Bradford (1976) method, with bovine serum albumin as the standard.

Data analysis

Data are presented as means ± standard error. For statistical analysis, a one-way analysis of variance (ANOVA) was used, followed by Duncan's multiple range test. We used the SPSS software (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant when $P < 0.05$.

Results

Glutathione (GSH)

Fig. 1 shows the GSH levels in liver, gills, and kidney of black rockfish *Sebastes schlegeli* and the interactions of water temperature and copper for 4 days. Although the kidney showed no significant change in GSH levels, liver and gill GSH levels were increased significantly at 28°C versus 18°C in the group not exposed to copper. Copper caused a significant increase in GSH levels in the liver, gills, and kidney of *S. schlegeli*, compared with the non-exposed groups at each test temperature. At 18°C, the GSH levels were significantly higher with 200 than 100 μg/L in both gill and kidney. However, acclimation to 23 and 28°C showed no difference between 100 and 200 μg/L in the GSH levels, although the levels were significantly higher than in the non-exposed groups in the gills and kidney (Fig. 1B, C).

Glutathione *S*-transferase (GST)

Fig. 2 shows the GST activities in the liver, gills, and kidney of *S. schlegeli* and the interactions of water temperature and copper for 4 days. Copper caused a significant increase in GST activities in the liver, gill, and kidney, compared with the non-exposed groups, regardless of water temperature. In particular, GST activities were significantly higher in the presence of 200 μg/L than 100 μg/L copper, in the liver, gill, and kidney at each water temperature.

Glutathione peroxidase (GPx)

Fig. 3 shows the GPx activities in the liver, gills, and kidney of *S. schlegeli* and the interactions of water temperature and copper for 4 days. The GPx activities in the liver and gills decreased significantly at 28°C compared with those at 18 and 23°C in the non-exposed groups. Copper caused significant inhibition of GPx activities in liver, gill, and kidney compared with the non-exposed groups at each test temperature. These reductions in GPx activities in the liver, gill, and kidney of

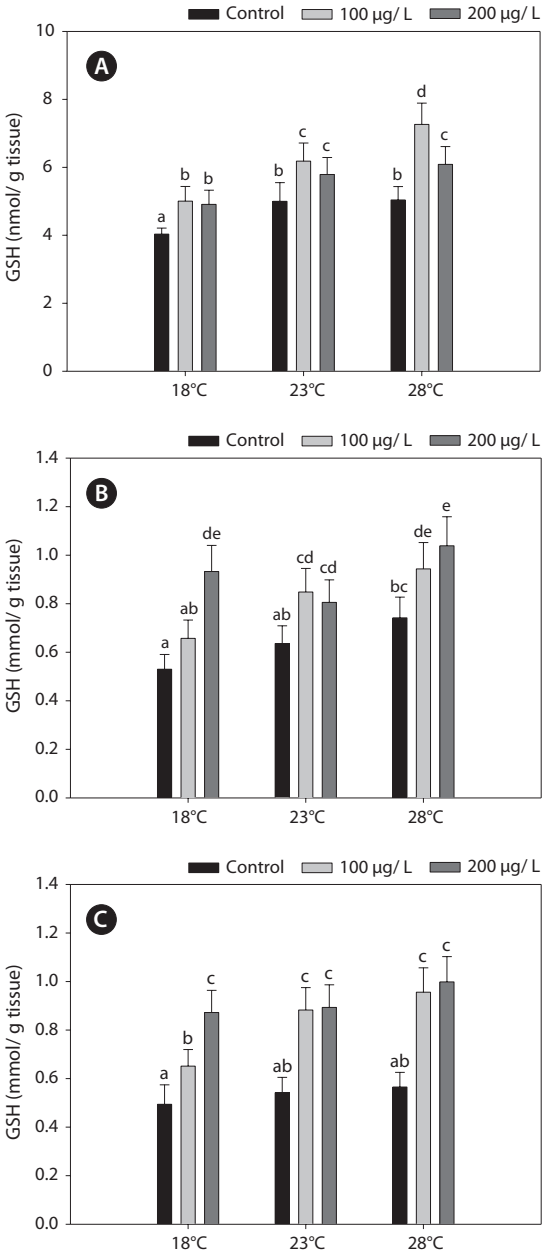

Fig. 1. Glutathione (GSH) levels in liver (A), gill (B) and kidney (C) of the black rockfish *Sebastes schlegeli* exposed to copper at 18, 23 and 28°C for 4 days. Vertical bar denotes one standard deviation of the mean. Different letters indicate significant difference ($P < 0.05$) between the groups (n=12).

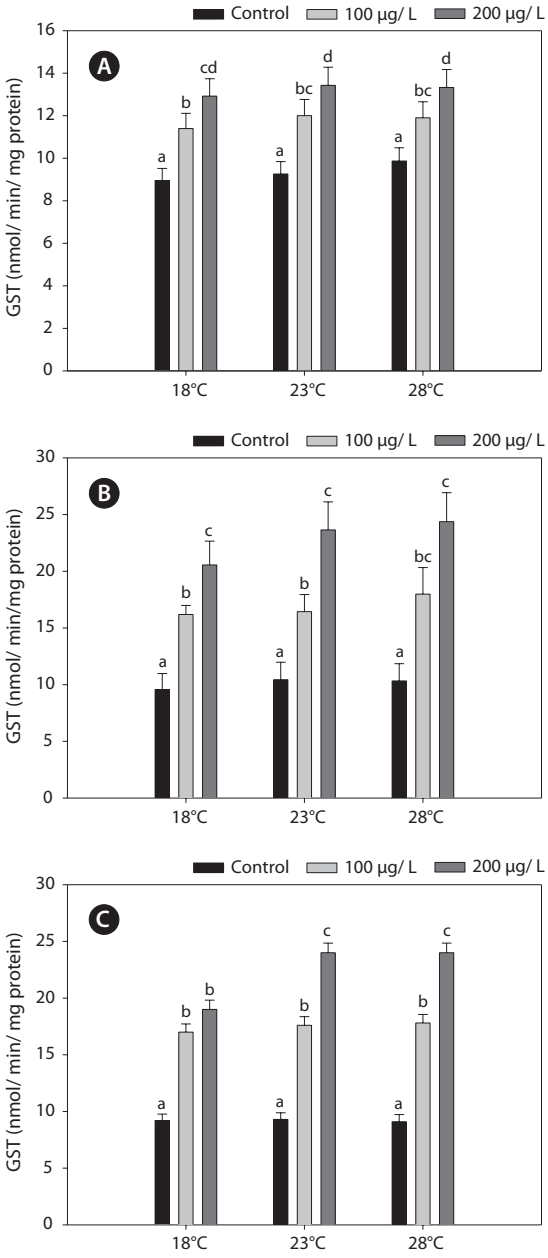

Fig. 2. Glutathione *S*-transferase (GST) activities in liver (A), gill (B) and kidney (C) of the black rockfish *Sebastes schlegeli* exposed to copper at 18, 23 and 28℃ for 4 days. Vertical bar denotes one standard deviation of the mean. Different letters indicate significant difference ($P < 0.05$) between the groups (n=12).

fish exposed to copper were promoted by acclimation to 28°C.

Glutathione reductase (GR)

The GR activities in the liver and gill were similar between 18°C and warm (23 and 28°C)-acclimated fish (Fig. 4). Also, the GR activities in the liver and gill of non-exposed and copper-exposed fish did not differ ($P > 0.05$). However, GR activities in the kidney were increased significantly after exposure to copper, but were unaffected by temperature acclimation. Copper caused a significant increase in GR activities in the liver, gill, and kidney of *S. schlegeli* compared with the non-exposed groups, regardless of water temperature. In particular, GR activities were significantly higher in the presence of 200 μg/L than 100 μg/L copper, but were unaffected by temperature acclimation.

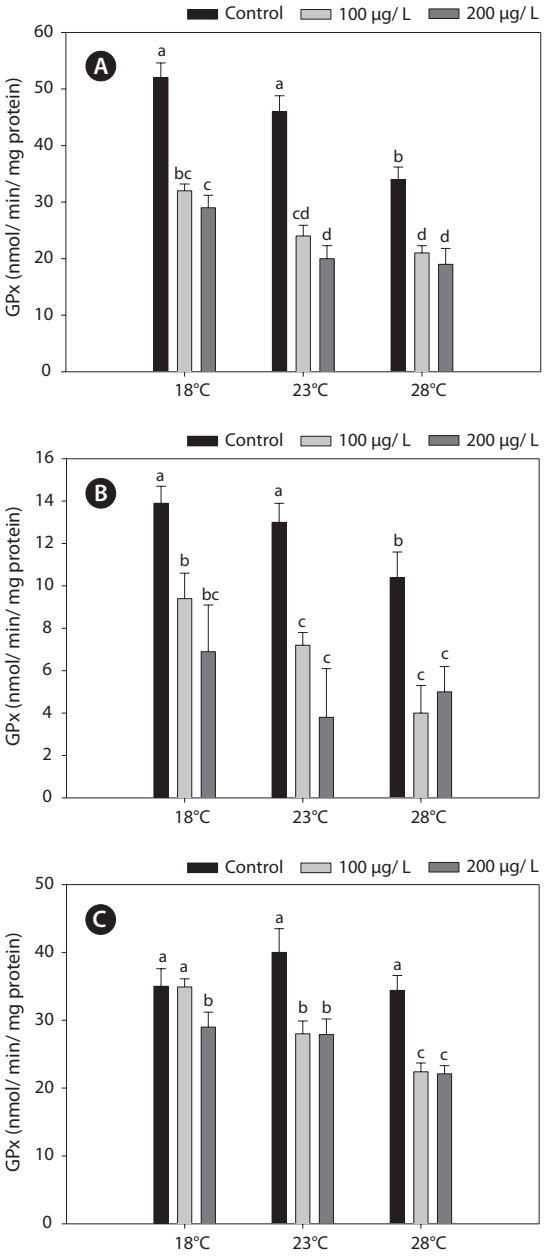

Fig. 3. Glutathione peroxidase (GPx) activities in liver (A), gill (B) and kidney (C) of the black rockfish *Sebastes schlegeli* exposed to copper at 18, 23 and 28℃ for 4 days. Vertical bar denotes one standard deviation of the mean. Different letters indicate significant difference ($P < 0.05$) between the groups (n=12).

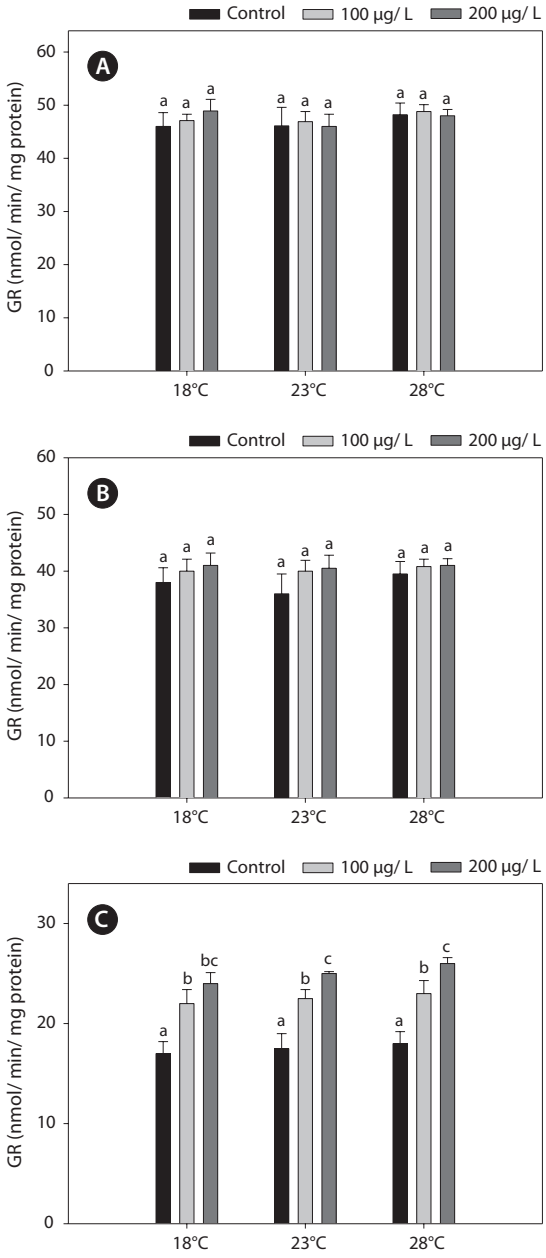

Fig. 4. Glutathione reductase (GR) activities in liver (A), gill (B) and kidney (C) of the black rockfish *Sebastes schlegeli* exposed to copper at 18, 23 and 28℃ for 4 days. Vertical bar denotes one standard deviation of the mean. Different letters indicate significant difference ($P < 0.05$) between the groups (n=12).

Superoxide dismutase (SOD)

In this study, SOD levels did not differ significantly between 18°C- and warm (23°C and 28°C)-acclimated fish. SOD activities in liver and gills decreased significantly after copper treatment, compared with the non-exposed group, and these reductions were enhanced by acclimation to 28°C (Fig. 5).

Discussion

Dissolved copper—as $CuSO_4$—at low molecular concentrations has been reported to be toxic to zebrafish *Danio rerio* and rainbow trout *Oncorhynchuss mykiss* cells exposed to copper while subjected to oxidative stress. Antioxidants could protect these cells from the effects of the metal (Manzl et al., 2004; Olivari et al., 2008). An important feature of antioxidant

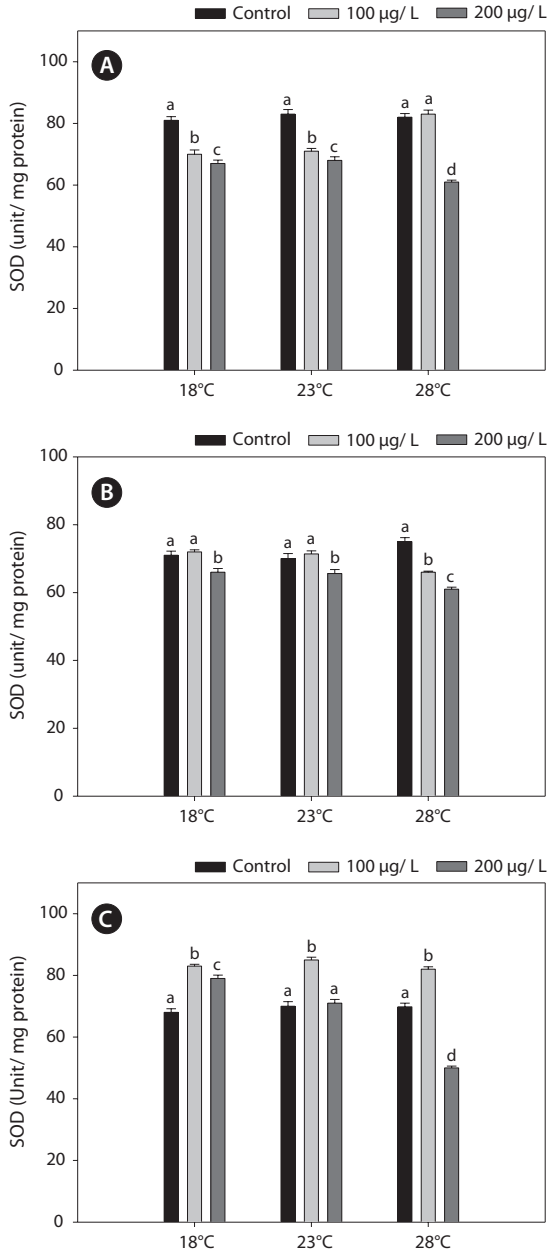

Fig. 5. Superoxide dismutase (SOD) activities in liver (A), gill (B) and kidney (C) of the black rockfish *Sebastes schlegeli* exposed to copper at 18, 23 and 28℃ for 4 days. Vertical bar denotes one standard deviation of the mean. Different letters indicate significant difference ($P < 0.05$) between the groups (n=12).

enzymes is their induction by slight oxidative stress. Severe oxidative stress, however, suppresses the activities of these enzymes due to damage and loss of compensatory mechanisms (Zhang et al., 2004). Several studies have reported that acute exposure to elevated temperatures in fishes and marine invertebrates results in an increase in oxidative damage (Estevez et al., 2002; Lushchak and Bagnyujova, 2006b; Mueller et al., 2012). Additionally, it has been shown that seasonal acclimation to warm temperatures results in higher oxidative stress indices (Bagnyukova et al., 2007; Heise et al., 2007). Overall, these findings support the notion that higher levels of antioxidant defense are necessary in many ectotherms at warmer body temperatures, possibly in response to increased rates of ROS production and/or increased ROS-induced damage. In fact, water temperature affects many chemical and biological processes, including the amount of dissolved oxygen in water, the rate of chemical reactions, and the mobility and metabolism of organisms as well as their sensitivity to toxic substances, parasites, and disease (Bolton and Havenhand, 2005; Mubiana and Blust, 2007).

Anti-oxidant enzyme activities are significantly increased in copper-exposed fish, indicating that copper causes oxidative stress in fish (Florence et al., 2002; Sanchez et al. 2005; Varo et al., 2007; Vieira et al., 2009). However, we suggest that after temperature acclimation heavy metals have no toxic effects on the components of the glutathione antioxidant defense system. Indeed, in the present study, we demonstrated the combined effects of thermal and copper stress on five antioxidant enzymes in black rockfish *Sebastes schlegeli*. The 'no observed effect concentrations' (NOECs) of copper in 14 freshwater fish species were 4–120 Cu μg/L (Grosell et al., 2002). Thus, we used copper concentrations of 100 and 200 μg/L, although a seawater fish, the black rockfish, was assessed in this study.

Acute copper toxicity is believed to result primarily from an imbalance in ion regulation in the gill (Wood, 2001). Temperature affects ion regulation processes in fish and may increase copper toxicity at higher temperatures (Lemus and Chung, 1999). Changes in membrane permeability and increased breathing frequency, which may accelerate copper absorption, are the main reasons for the increased toxicity of copper at higher temperatures. Several mechanisms have been proposed to explain copper-induced cellular toxicity. Most commonly, the basis of these theories is the propensity of free copper ions to participate in the formation of ROS. Copper ions participate in oxidation and reduction reactions. In the presence of superoxide or reducing agents, such as GSH, Cu^{2+} can be reduced to Cu^{+}, which is capable of catalyzing the formation of hydroxyl radicals from hydrogen peroxide via the Haber-Weiss reaction (Kadiiska et al., 1993; Bremner, 1998).

Endogenous GSH plays a role in cells as an antioxidant, as a co-factor of GPx, and participates in the reduction of peroxides, with concomitant formation of oxidized glutathione disulfide (GSSG). Under normal physiological conditions, GSSG is reduced to GSH by GR at the expense of reduced NADPH, thereby forming a redox cycle (Parke and Piotrowski, 1996). GSH is central to the detoxification of ROS, but in the absence of an enzyme system to catalyze the multiple detoxification reactions, GSH cannot function as an intracellular antioxidant. The production of GSSG by the reduction of GPx or as a consequence of free-radical scavenging is potentially highly cytotoxic. Subsequently, GSSG is reduced by GR,

using NADPH as a reductant, or exported by the multidrug resistance-associated protein (Dringer, 2000). Accordingly, the activity of GR represents one of the most important determinants of cellular protection against oxidative stress (Hayes and McLellan, 1999). In this regard, our study showed that both increased GSH and GR and decreased GPx activities are of importance in protecting against copper-induced oxidative stress. Furthermore, fluctuations in GSH activities in organisms exposed to metals are generally accompanied by variations in GST, which conjugates GSH to various xenobiotic compounds (Sheweita et al., 1998). Also, the S-conjugation reaction by GSH is accelerated significantly by GST (Strange et al., 2000). These findings support the results of our study; i.e., the interactions between GSH and glutathione-related enzyme activities through redox cycles in the black rockfish. Specifically, changes in the activities of GPx, GR, and GST can alter the GSH concentration.

In this study, copper alters the activities of these enzymes, resulting in modulated antioxidant capacity. Also, this effect of copper was particularly pronounced at elevated temperatures for 96 h. In detail, GST and GR activities were significantly higher in copper-exposed fish than control, non-exposed fish (Figs. 2, 4), and remained elevated even upon exposure to copper, with the exception of GR activity, in which no significant difference was observed between fish acclimated to temperatures of ≥18°C.

Regarding the basis of the enzymatic activities in the liver and kidney, the increases in the antioxidant capacity in copper-exposed fish were probably linked to the increased metabolic activity in warmer temperatures. This activation was related to an adaptive mechanism against stress (Schreck, 1981). In this study, while copper modulated other antioxidant enzyme activities in liver and gills, it had no effect on GR activity in the liver or gill. However, kidney GR activities were increased significantly, in a copper-concentration-dependent manner (Fig. 4). This discrepancy between the liver and kidney antioxidant capacities highlights the central position of the liver in the antioxidant responses of fish. Although copper is an essential micronutrient, it can accumulate and overload hepatocytes, causing liver injury (Pourahmad and O'Brien, 2000).

Kaur et al. (2005) showed that 3-h heat treatment (from 20 to 32°C) of *Channa punctata* modulated antioxidant enzymes – GSH, GST, GR, and CAT – in the liver, gill, and kidney. The authors suggested that although the patterns of the increases/decreases varied among tissues, the changes were unambiguous. Modulation of antioxidants by heat stress has been reported in broiler chickens and marine invertebrates (Heise et al., 2003; Mahmoud and Edens, 2003). Regarding the antioxidant response under temperature stress, Prakash et al. (1998) reported ROS production in response to temperature stress of 12°C in the gills of the catfish *Heteropneustes fossilis*.

In the present study, GST and GR activities were increased, and that of SOD was decreased, by copper in the liver, gill, and kidney, regardless of the water temperature (Figs. 2, 4, 5). Although acclimation to warm conditions had no effect on GST, GR, and SOD activities, both GSH and GPx were affected by temperature (Figs. 1, 3). Grim et al. (2013) suggested that because activities of glutathione-dependent enzymes were unaffected by temperature acclimation, the protein levels of glutathione-dependent antioxidants may limit the activity of the enzymatic system at lower temperatures. Similarly, in other studies, SOD and CAT activities were unchanged in fishes following temperature acclimation (Leggatt et al., 2007; Grim et al., 2010). Furthermore, there is no consensus as to whether changing body temperature impacts oxidative stress in fishes (Grim et al., 2010; Kammer et al., 2011; Mueller et al., 2012). However, Cherkasov et al. (2007) reported that Cd-induced oxidative stress in oyster mitochondria was particularly pronounced at 30°C versus 20°C. They suggested that antioxidants conferred adequate protection against the Cd-induced increase in ROS production at 20°C. In contrast, at 30°C, antioxidant systems appeared to be incapable of coping with Cd-induced ROS generation, and considerable oxidative damage resulted. Lanning et al. (2006) also suggested that elevated ROS production in response to Cd might be the mechanism underlying the increased lipid peroxidation at 24 compared to 28°C. These findings are consistent with the results of the present study, in that exposure of black rockfish to copper resulted in significant modulation of GSH and GPx activities at elevated temperatures (23 and 28°C). This study also supports the notion that elevated temperatures can exacerbate copper-induced oxidative stress in the black rockfish, and emphasizes the importance of the thermal context when assessing metal toxicity.

In conclusion, our data indicate that a higher water temperature may increase the toxicity of copper, so that antioxidant defenses in various tissues become overwhelmed, leading to elevated oxidative stress. This provides another mechanism for the synergistic effects between environmental temperature and pollutants, which may have important implications for the survival of fish in polluted environments during seasonal warming and/or the temperature increase caused by global climate change.

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