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Biochemical Changes in Chinese Pond Mussel *Anodonta woodiana* Following Exposure to Copper

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We evaluated the potential utility of biochemical parameters as indicators of exposure to water–borne copper (Cu) in the freshwater mussel *Anodonta woodiana*. Semi–static renewal tests showed that the 96 h median lethal concentration of Cu was 3.4 mg/L (95% confidence interval [CI]: 2.1–6.5 mg/L) for juveniles and 22.1 mg/L (CI: 13.8–28.6 mg/L) for adult *A. woodiana*. In vivo bioassays were performed to determine the activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), and the levels of glutathione (GSH) in the adductor muscle, foot, gill, mantle, and visceral mass of *A. woodiana* after 48 and 96 h of exposure to Cu concentrations of 0.125 and 0.25 mg/L for juveniles, and 0.75, 1.5, 3 mg/L for adults. The levels of the lipid peroxidation product, malondialdehyde (MDA), were evaluated in five adult tissues. Our results indicated that SOD and CAT activity, and GSH levels in juvenile mussel foot and adductor muscle of adults might be sensitive to Cu exposure. This antioxidant defense system (CAT, GSH) was significantly enhanced in the mantle of juvenile mussel exposed to 0.25 mg Cu/L. The effect of Cu on the foot and mantle of juveniles, and the adductor muscle of adults seemed to be directly correlated with the levels of reactive oxygen species and antioxidant defense system components. Our data suggest that the biochemical responses (SOD and/or CAT, GSH) in the foot and mantle of juveniles, and the adductor muscle of adult *A. woodiana* might be good biomarkers of Cu contamination.

Key words: *Anodonta woodiana*; copper; tissue; biochemical changes; biomarker

INTRODUCTION

Copper (Cu) is an important micronutrient and essential element for bivalve development; however, it is also a widespread pollutant found in aquatic environments (Lorenzo *et al*., 2005; Rivas *et al*., 2007), ranking as the metal of highest concern for aquatic organisms (Donnachie *et al*., 2014). At low levels of exposure (<0.1 mg/L) mussels can bioaccumulate Cu to various extents in different tissues (Amiard–Triquet *et al*., 1986). Excess Cu is frequently found following chemical treatments, such as fungicide application in aquaculture ponds to eradicate filamentous algae; application of copper sulfate as high as 2 mg/L has been reported (Li *et al*., 2007). Such high concentration may result in serious injury to aquatic organisms through the production of reactive oxygen species (ROS) and oxidative damage.

Antioxidant defenses are present in all aerobic organisms, and involve enzymatic and non–enzymatic constituents (Szaleczky *et al*., 1999). Antioxidant enzymes (superoxide dismutase [SOD] and catalase [CAT]) and free radical scavengers (reduction of superoxide anion), which remove ROS and maintain cell homeostasis, represent the first line of defense against the toxic effects of xenobiotics that may occur following exposure to pollutants (Doyotte *et al*., 1997). Nevertheless, when organisms are exposed to high levels of pollutants, antioxidant systems may be inhibited and oxidative stress may lead to enzymatic inactivation and lipid peroxidation (LPO) (Al–Subiai *et al*., 2011; Doyotte *et al*., 1997).

The development of biochemical biomarkers of non-lethal exposure may provide sensitive and rapid indicators that can be used to assess the cellular effects of contaminants. A recent study that measured oxidative stress and the antioxidant response of mussel to environmental chemicals was able to demonstrate that mussel condition can be rapidly documented (Farris and van Hassel, 2007). SOD, CAT, glutathione (GSH), and malondialdehyde (MDA) are the most common markers of antioxidant defense or oxidative damage that are affected by metal exposure (Eertman *et al*., 1995; Goswami *et al*., 2014; Radak *et al*., 2017). The antioxidant defense system involved in the detoxification of xenobiotics and their metabolites (biotransformation and antioxidant enzymes) has been extensively studied (Cossu *et al*., 1997, 2000; Doyotte *et al*., 1997), and has well–defined impacts on mussels.

The nature of antioxidant defense may differ for euryoxic and stenoxic organisms (Livingstone *et al*., 1992). However, few investigators have successfully used these reliable, sensitive, and definitive measures for freshwater bivalves. In fact, in the freshwater mussel, *Unio tumidus*, reduced glutathione was shown to be the most sensitive parameter indicating toxicant exposure (Doyotte *et al*., 1997). Therefore, freshwater mus-

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sels are potentially suitable species for assessing aquatic contaminants and biochemical responses, allowing freshwater environments to be monitored.

The swan mussel *Anodonta woodiana* Lea, 1834 is typical among unionid bivalves; it has largely expanded its worldwide distribution and is one of the most successful invasive mussel species (especially in Europe) (Douda *et al*., 2012; Guarneri *et al*., 2014). This species easily meets the requirements for environmental monitoring due to its wide distribution, sessile lifestyle, and high tolerance to chemical contaminants (Chen and Xie, 2005; Liu *et al*., 2010; Yokoyama and Park, 2002). *A. woodiana* has been successfully bred and cultured under artificial conditions in our laboratory (Chen *et al*., 2015). Since artificially–propagated *A. woodiana* displayed low background levels of heavy metals (Chen *et al*., 2012), it is suitable for standardized monitoring or toxicity testing of waterborne contaminants. Thus far, the toxicity and bioaccumulation of organotins (Yang *et al*., 2008), organochlorines (Bian *et al*., 2009), and heavy metals (Liu *et al*., 2010) have been studied using *A. woodiana* from freshwater fisheries and aquaculture. However, less is known about the biochemical response of these contaminants or the potential of target tissues in the mussel at different life history stages.

Since we hypothesize that Cu exposure will lead to oxidative stress and induce LPO (Regoli, 1998), and that such disruption may have age–, tissue–, and dose–specific tendencies in *A. woodiana*, the aim of this study was to investigate the biochemical responses of five tissues of *A. woodiana* (i.e., adductor muscle, gill, foot, mantle, and visceral mass) following exposure to nonlethal Cu concentrations. The impaired response was assessed by measuring the levels of biomarkers of oxidative damage, namely SOD, CAT, GSH, and LPO. In this study, LPO was determined by measuring MDA. MDA is a common end–product of LPO, and is highly reactive and can form simple and complex adducts with proteins (Que *et al*., 2013). This study was performed to elucidate the biochemical response of bivalves to Cu exposure and to develop biomarkers for the assessment of Cu ecotoxicity in this freshwater bivalve mollusk.

MATERIALS AND METHODS

Chemicals

American Standard Testing Method (ASTM)–reconstituted water (ASTM, 2006) was prepared by adding reagent–grade salts ($NaHCO_3$, $MgSO_4$, $CaSO_4 \cdot 2H_2O$, and KCl; analytical reagent grade quality) to deionized water, and was used in all reference toxicant tests. The nominal concentrations of Cu, sodium (Na), magnesium (Mg), potassium (K), and calcium (Ca) were 0, 30, 15, 2, and 10 mg/L, respectively. Copper sulfate ($CuSO_4 \cdot 5H_2O$, analytical reagent grade quality) was chosen as the test chemical and was prepared by adding and dissolving adequate chemical stock to the ASTM–reconstituted water. The chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China.

Sampling

Six–month–old (mean ± standard deviation: shell length 25.0 ±1.5 mm, shell height 15.2 ± 1.5 mm, shell width 6.6 ± 0.9 mm) and 4–year–old adults (shell length 85.3 ± 3.6 mm, shell height 53.4 ± 2.8 mm, shell width 33.7 ± 1.9 mm) of *A. woodiana* were collected from our culture pond at Nanquan Aquatic Base, Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences. The culture contains an artificially–propagated, standardized, healthy *A. woodiana* population with successful recruitment. Live mussels were transported directly to the laboratory and kept in sediment–free container (1×1×1 m) supplied with 30 L of dechlorinated tap water. Aeration was provided continuously by plastic tubes with a gentle air flow. The mussels were acclimatized to laboratory conditions for 5 days prior to the experimental period. Mussels were fed with 1 g/L of *Chlorella* sp. (Chlorella Industry Co., Tokyo, Japan) once every 2 days and maintained under a 16:8 h light/dark photoperiod. Mussel survival exceeded 95%. The criteria required for a death record were prolonged valve opening and no response to tactile stimulation (APHA, 1999).

Toxicity experiment

Juvenile mussel treatment

The test treatments (acute toxicity tests) were performed according to the methods set forth in the Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels of the American Society for Testing and Materials (ASTM, 2006). Five juvenile mussels were placed in a 200 mL glass crystallizing dish (122 mm diameter, 22 mm deep) containing 150 mL ASTM–reconstituted water with a Cu concentration of 0, 0.625, 1.25, 2.5, 5, or 10 mg/L (nominal concentration hereinafter). Each concentration was tested in three dishes (five mussels per dish) and the 96 h EC_{50} value (Wang *et al*., 2009, 2011) was calculated via a probit analysis.

Based on the results of the acute toxicity tests, the effects of nonlethal Cu concentrations on antioxidant enzyme activity and GSH in the tissues of juvenile mussels were investigated. After a 48 h acclimation period, mussels were placed into glass aquaria (20 × 20 × 27.5 cm) with 2 L of ASTM–reconstituted water and exposed to different concentrations of Cu (control, 0.125, and 0.25 mg/L), with three replicates per treatment for 48 and 96 h. Approximately 75% of the test solution was changed after 48 h. Mussels were not fed during the test.

Adult mussel treatment

The experimental procedures followed the ASTM E2455–06R13 guideline for testing chemicals with mussels (ASTM, 2006). Before the experiments, 90 adult mussels were randomly selected and introduced into 18 glass aquaria (20 × 20 × 27.5 cm) containing 3 L of ASTM–reconstituted water and acclimated for 48 h. No mortality was observed during the acclimation period. Test mussels were not fed for 24 h prior to Cu exposure. Copper sulfate was prepared at concentrations of 0, 18.75, 37.5, 75, 150, and 300 mg/L (nominal concentrations) in ASTM–reconstituted water, and each set–up

consisted of three replicate containers. Photoperiod, duration of toxicity tests, test water changes, pH range, and water temperature were the same as those used in the juvenile toxicity tests. Dead mussels were removed daily whenever detected.

The effects of nonlethal concentrations of Cu on biochemical responses were bioassayed in a similar way. Mussels were exposed to Cu at concentrations of 0 (control), 0.75, 1.5, and 3 mg/L for 96 h. Renewal of test solutions, and water quality and photoperiod conditions were the same as those described for the juvenile mussel toxicity tests.

Water chemical analysis

Water samples (20 mL) were collected from each test concentration after 48 h of exposure. Copper analyses were performed with an inductively–coupled plasma mass spectrometer (ICP–MS, Agilent 7500ce, Agilent Technologies, USA) as described by Chen *et al*. (2012). Quality assurance and quality control was performed by spike recoveries; ranged from 99.7% to 101.5%.

The measured Cu, Na, Mg, K, and Ca concentrations were 0.00, 28.16, 13.25, 2.31, and 8.39 mg/L in ASTM–reconstituted waters, respectively. Water Cu concentrations in the toxicity test were 0.00, 0.126, and 0.23 mg/L for juveniles, and 0.00, 0.59, 1.42, and 2.84 mg/L for adults. The observed results will be presented based on the nominal concentration of Cu.

Tissue collection and determination of biochemical parameters

The adductor muscle, foot, gill, mantle, and visceral mass from each individual were obtained and rinsed with cold saline solution for further analysis. Tissues were weighed and homogenized in 9–fold (w/v) physiological saline. After centrifugation at 2500 × g for 10 min at 4°C, the supernatant was collected and SOD and GSH were measured using a microplate reader (Multiscan MK3; Thermo Fisher Scientific, Waltham, MA, USA), and CAT and MDA were measured using a spectrophotometer (722, Shanghai Jingke Instrument Plant, China) with commercially–available kits (SOD, A001–3; CAT, A007–1; GSH, A006–2; MDA, A003–1; Nanjing Jiancheng Institute of Biotechnology, Nanjing, China), according to the manufacturer's instructions. Briefly, SOD activity was assayed at 450 nm using xanthine oxidase as a superoxide generator and expresed as U/mg protein (Moustafa *et al*., 2010). CAT activity was measured following the method of Shimizu (Shimizu *et al*., 1984), and changes in absorbance were recorded at 405 nm. Results were expressed as U/mg protein. The GSH content was estimated using the method described by Jollow *et al*. (1974). The developed yellow color was read immediately at 405 nm and the content was expressed as µmol/mg protein. The amount of malonaldehyde was assayed in the form of thiobarbituric acid reacting substances (TBARS) using the method described by Esterbauer and Cheesman (1990), and the MDA formed in each sample was measured at 532 nm. The final concentration of MDA was expressed as nmol/mg protein.

Statistical analysis

The Cu EC_{50} at 96 h was calculated by probit analysis based on the nominal concentrations. EC_{50} was presented with 95% confidence intervals (CI) as EC_{50} (95% CI). The no–observable effect concentration (NOEC) and the lowest–observable effect concentration (LOEC) were determined using the method described by Wright and Welbourn (2002). The maximum allowable toxicant concentration (MATC) was calculated by taking the geometric means of the NOEC and LOEC (Wright and Welbourn, 2002).

The activities of Cu/Zn–SOD and CAT and the levels of GSH and MDA in treated swan mussel were compared with those of the control group at different sampling times (48 and 96 h) and data were expressed as means ± standard deviation (n = 3). Dunnett's test of one–way analysis of variance (ANOVA) was used to compare differences between the control and treatment groups. Linear regression analysis was also used to describe the relationship between biochemical parameters and Cu concentrations. All statistical analyses were performed using SPSS Statistics v.16.0 software (IBM Corp., Armonk, NY, U.S.A.) and only $P < 0.05$ was accepted as significant.

RESULTS

Copper exposure test

The results of acute Cu^{2+} toxicity tests are listed in Tables 1 and 2. The 96 h acute Cu EC_{50} was 3.4 mg/L for juvenile *A. woodiana* and 22.1 mg/L for adult *A. woodi-*

Table 1. Number of juvenile *Anodonta woodiana* deaths (mean ± standard error [SE]) following exposure to Cu for different durations (mean ± SE), and 48 and 96 h EC_{50} values of Cu for juvenile *A. woodiana* (mg/L)

Cu concentration	48 h	96 h
0	0	0
0.625	0.25 ± 0.50	1.00 ± 0.82
1.25	0.50 ± 0.58	1.25 ± 0.96
2.5	1.25 ± 0.96	2.25 ± 0.96
5	1.25 ± 0.96	2.75 ± 0.50
10	1.75 ± 0.96	3.75 ± 0.96

Table 2. Number of adult *Anodonta woodiana* deaths (mean ± SE) following exposure to Cu for different durations (mean ± SE), and 48- and 96- h EC_{50} values of Cu for adult *A. woodiana* (mg/L)

Cu concentration	48 h	96 h
0	0	0
18.75	1.33 ± 0.58	2.00± 0.00
37.5	1.67 ± 0.58	4.00 ± 0.00
75	4.67 ± 0.58	5.00 ± 0.00
150	5.00 ± 0.00	5.00 ± 0.00
300	5.00 ± 0.00	5.00 ± 0.00

ana. The 96 h LOEC and MATC were 0.625 and 0.25 mg/L for juveniles, and 18.75 and 5.63 mg/L for adults, respectively. Based on these data, the Cu gradient of sublethal exposure was selected as 0.125 and 0.25 mg/L for juvenile *A. woodiana*, and 0.75, 1.5, and 3 mg/L for adult *A. woodiana*. No mortality was observed in mussels exposed to a Cu gradient during the experiment.

Biochemical responses

Different response patterns for SOD and CAT activity, and GSH levels were observed in the adductor muscle (Fig. 1A, B, C), foot (Fig. 1D, E, F), gill (Fig. 1G, H, I), mantle (Fig. 1J, K, L), and visceral mass (Fig. 1M, N, O) of juvenile mussels after 48 and 96 h of Cu exposure.

After 96 h, SOD activity was decreased in each tissue, CAT activity was increased only in the mantle, and GSH levels were increased in the foot and mantle. Furthermore, the mantle exhibited significantly higher CAT activity (569% of control, $P = 0.005$) and GSH level (3081.3% of control, $P = 0.005$) compared with the control after 96 h of exposure to 0.25 mg/L Cu.

Additionally, adverse changes were found in antioxidant enzyme activities and GSH levels in the foot between mussels exposed to Cu for 48 and 96 h. SOD and CAT activities, and GSH levels were maximum (SOD_{48h}: 3275.8 ± 636.1 U/mg protein; CAT_{48h}: 537.2 ± 39.8 U/mg protein; GSH_{48h}: 556.2 ± 156.4 μmol/L protein) after 48 hours of exposure to 0.25 mg/L Cu, followed by a rapid decrease (SOD_{96h}: 79.9 ± 20.6 U/mg protein; CAT_{96h}: 15.5 ± 3.7 U/mg protein; GSH_{96h}: 36.5 ± 13.3 μmol/L protein) after 96 h of exposure.

Conversely, moderate variations in SOD and CAT activity, and GSH and MDA contents were observed in adult mussels collected from the groups exposed to 0.75 and 1.5 mg/L Cu. Although no significant changes in biochemical parameters were found in adult mussel tissue after 96 h of exposure to 3 mg Cu/L, considerable augmentation of SOD and CAT activity, and GSH levels (SOD_{48h}: 297.9% of control, $P = 0.012$, Fig. 2A; CAT_{48h}: 241.3% of control, $P = 0.034$, Fig. 2B; GSH_{48h}: 660.1% of control, $P = 0.005$, Fig. 2C) was recorded at 48 h in the adult adductor muscle compared with the control.

Overall, maximum variation in antioxidant enzyme activity and GSH levels was observed in juvenile mussel foot. CAT activity and GSH levels were significantly enhanced in the mantle of juvenile mussels in the group exposed to 0.25 mg Cu/L.

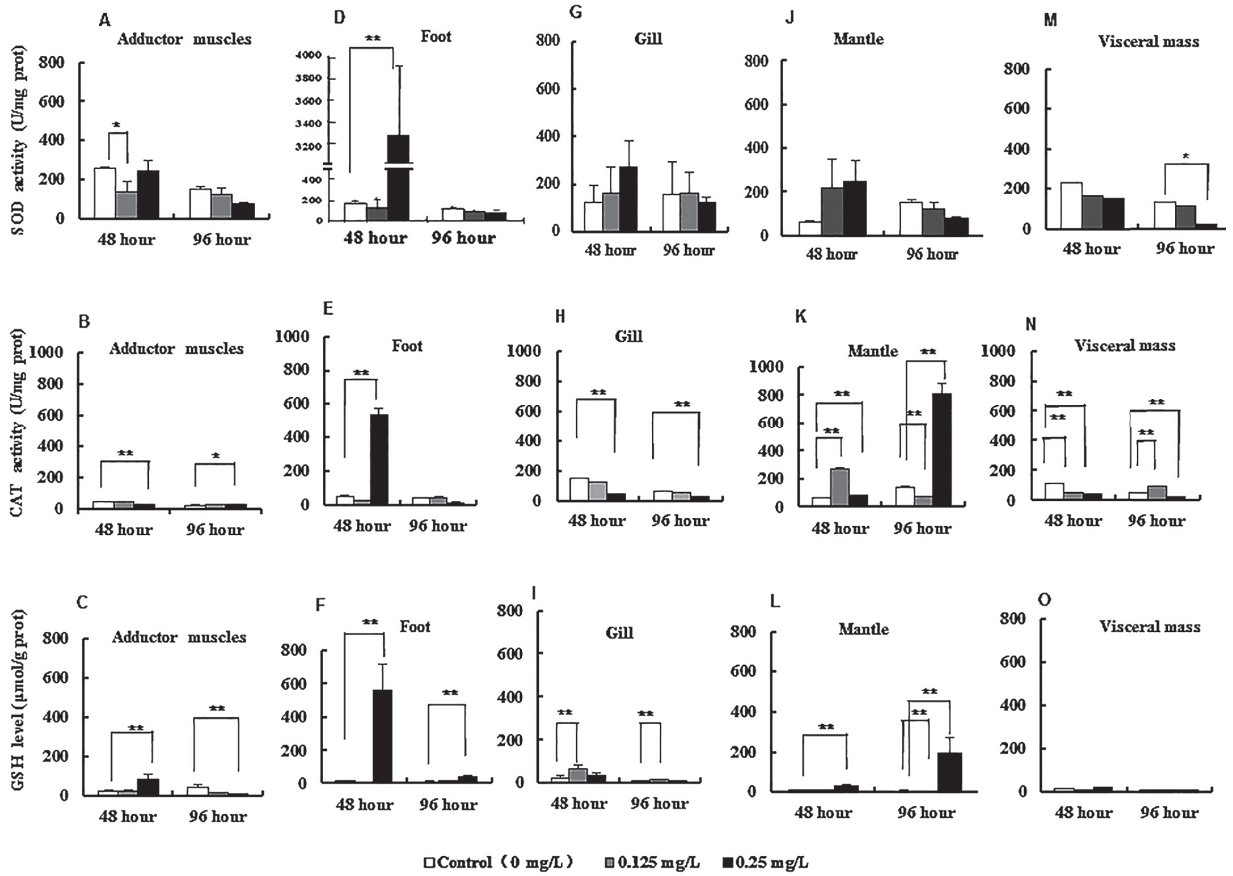

Fig. 1. Superoxide dismutase (SOD) and catalase (CAT) activities, and glutathione (GSH) levels in the tissues of juvenile *Anodonta woodiana* exposed to nonlethal concentrations of Cu (mg/L). ** Indicates a significant difference between the experimental and control groups according to Dunnett's *t*-tests, $P < 0.01$; * indicates a significant difference between the experimental and control groups according to Dunnett's *t*-tests, $P < 0.05$. Adductor muscle: Figs. 1A–C; foots: Figs. 1D–F; gill: Figs. 1G–I; mantle: Figs. 1J–L; visceral mass: Figs. 1M–O.

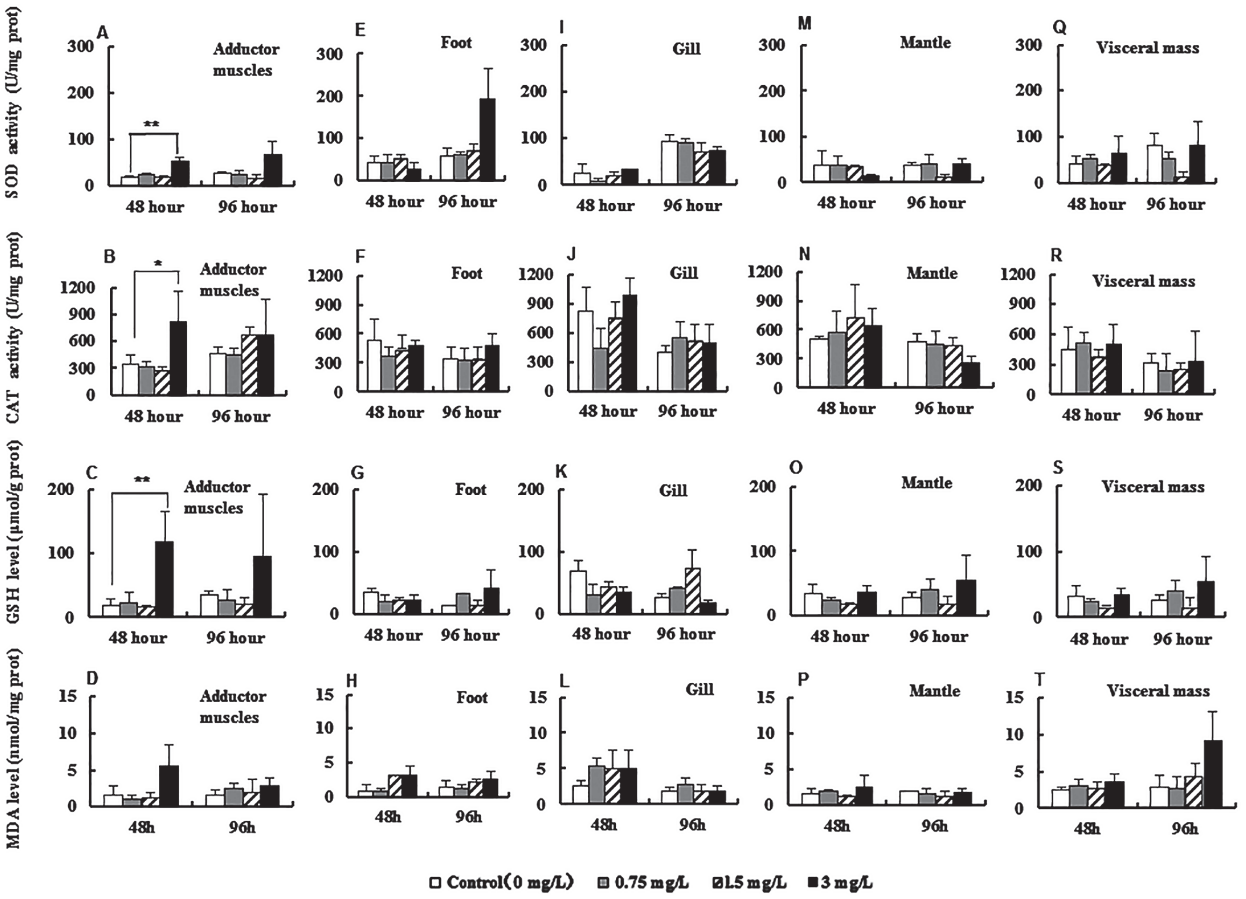

Fig. 2. Superoxide dismutase (SOD) and catalase (CAT) activities, and glutathione (GSH) and malondialdehyde (MDA) levels in the tissues of adult *Anodonta woodiana* exposed to nonlethal concentrations of Cu (mg/L). ** Indicates a significant difference between the experimental and control groups according to Dunnett's *t*-tests, $P < 0.01$; *indicates a significant difference between the experimental and control groups according to Dunnett's *t*-tests, $P < 0.05$. Adductor muscle: Figs. 2A–D; foots: Figs. 2E–H; gill: Figs. 2I–L; mantle: Figs. 2M–P; visceral mass: Figs. 2Q–T.

DISCUSSION

The results of the toxicity test demonstrated that the EC_{50} following 96 h of Cu exposure was much lower for the juvenile *A. woodiana* (3.4 mg/L) that for the adults (22.1 mg/L).

Acute toxicity studies have demonstrated that chemical toxicity can vary by an order of magnitude among life stages and species (Cherry *et al*., 2002; Augspurger *et al*., 2003). The early life stages of freshwater mussels are more vulnerable to some pollutants (e.g., Cu) (Keller and Zam *et al*., 1991; Wang *et al*., 2011) than adult mussels, because adult mussels can close their valves for a long time to reduce harmful exposure (Harrison *et al*., 1984). Conversely, the EC_{50} for the adults in the present study was substantially higher than that for other mussel species, such as *Lamellidens corrianus* Lea, 1834, *Mytilus coruscus* Gould, 1861, and *Mytilus galloprovincialis* Lamarck, 1819 (Bat *et al*., 2013; Harrison *et al*., 1984; Patil and Mahajan, 2012). Notably, the 96 h EC_{50} value for Cu in the adult marine gastropod, *Onchidium struma* was 74.8 mg/L (Li *et al*., 2009), which was comparable to that reported here for adult *A. woodiana*. Species–specific differences in tolerance to pollutants may contribute to their diversity under different environmental conditions (e.g., temperature, test medium) (Harrison *et al*., 1984), the type of exposure in the field or laboratory (Goswami *et al*., 2014), and the metabolic capacity for pollutants (Salánki and V–Balogh, 1989). Our observations indicate that the response of swan mussels to Cu is dependent on life stage, with the adult *A. woodiana* being insensitive to Cu compared with adults of other marine bivalves.

Antioxidant defense systems and LPO have been used to monitor oxidative damage induced by heavy metals in mollusk (Company *et al*., 2006; Farris *et al*., 1994). However, knowledge of such damage in freshwater mussels remains scarce. The present study is the first to report data on the antioxidant enzymes SOD and CAT, the free radical scavenger GSH, and the peroxidation of the cell constituent MDA in cultured juvenile and adult *A. woodiana*. Exposure of bivalves to Cu resulted in increased CAT and/or SOD activity accompanied by increased GSH content in the foot, mantle of juveniles, or GSH content and MDA product in the adductor muscle of adults. Variations in the antioxidant defense systems illustrate a hormesis effect in the aforementioned tissues.

According to the hormesis theory, there is a non–monotonic response of biological systems to chemicals and toxins exposures (Radak *et al*., 2017). At low– concentrations (0.125 mg/L for juveniles; 0.75 and 1.5 mg/L for adults, respectively), relatively low levels of ROS may

be rapidly eliminated by an array of anti–oxidant enzymes in order to alleviate oxidative stress and maintain antioxidant enzyme activity at near–normal levels. At a higher concentration (0. 25 mg/L for juvenile; 3 mg/L for adult), Cu clearly stimulated the generation of ROS (MDA) in the adult adductor muscle, and significantly increased the activity of antioxidant defense systems (SOD and/or CAT, GSH) in the foot of juveniles at 48 h, in the mantle of juveniles after 96 h, and in the adult adductor muscle throughout the period of exposure. In a comparable study (Eertman *et al*., 1995), contaminated sediment caused a similar non–dose–dependent induction of SOD and CAT activities in blue mussel *Mytilus edulis*.

Our data suggest that Cu levels in the foot, mantle of juveniles, and the adductor muscle of adults are good indicators of Cu pollution, although enzyme activity levels in the gill have been widely used as biomarkers of metal pollution, including Cu (Rajalakshmi and Mohandas, 2005; Xia *et al*., 2016). Differential expression of antioxidant enzymes in various tissues of *A. woodiana* is likely associated with the different physiological functions of those tissues. The adductor muscle is the main muscular system in bivalve mollusks and it enables the animal to close its valves tightly when necessary. The foot serves as a fleshy anchor when the animal is stationary or moving forward. The gill and mantle are the main interface between the organism and its environment, and may therefore concentrate contaminants in their tissues (Li *et al*., 2015). The visceral mass generally holds the bulk of the digestive, reproductive, and excretory systems, including the hepatopancreas, which is involved in digestion, neutralizes large amounts of toxicants that are ingested by the organism (Xia *et al*., 2016). The lysosomal system in hepatopancreatic cells plays an important role in the detoxification of toxic metals (Rajalakshmi and Mohandas, 2005). Therefore, it is not surprising that the activities of SOD and CAT were markedly low in the visceral mass of juveniles exposed to Cu. Similar variations in antioxidant enzymes have been observed in the hepatopancreas of a freshwater mussel *Lamellidens corrianus* (Lea) exposed to different concentrations of Cu (Rajalakshmi and Mohandas, 2005). More pronounced changes in enzymatic (SOD and/or CAT) activity and GSH levels in juveniles, and in the adult adductor muscle can be attributed to a more sensitive response to Cu during the early–life stage, or to increased levels of oxidative stress in the adult muscular system in response to the metal. Conversely, the absence of any significant change in the foot, gill, mantle, visceral mass of adult *A. woodiana* may be due to valve closure elicited by the high ambient metal concentration, or to metallothionein (MT) synthesis and thus the sequestration of accumulated metal, maintaining the near–normal activity of the antioxidant defense system (Rajalakshmi and Mohandas, 2005).

The response of antioxidant defense systems (SOD and CAT) to Cu might result in the formation of complexes (especially MDA) (Company *et al*., 2006; Doyotte *et al*., 1997) or the oxidation of GSH (Livingstone *et al*., 1992; Regoli, 1998). Differences in response patterns might be related to different pathways of ROS production in tissues (Company *et al*., 2006), the physiologic function of tissues, and their susceptibility to oxidative stress or properties of the enzymes present in the analyzed tissues (Goswami *et al*., 2014). Our results demonstrated that antioxidant responses of *A. woodiana* to Cu stress varied amongst tissues and life stages. The foot, mantle of juveniles, and the adductor muscle of adults might be more susceptible to Cu toxicity because marked fluctuations in biochemical indices and strong antioxidant responses were observed in those tissues.

Contaminants can influence unionid bivalves on many different levels (Farris and van Hassel, 2007). An ideal approach would include the use of multiple biomarkers to assess the condition of unionids. A number of related studies have demonstrated the combined effects of environmental stressors on the immune response of mussels (Al–Subiai *et al*., 2011; Banni *et al*., 2010; Goswami *et al*., 2014; Netpae *et al*., 2012). However, we detected that Cu exerted its effects on a cellular level. To better overcome this shortcoming, other biomarkers, such as molecular, cytological, physiological, and autecological markers, should be taken into consideration, and investigated in further studies (Bourgeault *et al*., 2010; Farris and van Hassel, 2007). In addition to metal, other biotic and abiotic factors, including sex, salinity, temperature, and season of sampling, can contribute to variations in the antioxidant defense system in mussels (Baraj, 2011). The benefits of using a multi–assay approach to monitor environmental pollution using "standardized" *A. woodiana* are highlighted.

AUTHOR CONTRIBUTIONS

H. Liu designed the study, performed the toxicity experiment, analyzed the data and wrote the paper. X. Chen assisted the toxicity experiment, analyzed the data and wrote the paper. Y. Oshima designed the study, supervised the work and wrote the paper. Y. Shimasaki supervised the work. T. Jiang assisted the water chemical analysis. J. Yang designed the study, supervised the work, analyzed the data and wrote the paper. All authors assisted in editing of the manuscript and approved the final version.

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