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Effects of water-borne copper on the survival, antioxidant status, metallothionein-I mRNA expression and physiological responses of the Chinese mitten crab, *Eriocheir sinensis* (Decapoda: Brachyura) larvae

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Summary: The lethal concentration of water-borne copper in Chinese mitten crab *Eriocheir sinensis* larvae was tested by exposing the animals to 0, 0.1, 0.2, 0.3, 0.5 and 0.8 mg Cu L^{-1} at 20°C for 96 h. The 96-h median lethal concentration (LC_{50}) and its corresponding 95% confident interval was estimated on zoea 1 larvae and megalopa larvae, respectively. Acute dissolved copper toxicity was higher for zoea 1 larvae (0.16 mg L^{-1}) than for megalopa larvae (0.21 mg L^{-1}). The antioxidant status, metallothionein-I mRNA expression and physiological response of the crab to copper toxicity was further investigated by exposing the megalopa larvae to 0, 0.08 and 0.16 mg Cu L^{-1} for 96 h. The superoxide dismutase activity, catalase activity, glutathione S-transferase (GST) activity and lipid peroxidation content of megalopa larvae increased concomitantly with the exposure time and copper concentration. MT-I mRNA expression levels were positively correlated with both the concentration and duration of copper exposure. The oxygen consumption and respiratory quotient of megalopa larvae in response to 0.16 mg L^{-1} copper were significantly higher than those in the control group after 96 h of exposure ($P<0.05$). The results of this study highlight the potential effects of copper as a common stressor in *E. sinensis* larvae. MT-I and GST appear to be suitable biomarkers of environmental copper exposure stress in *E. sinensis* larvae.

Keywords: *Eriocheir sinensis*; copper toxicity; physiological; larvae; antioxidant enzyme; metallothionein.

Efectos del cobre disuelto sobre la supervivencia, estado antioxidante, expresión de la metalotionina-I mRNA y la respuesta fisiológica de las larvas del cangrejo de Shangai, *Eriocheir sinensis* (Decapoda: Brachyura)

Resumen: La concentración letal de cobre disuelto se determina mediante la exposición de larvas del cangrejo de Shangai (*Eriocheir sinensis*) a dosis de 0, 0.1, 0.2, 0.3, 0.5 y 0.8 mg Cu L^{-1}, a 20°C durante 96 h. La concentración letal media (LC_{50}) y su correspondiente intervalo de confianza del 95% fueron estimados en los estadios larvales zoea I y megalopa. La toxicidad aguda del cobre disuelto fue mayor sobre larvas de zoea I (0.16 mg L^{-1}) que sobre las de megalopa (0.21 mg L^{-1}). El estado antioxidante, la expresión del mRNA de la metalotionina–I y la respuesta fisiológica del cangrejo a la toxicidad del cobre fueron posteriormente investigados exponiendo las larvas de megalopa a dosis de 0, 0.08 y 0.16 mg Cu L^{-1} durante 96 h. La actividad de la superóxido dismutasa (SOD), de la catalasa (CAT), de la glutatión-S-transferasa (GST) y la peroxidación lipídica de las larvas de megalopa se incrementó en relación con el tiempo de exposición y la concentración de cobre. Los niveles de expresión de MT-1 mRNA se correlacionaron positivamente con la concentración de cobre y el tiempo de exposición. El consumo de oxígeno y la tasa respiratoria de las larvas de megalopa fueron significativamente más elevadas que las del grupo control ($P<0.05$) en respuesta a dosis de 0.16 mg Cu L^{-1}, durante 96 h de exposición. Los resultados presentados en este estudio ponen de manifiesto los efectos potenciales del cobre como factor de estrés sobre las larvas de *E. sinensis*. MT-I y GST parecen ser adecuados biomarcadores de estrés a la exposición de Cu ambiental en larvas de *E. sinensis*.

Palabras clave: *Eriocheir sinensis*; toxicidad del cobre; fisiológica; larvas; enzima antioxidante; metalotionina.

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INTRODUCTION

Copper sulphate is an important biocide commonly used in aquaculture to reduce the abundance of phytoplankton, including *Microcystis* and other blue-green algae (Yang et al. 2007). Aquatic animals acquire an unpleasant flavour when kept in water containing blue-green algae (Yeh et al. 2004). Therefore, Chinese farmers often use copper sulphate to eradicate filamentous algae during pond management of the Chinese mitten crab (*Eriocheir sinensis* Milne-Edwards, 1853), which is one of the most commercially important crustaceans used in aquaculture. In addition, multiple human activities have considerably expanded the input of copper into estuaries and marine environments around the world (D'Adamo et al. 2008). For example, an increase in copper concentration was observed near the Yangtze River and the Liaohe River estuary in China (Lin et al. 2002, Hou et al. 2011), where megalopa larval stages of *E. sinensis* migrate to freshwater to grow. The average copper concentration in these areas can reach 0.07 mg L^{-1}, which is seven times higher than levels stipulated by the Chinese National Water Quality Standard for Fisheries (GB 11607-89) (Li and Li 2003). Copper can be potentially toxic to aquatic organisms when the internally available concentration exceeds the capacity of the physiological and biochemical detoxification abilities of aquatic animals (Sunda and Hanson 1987, Rainbow 1992). This results in protective mechanisms (metallothioneins [MTs] and antioxidant enzymes) failing to protect against lipid peroxidation (LPO) of cell membranes. Numerous investigations have shown the inhibitory effects of copper overdoses on the metabolic processes of crustaceans (Zapata et al. 2001, Yang et al. 2006a, 2006b, Li et al. 2008, Amin and Comoglio 2010), including effects related to aerobic metabolism and oxidative stress. As the reactive oxygen species (ROS), such as the superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-), are also continuously being formed during normal aerobic metabolism and many oxidative stress processes, all organisms have generated antioxidant defences with both enzymatic and non-enzymatic components. Antioxidant enzymes represent the enzymatic defences that are mainly involved in this context: superoxide dismutase (SOD) and catalase (CAT) remove O_2^- and H_2O_2 directly, respectively, while glutathione S-transferase (GST) catalyses the conjugation of the reduced form of glutathione to xenobiotic substrates for the purpose of detoxification (Hotard and Zou, 2008).

The lethal effect of copper on different growth stages of crustaceans has been studied extensively, for example in adult blue crabs (*Callinectes sapidus*) (Martins Cde et al. 2011), larval southern king crabs (*Lithodes santolla*) (Amin and Comoglio 2010) and juvenile freshwater crabs (*Barytelphusa cunicularis*) (Chourpagar and Kulkarni 2011). However, no study has yet evaluated the acute toxicity of copper on Chinese mitten crabs (*E. sinensis*) zoea 1 and megalopa larvae. Their habitats are strictly confined to water bodies, and they are more commonly exposed to toxic compounds dissolved in estuaries (Agrahari 2009). Considering the fact that the same species could show a differential susceptibility to pollutants throughout its various life stages (Bambang et al. 1995), we used *E. sinensis* larvae as experimental models to test their physiological responses to acute toxicity of copper.

The MT protein family has multiple physiological functions and diverse regulatory activities, which include metal homeostasis, heavy-metal detoxification and antioxidant protection from free radicals (Coyle et al. 2002, Haq et al. 2003). Three MT isoform-encoding genes have been identified in crustaceans: MT-I, which is inducible by cadmium, zinc, and copper; MT-II, which is inducible by zinc and cadmium; and MT-III, which is inducible by copper only (Brouwer et al. 1992, 1995, Syring et al. 2000). For our study, we chose only the MT-I gene as no copper-induced MT-III is present in intermoult-stage crustaceans (Brouwer et al. 2002). MT has been suggested to be a biomarker for evaluating heavy-metal pollution (Leung and Furness 1999, Correia et al. 2002, De Boeck et al. 2003). However, no attempt has yet been made to study MT gene expression levels in *E. sinensis* larvae.

In this study, we focused on the toxic effects of copper sulphate pollution on the antioxidant enzyme activity and respiration metabolism of *E. sinensis* larvae. We also aimed to assess the sensitivity of MT mRNA expression levels and the extent of LPO as effective environmental indicators for copper exposure in *E. sinensis* larvae.

MATERIALS AND METHODS

Animals and rearing conditions

Zoea 1 larvae and megalopa larvae of *E. sinensis* were obtained from the Guanghe Co. Fishery Co. Ltd., China. They were immediately transferred to an aquatic laboratory and held in a plastic tank (100×80×50 cm). During acclimation and toxicity bioassays, the physico-chemical water parameters of the pre-aerated filtered seawater (size mesh, 0.22 μm) in the tank were as follows: temperature, 20±1°C; pH, 8.35±0.08; salinity, 20-21; dissolved oxygen, 5.16-6.53 mg L^{-1}; and total ammonia-nitrogen, below 0.08-0.09 mg L^{-1}. The water exchange rate was 50% per day, and the photoperiod was maintained at 14L:10D with white fluorescent tubes.

Acute lethal concentration of copper

The copper solution was prepared with $CuSO_4 \cdot 5H_2O$ (Analytical Reagent, Shanghai Chemical Co., Shanghai, China). After several pre-trials, six levels of copper sulphate (0, 0.1, 0.2, 0.3, 0.5 and 0.8 mg Cu L^{-1}) were used to estimate the median lethal concentration (LC_{50}) of zoea 1 larvae and megalopa larvae, respectively. All test solutions were refreshed daily. Only actively swimming larvae were selected for the assays, and no feed was supplied to the larval crabs during the test period. In total, 9000 zoea 1 larvae and 360 mega-

lopa larvae were randomly selected for the 96-h acute toxicity test. At each copper level, 150 zoea 1 larvae were stocked in triplicate 800-ml beakers containing 500 mL filtered seawater, and 60 megalopa larvae were stocked in triplicate 2000-mL beakers containing 1500 ml filtered seawater. Observations on mortality and abnormal behaviour of the larval crabs were conducted at least twice each day. During the test, water quality conditions were the same as during the acclimatization period. At 24, 48, 72 and 96 h, we separately counted the number of crabs killed and the number of crabs exposed to each copper concentration. Larvae were considered dead when there was no movement of the appendages and when they did not respond to prodding with a glass pipette. The 96-h LC_{50} and its 95% confidence limits were estimated using a Probit analysis (Finney 1971). Measured concentrations of copper in the test solutions were accordant with calculated concentrations.

Water-borne copper exposure

Based on the LC_{50} estimate, 12 rectangular PVC tanks containing 30 L filtered seawater at an approximate stocking density of 50 megalopa larvae L^{-1} were exposed to 0 (control), 0.08 and 0.16 mg Cu L^{-1} for 96 h with four replicates in each treatment group. Three hundred megalopa larvae were taken randomly from each tank for an exposure period of 12, 24, 48 and 96 h, respectively, and were immediately frozen afterwards in liquid nitrogen and stored at –80°C for further enzyme assays.

Effect of water-borne copper on antioxidant enzyme activities and LPO

To assess the enzyme activities, samples stored at –80°C were washed by stirring in 10 volumes (v/w) of ice-cold distilled water, and homogenized in a phosphate buffer (0.025 M KH_2PO_4, 0.025 M $Na_2PO_4 \cdot 12 H_2O$, pH 7.5) with a vortex mixer at maximum speed for 30 s. The homogenate was centrifuged at 1000× g for 10 min at 4°C. The supernatant was collected to determine the SOD activity. SOD activity was determined using the method of Marklund and Marklund (1974) based on the autoxidation of pyrogallol at 325 nm as modified by Jing and Zhao (1995). One unit of SOD was defined as the amount of enzyme that inhibits the autooxidation of pyrogallol by 50% per minute. CAT activity was determined by measuring the decrease in H_2O_2 concentration at 240 nm according to Aebi (1984). The reaction mixture contained 3 mL potassium phosphate buffer (pH 7.0) and freshly prepared 10.6 mM H_2O_2. One unit of CAT was defined as the amount of enzyme needed to reduce 1 μmol of H_2O_2 per minute. GST activity was evaluated according to the method of Habig and Jakobi (1981), using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The formation of *S*-2,4-dinitro-phenyl glutathione conjugate was monitored by following the resulting absorbance at 340 nm. One unit of activity was defined as the amount of enzyme that synthesizes 1 μmol of product per minute. Total soluble protein contents were determined according to Lowry et al. (1951), using bovine serum albumin as a standard. All enzyme activities were separately expressed as a relative unit per milligram of soluble protein (U/mg).

LPO was determined according to Beuge and Aust (1972). Briefly, 100 megalopa larvae in each tank at each time point were homogenized in Tris buffer (0.1 M, pH 7.8) with butylhydroxytoluene (BHT) added at a final concentration of 0.01% w/v, and centrifuged. The supernatant was mixed with TCA-TBA-HCl solution (15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid, 9.125% w/v hydrochloric acid) and placed in a boiling water bath. The formation of thiobarbituric acid-reactive components in the reaction was determined at 535 nm, using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} to calculate malondialdehyde equivalents (MDA) expressed as μM MDA mg $protein^{-1}$.

Real-time quantitative polymerase chain reaction (RT-PCR)

Total RNA was extracted from the megalopa larvae in different treatment groups by using an RNA Extraction Kit (Axygen, Union City, USA) according to the manufacturer's protocol. RNA concentration and quality were estimated by spectrophotometry at an absorbance at 260 nm (Eppendorf Biophotometer plus) and agarose gel electrophoresis (Bio-Rad PowerPac™ Basic), respectively. Two micrograms of total RNA was reverse-transcribed using the PrimeScript™ RT-PCR Kit (TaKaRa, Japan). Real-time quantitative RT-PCR was performed in a final volume of 25 μl containing 12.5 μl SYBR Premix Ex Taq (Perfect Real Time, TaKaRa, Japan), 2 μl of cDNA template and 9.5 μl or 0.5 μl of primer for MT-I or actin, respectively (primer pairs MT-I-R and MT-I-F or Actin-R and Actin-F, respectively; Table 1). Amplification was performed in a C1000™ Thermal Cycler (Bio-Rad CFX 96™ Real-Time System) under the following conditions: 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 30 s, followed by an incremental increase from 60°C to 95°C at a rate of 0.1°C/s. All samples were run in four replicates. MT-1 expression levels were analysed with the $2^{-\Delta\Delta CT}$ comparative CT method (Livak and Schmittgen 2001).

Table 1. – Sequences of the primers used in this study.

Primer	Sequence (5'-3')	GenBank accession no.
Actin-F (forward)	GCATCCACGAGACCACTTACA	HM053699
Actin-R (reverse)	CTCCTGCTTGCTGATCCACATC	HM053699
MT-I -F (forward)	GCATCTCCTTCCCAACG	GU479376
MT-I –R (reverse)	GTCATCACAGCAGCCAGC	GU479376

Respiration trial

Eighty megalopa larvae from each tank were used after 96 h of copper exposure, and randomly allotted into four 400-mL containers with 20 megalopa larvae each; one container without megalopa larvae was used as a control. The surface was sealed with liquid wax when all megalopa larvae had settled in the tank. After 1 h incubation, dissolved oxygen and CO_2 concentrations were determined (Chen 1998). The oxygen consumption rate (R) and CO_2 production rate (P) were calculated from the following equation:

$$R\ (mg\ h^{-1}\ g^{-1}) = (O_0 - O_t + O_c)\ V/W \times T$$
$$P\ (mg\ h^{-1}\ g^{-1}) = (C_t - C_0 - C_c)\ V/W \times T$$

where O_0 is the initial oxygen concentration (mg L^{-1}); O_t is the final oxygen concentration in the megalopa larvae container (mg L^{-1}); O_c is the final oxygen concentration in the control (mg L^{-1}); C_0 is the initial CO_2 concentration (mg L^{-1}); C_t is the final CO_2 concentration in the megalopa larvae container (mg L_{-1}); C_c is the final CO_2 concentration in the control (mg L^{-1}); V is the volume of the container (l); W is the wet weight of the megalopa larvae (g); and T is the duration (h). The respiratory quotient (RQ) was calculated as RQ = (R/32)/(P/44) (Li et al. 2007).

Statistical analysis

Data were expressed as mean ± standard error (SE). The effects of water-borne copper and exposure time on antioxidant enzyme activities and LPO values were analysed using a two-way analysis of variance (ANOVA). When significant interactions of the two factors were observed, the main effects were not further discussed, but all data were subjected to one-way ANOVA. Tukey's test was applied to detect significant differences between means ($P<0.05$). All statistical analyses were conducted using SPSS 16.0 for Windows.

RESULTS

LC_{50} of water-borne copper in *E. sinensis*

The estimated 24-, 48-, 72- and 96-h LC_{50} values and 95% confident intervals of water-borne copper for zoea 1 larvae and megalopa larvae are shown in Figure 1. The lethal effect of copper was greater for zoea 1 larvae, suggesting that they are more sensitive than megalopa larvae to different levels of water-borne copper.

Effects of copper on SOD, CAT and GST activities and LPO content of *E. sinensis* larvae

A two-way ANOVA showed that increased copper concentrations exert a significant effect on antioxidant enzyme activities and LPO content. The SOD, CAT and GST activities in the groups exposed to the highest copper concentrations (0.08 mg L^{-1} and 0.16 mg L^{-1}) increased significantly compared with the control group after 96 h of exposure (Fig. 2A-C). Antioxidant enzyme activities for each treatment showed an increase in a time-dependent way. For each time point of copper exposure, the antioxidant enzyme activities in megalopa larvae were increased in comparison with the control group. After megalopa larvae had been exposed to 0.16 mg Cu L^{-1}, the highest activities as-

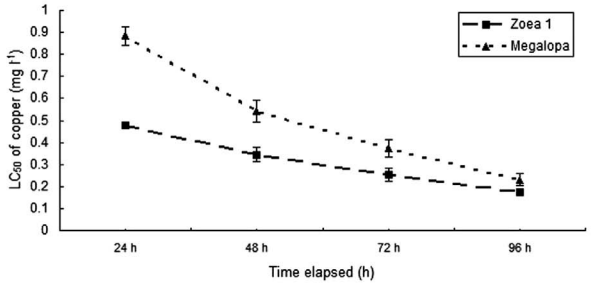

Fig. 1. – LC_{50} values (95% confidence limits) of copper versus exposure time for *Eriocheir sinensis* zoea 1 and megalopa larvae. Values are means ±SE (n=3).

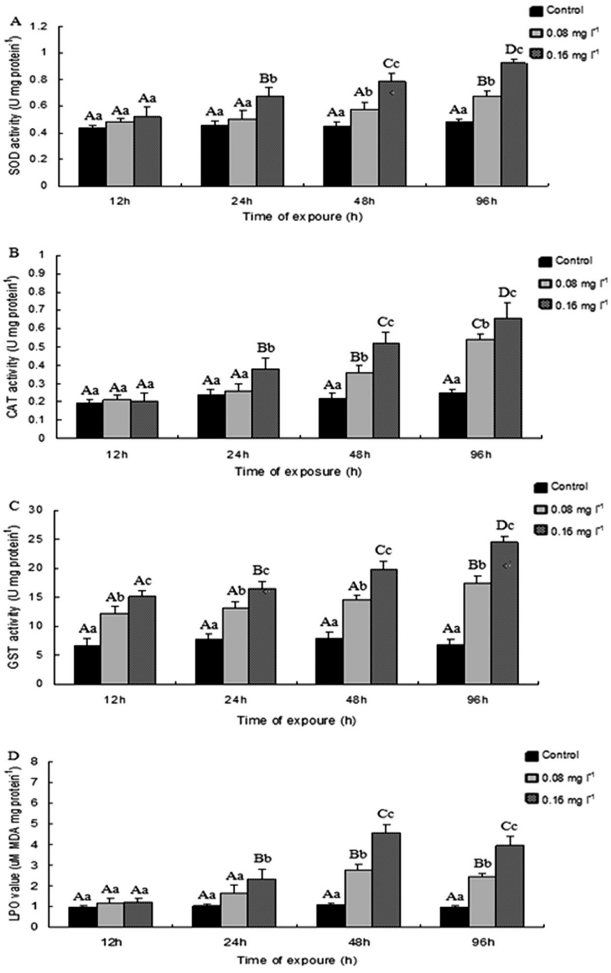

Fig. 2. – Superoxide dismutase (SOD) activity (A), catalase (B) activity, glutathione S-transferase (GST) activity (C) and lipid peroxidation (LPO) value (D) in megalopa larvae after copper treatment (0, 0.08 mg L^{-1}, 0.16 mg L^{-1}). Values are means ±SE (n=4). Bars sharing different letters in each index are significantly different ($P<0.05$). Different lowercase letters indicate significant differences between the treatment and control groups for each exposure time. Different capital letters indicate significant differences between different exposure times for the same copper concentrations.

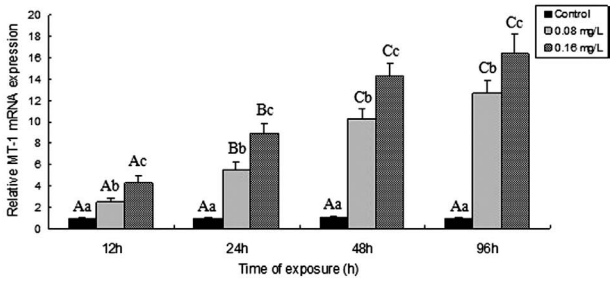

Fig. 3. – MT-1 mRNA expression in megalopa larvae after copper treatment (0, 0.08 mg L^{-1}, 0.16 mg L^{-1}). Expression was determined by real-time quantitative RT-PCR. Values are means ±SE (n=4). MT-1 expression was normalized to β-actin expression. Bars with different letters differed significantly ($P<0.05$). Different lowercase letters indicate significant differences between the treatment and control groups for each exposure time. Different capital letters indicate significant differences between different exposure times for the same copper concentrations.

sayed were observed at 96 h. Moreover, the LPO of megalopa larvae exposed to 0.08 and 0.16 mg Cu L^{-1} showed significant differences from the control group. In particular, after megalopa larvae were exposed to 0.16 mg Cu L^{-1}, the highest LPO content assayed was observed at 48 h.

Effect of copper on the MT-I mRNA expression levels in *E. sinensis* larvae

Changes in MT-I mRNA expression levels in megalopa larvae exposed to different copper concentrations for each time point are shown in Figure 3. Increased expression levels of MT-I in megalopa larvae occurred in response to increased water-borne copper levels, and the expression levels continually increased from 12 to 48 h. The expression level of MT-I after 96 h of exposure to 0.16 mg L^{-1} copper was 15-fold higher than that in controls.

Effects of copper on oxygen consumption rates and CO_2 production rates in *E. sinensis* larvae

The oxygen consumption rates, CO_2 production rates and RQs of megalopa larvae at different salinities are shown in Figure 4. The oxygen consumption rates and RQs of the megalopa larvae exposed to 0.16 mg Cu L^{-1} for 96 h were significantly higher than in other treatments. No significant differences were observed in CO_2 production rates between all treatments.

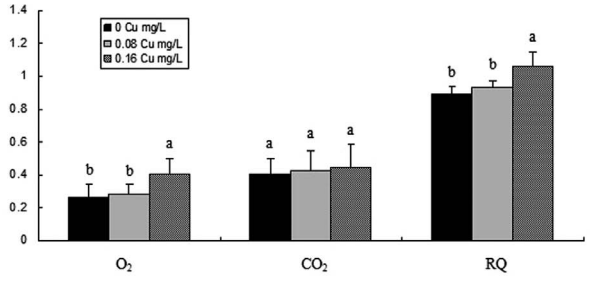

Fig. 4. – Oxygen consumption rates (mg g^{-1} h^{-1}), CO_2 production rates (mg g^{-1} h^{-1}) and respiratory quotient (RQ) of megalopa larvae exposed to copper. Values are means ± SE (n=4). Bars sharing different letters in each index are significantly different ($P<0.05$).

DISCUSSION

When assayed on *Lithodes santolla* and larval stages, copper was shown to be an order of magnitude more toxic than other metals, resulting in the following relative scale of acute lethal toxicity: Cu>Pb>Cd>Zn (Amin et al. 2003). The 96-h LC_{50} values of copper for *E. sinensis* larvae were 0.16 and 0.21 mg L^{-1} at different growth stages, respectively. Copper toxicity for *E. sinensis* was of the same order of magnitude as for other crustacean larvae (Munshi et al. 1996, López Greco et al. 2001, Ferrer et al. 2003, Amin and Comoglio 2010). The results obtained in this study showed that the lethal effects of copper were greater for zoea 1 larvae, suggesting that these larvae are more sensitive than megalopa larvae to water-borne copper. The differences between the 96-h LC_{50} of copper for *E. sinensis* zoea 1 larvae and megalopa larvae are considered to be mainly due to their different sizes (Liang et al. 1974). The mortality of *E. sinensis* increased with increasing duration of exposure. This result supports previous studies in which the biochemical response of *E. sinensis* to water-borne copper was investigated (Yang et al. 2005, 2006a, 2006b).

ROS are caused by free metal ions, and all organisms have developed antioxidant defences with both enzymatic and non-enzymatic components. These include SOD and CAT, which can detoxify O_2^- and H_2O_2, respectively (Fang et al. 2002), and non-enzymatic components, such as glutathione, selenium and vitamins C and E (El-Bahr 2013). Antioxidant enzymes are prone to oxidative modification and inactivation due to alteration by ROS and/or induction by their chemical environment (Sharonov and Churilova 1992). In this study, it is particularly interesting that we observed an increase in antioxidant enzyme activities: even at maximum copper exposure the enzymatic activity of SOD and CAT remained slightly higher than in the control. Our data agree with previous studies in aquatic animals (Hasspieler et al. 1994, Li et al. 2008), showing a positive relationship between copper exposure and copper-induced oxidative stress conditions in the tissues of organisms. Increased GST enzymatic activity at high copper concentrations could reflect a copper-induced increase in glutathione content, which plays a protective role in major detoxification processes, when the activity of the antioxidant enzyme is lowered in megalopa larvae (van der Oost et al. 2003). Our findings indicate that GST activity is sensitive to early copper exposure. Therefore, GST could be regarded as a more effective biomarker of the cellular defence system against the effects of environmental copper contamination on the physiological activities of *E. sinensis* larvae. In crustacean species, it has been shown that oxyradical production has a pollution-mediated mechanism of toxicity. LPO has been observed in individuals exposed to copper (Correia et al. 2002, Barata et al. 2005). This is consistent with our findings that megalopa larvae exposed to 0.08 and 0.16 mg L^{-1} treatments showed significantly higher levels of LPO than the control group. One reasonable explanation is that copper may act as a catalyst for the Fenton reaction, facilitating the conversion of superoxide anions and hydrogen per-

oxide to hydroxyradicals, a species frequently proposed to initiate LPO (Stohs and Bagchi 1995).

The presence of MT has been demonstrated in many crustaceans (Olafson et al. 1979, Moksnes et al. 1995). In general, the content of MT in healthy aquatic animals is low. However, its synthesis will be increased to excess if animals are exposed to higher doses of heavy metals; thus, it plays an important role in metal detoxification, sequestration and regulation. While MT-I gene mRNA expression was low in *E. sinensis* in the control group, it was increased in *E. sinensis* exposed to copper concentrations of 0.08 and 0.16 mg L^{-1}. Water-borne copper has been shown to increase MT-1 mRNA expression levels in *E. sinensis* juveniles (Ren et al. 2011), as our results, in which the maximum expression levels of MT-I was recorded in the groups exposed to 0.16 mg L^{-1} copper for 48 or 96 h. Our results suggest that MT-I mRNA expression levels could be used as a bioindicator for monitoring aquatic copper pollution for *E. sinensis* larvae.

In the present study, the oxygen consumption and RQs of the megalopa larvae exposed to water-borne copper (0.16 mg L^{-1} for 96 h) were significantly higher than in other treatments. Oxygen consumption and other indices related to respiration and metabolism have been used to assess physiological responses under various stressful environments in decapod crustaceans (Li et al. 2007, Dissanayake et al. 2008, Amin and Comoglio 2010). High oxygen consumption and RQs after copper exposure have been attributed to ultrastructural damage to the gill epithelium (Yang et al. 2007), and their increases reflect an extra metabolic demand in copper-exposed animals. In the present study, the approximate RQ of 0.9 indicates reliance on a mixture of carbohydrates, lipids and proteins for energy. This value is higher than the RQ of 0.7 found in another freshwater crab, *Potamonautes warreni*, after prolonged exposure to copper (Vosloo et al. 2002). The highest copper concentration employed in the physiological and biochemical analysis (0.16 mg L^{-1}), which represents about 70% of the estimated 96-h LC_{50}, had visible effects on the analysed parameters. Furthermore, a report on the Chinese fishery eco-environment emphasized that copper is the most commonly encountered heavy-metal pollutant. An average concentration of 0.07 mg L^{-1} water-borne copper has been documented in some fishing areas of the Yangtze River, Hangzhou Bay and the coast of Zhoushan on the East China Sea (Ministry of Agriculture and State Environmental Protection Administration 2004), which would be comparable to exposure levels analysed in our study (0.08 mg L^{-1}). However, further studies are needed to investigate the impact of such a high heavy-metal presence in the coastal zone of China on other aspects of crab larvae, such as moulting, bioaccumulation and immune responses.

In conclusion, acute dissolved copper toxicity (96-h LC_{50} and its corresponding 95% confidence interval) was determined for *E. sinensis* zoea 1 and megalopa larvae. This study showed that megalopa larvae are able to survive under high concentrations of copper through various functional adaptations, such as high-energy expenditure towards respiration, induced MT-I synthesis and stimulated antioxidant systems. Therefore, MT-I mRNA expression levels and GST activity could be used as an effective method to monitor water-borne copper stress in *E. sinensis* larvae in aquaculture.

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