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Effects of copper on the physiological responses of the commercial crab *Lithodes santolla* (Decapoda: Anomura) larvae

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SUMMARY: Effects of copper toxicity on zoea I of *Lithodes santolla* (Decapoda: Anomura) were analysed. The 96-h LC_{50} was estimated, resulting in 298.5 μg L^{-1}. Groups of larvae were exposed to sublethal concentrations (40, 80 and 160 μg L^{-1}) for 96 h. Oxygen consumption, ammonia excretion, O:N atomic ratio, lipid peroxidation (LPO) and body water content were measured. Oxygen consumption of treated groups (mean 46.92 ± 8.03 μg-atom O_2 h^{-1} mg^{-1}) did not differ significantly with control. Ammonia excretion decreased by 60% at higher Cu concentration (1.61 ± 0.65 μg-atom N-NH_3 h^{-1} mg^{-1}), leading to a 117% increase in the O:N ratio. LPO values during the exposure time were higher in all treatments than in the controls. The water content was significantly higher in treatments than in controls. The highest concentration assayed, which represents about 50% of 96-h LC_{50}, had evident effects on the parameters analysed. The values of copper in water reported for the coastal zone of Ushuaia bay exceed the value established by the United States Environmental Protection Agency (USEPA) for ambient water quality criteria. Therefore, the results obtained in the present study are a contribution to the study of potential effects of copper as a common stressor in the first larval stage of this commercial species of the Beagle Channel.

Keywords: crustacean larvae, oxygen consumption, ammonia excretion, O:N atomic ratio, lipid peroxidation, copper.

RESUMEN: Impacto del cobre sobre las respuestas fisiológicas de la larva del cangrejo comercial *Lithodes santolla* (Decápoda: Anomura). – Los efectos de la toxicidad del cobre sobre la zoea I de *Lithodes santolla* (Decapoda: Anomura) fueron analizados. La CL50 96-h fue estimada en 298,5 μg L^{-1}. Grupos de larvas fueron expuestos a concentraciones subletales (40; 80 y 160 μg L^{-1}) durante 96 horas. El consumo de oxígeno, excreción de amonio, relación atómica O:N, lipoperoxidación (LPO) y contenido de agua corporal fueron medidos. El consumo de oxígeno de los grupos tratados (media 46.92 ± 8.03 μg-átomo O_2 h^{-1} mg^{-1}) no difirió significativamente del control. La excreción de amonio disminuyó un 60% en la mayor concentración de Cu (1.61 ± 0.65 μg-átomo N-NH_3 h^{-1} mg^{-1}) produciendo un incremento del 117% en la tasa O:N. Los valores de LPO a lo largo de la exposición fueron mayores en todos los tratamientos respecto al control. El contenido de agua corporal fue mayor en los tratamientos que en el control. La mayor concentración ensayada, que representa alrededor del 50% de la CL50 96-h estimada produjo efectos evidentes sobre los parámetros analizados. Los valores de cobre en agua reportados para la zona costera de bahía Ushuaia exceden los establecidos por la Agencia de Protección Ambiental de Estados Unidos (USEPA) como criterio de calidad ambiental. Por esta razón, los resultados obtenidos en el presente estudio se convierten en una contribución sobre el impacto potencial del cobre como un estresor común durante el primer estadio larval de esta importante especie comercial del Canal Beagle.

Palabras clave: larva de crustáceos, consumo de oxígeno, excreción de amonio, relación atómica O:N, lipoperoxidación, cobre.

INTRODUCTION

Heavy metals have been described as very dangerous pollutants for several aquatic species. Among them, copper is an essential metal which is a widespread contaminant and its anthropogenic input can occur from a variety of sources (Marcovecchio, 2000). The copper concentration in marine water

varies within a broad range, from 0.05 μg L^{-1} in unpolluted marine waters to 810–1000 μg L^{-1} in highly polluted areas (Soegianto *et al*., 1999). In spite of its properties as an essential metal for crustaceans, copper can alter metabolic processes (Fingerman *et al.*, 1996; Zapata *et al.*, 2001; Espina and Vanegas Perez, 2006), including those related to aerobic metabolism and oxidative stress (Geracitano *et al.*, 2002). Studies have shown that metals such as copper exhibit the ability to produce reactive oxygen species, resulting in lipid peroxidation, DNA damage, depletion of sulfhydryls, and altered calcium homeostasis (Stohs and Bagchi, 1995; Barata *et al.*, 2005). Sensitivity to copper can also depend on the homeostatic regulation of its uptake, storage and excretion (Depledge and Rainbow, 1990), regulation of membrane permeability and the amount of permeable membrane to body size through which copper can be absorbed (Newman and Heagler, 1991).

High concentrations of metals have been measured in coastal waters of Ushuaia Bay (54º48'S, 68º19'W, Beagle Channel), mainly from urban effluents, industrial waste and the intensive maritime traffic in the local port, with copper reaching a peak level of 60.68 μg L^{-1} (Amin *et al*., 1997). The southern king crab, *Lithodes santolla* (Molina, 1872) is the most important shellfish currently exploited commercially in the Beagle Channel. Shallow waters like bays have been mentioned as a possible recruitment area (Lovrich, 1997). Since larvae begin to moult to zoea II on the fifth to sixth day after hatching (Comoglio and Vinuesa, 1991), bioassays are always conducted within 96 h of exposure. This study, carried out under semi-static conditions, as previously done for other metals by Amin *et al*. (1998, 2003), attempts to evaluate the effects of copper on survival and selected responses during the first larval stage (Zoea I). It thus offers a useful contribution to the knowledge of how this common stressor affects a conspicuous sub-Antarctic species.

MATERIALS AND METHODS

Larvae were obtained from ovigerous females collected in the Beagle Channel during the spring months, and kept in the laboratory until zoeae hatching. Larvae were maintained at a water temperature of 7.5 ± 0.5ºC, a salinity of 28 and a photoperiod of 12L:12D (fluorescent light). Marine water used in the experiments was carried from open waters known as a pristine zone, filtered and UV-light lamp-treated prior to use. Metal stock solution, from which small aliquots were added to the dilution water, were prepared from analytical grade reagent Cl_2Cu_2 salt (Timper ®, 99% purity), with no addition of acid. Test solutions and water control for each experiment were renewed daily. Only actively swimming larvae were selected for the assays and no food was given to the larvae during the experiments.

The 96-h LC_{50} and its 95% confidence limits were estimated using Probit analysis (Finney, 1971). The concentration series used (expressed in μg L^{-1}) was 160; 230; 350; 510; 740; 1100; 1160. Groups of 10 organisms were placed in 150 mL of test solution. Each series was run in triplicate, including a water control. The cessation of movements was considered as the mortality criterion. Dead larvae were recorded daily and surviving animals were transferred to corresponding fresh test solution.

To analyse the effect on oxygen consumption and ammonia excretion, groups of 100 larvae each (divided into 4 subgroups of 25 individuals) hatched during the previous night were exposed for 96 h to 0 (control), 80 and 160 μg L^{-1} of copper. At the end of the exposure period each experimental test group was placed in 72-mL respirometer chambers, covered with dark paper to reduce the swimming activity, under a flow-through system (12 ml min^{-1}, by gravity). One chamber without organisms was added as a control. After a 2 h acclimation period, a water sample from each chamber was taken to determine the initial concentration of both oxygen (polarographic electrode YSI 5100) and ammonia (Strickland and Parsons, 1972). The flasks were sealed for 2 h and new samples were taken to measure the final concentrations. Consumed oxygen and excreted ammonia were calculated as the net difference between the initial and final value of the sealed period, corrected by the chamber capacity and dry weight of organisms in each one. The O:N ratio was estimated according to Taboada *et al*. (1998), using the individual values of oxygen consumption and ammonia excretion transformed to μg-atom g^{-1} h^{-1}.

To determine the effects on lipid peroxidation (LPO), larvae were exposed to 0 (control), 40, 80 and 160 μg L^{-1} for 96 h and daily subsamples of 50 larvae were taken in triplicate. LPO was determined according to Beuge and Aust (1972). Briefly, subsamples were homogenised in buffer Tris 0.1M pH 7.8, with 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, 0.25 N 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 $protein^{-1}$. The protein content was measured according to Markwell *et al*. (1978) and the optical density was read at 750 nm in a spectrophotometer using bovine albumin as standard. All measurements were made in triplicate.

Body water content was determined in subsamples of 50 larvae in quadruplicate at 24 and 96 h of exposure for each treatment, dried at 60ºC until constant weight.

Statistical analysis

Probit analysis (Finney, 1971) was employed to estimate the LC_{50} value and its 95% confidence limits, with Abbot's correction for mortality in controls. To compare LC_{50} values, differences were considered to be statistically significant when the *higher* LC_{50}*/lower* LC_{50} ratio exceeded the corresponding critical value established by the American Public Health Association *et al.* (1995).

To determine significant differences among treatments, normally distributed data were analyzed by one-way analysis of variance (ANOVA) and post hoc, least significant difference (LSD) range test (Sokal and Rohlf, 1981). Data not normally distributed were evaluated through the Kruskal–Wallis test and Dunn's multiple comparisons test (Daniel, 1978). In all statistical tests, results were considered significant when $p<0.05$. All statistical analyses were performed using STATISTICA (Statsoft).

RESULTS

No mortalities were recorded in the control group and no moulting was observed in either copper-exposed or control groups. The estimated 96-h LC_{50} was 298.5 μg L^{-1}. No significant differences ($p>0.05$) between estimated 72 and 96 h LC_{50} indicate an asymptotic trend in the acute toxicity and could be used as a threshold concentration (Table 1). Increased mortality was observed between 48 and 72 h, particularly at the concentrations of 510 and 740 μg L^{-1}, reaching 100% mortality after 72 h of exposure (Fig. 1).

TABLE 1. – Parameters of acute lethal toxicity bioassays for Zoea I of *Lithodes santolla* exposed to copper. Same superscript letters indicate homogeneous group ($p>0.05$)

Time of exposure (h)	LC_{50} (μg Cu L^{-1})	Confidence limits (95%)	Slope	R^2
24	1160.4 [a]	1071.5 - 1255.5	11.25	0.95
48	853.8 [b]	794.8 - 919.7	13.77	0.99
72	302.5 [c]	279.5 - 324.2	15.33	0.99
96	298.5 [c]	275.8 - 319.7	16.09	0.99

Oxygen consumption showed a non-significant increase in treatments (mean value= 52.62 ± 5.32 μg-atom O_2 h^{-1} mg^{-1}) in comparison with the control group (45.27 ± 6.76 μg-atom O_2 h^{-1} mg^{-1}) (Fig. 2a, ANOVA, $F_{2;10}=2.14$, $p=0.17$). Ammonia excretion decreased significantly with increased copper concentration (Fig. 2b, $F_{2;8}=4.89$, $p=0.04$), a 60% decrease was recorded between control and 160 μg L^{-1} (1.61 ± 0.65 μg-atom N-NH_3 h^{-1} mg^{-1} and 0.65 ± 0.08 μg-atom N-NH_3 h^{-1} mg^{-1}, respectively), so the O:N atomic ratio increased significantly at the higher experimental copper concentration (O:N atomic ratio: 72.96; 117% higher than control), being homogeneous in the other tested groups (Fig. 2c, ANOVA, $F_{2;9}=9.54$, $p=0.006$).

For each time of exposure, the trend of response of LPO in exposed organisms was to increase in comparison with the control group (Fig. 3). At the end of exposure MDA levels of organisms exposed to 80 and 160 μg L^{-1} treatments (3.77 ± 2.72 and 4.27 ± 2.25 μM MDA mg $protein^{-1}$) showed significant differences from the control group (0.88 ± 0.13 μM MDA mg $protein^{-1}$). In particular, the highest concentration assayed was always significantly different from the respective control (Kruskal-Wallis,

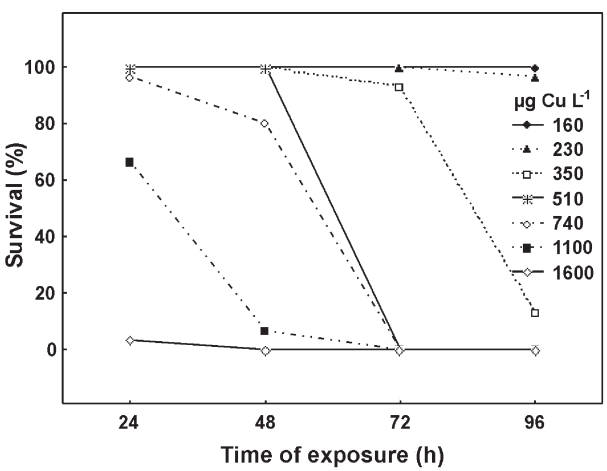

FIG. 1. – Survival (%) of Zoea I of *Lithodes santolla* exposed to copper.

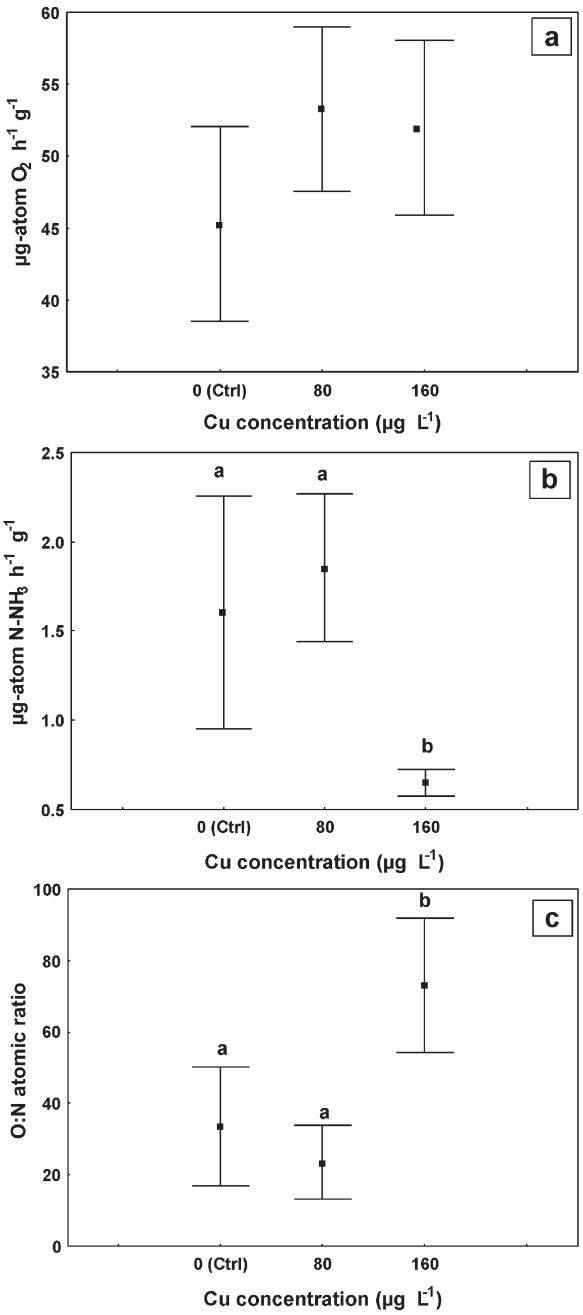

FIG. 2. – Oxygen consumption (a); ammonia excretion (b) and O:N atomic ratio (c) of Zoea I of *Lithodes santolla* exposed to copper. Mean ± S.D. Treatments sharing the same letters are not significantly different (ANOVA, $p>0.05$). (0 (Ctrl) = control group; 80 and 160 expressed as µg L^{-1}; S.D. = standard deviation).

H_{24h}=11.03, H_{48h}=15.23, H_{72h}=27.25, H_{96h}=24.40; $p\leq0.01$ for all tests). Moreover, LPO for each treatment showed significant increases in a time-dependent way. This was evident in the lower concentration at 48-72 h and the higher concentrations at 72-96 h (Kruskal-Wallis, $H_{0.04}$=10.93, $H_{0.08}$=14.89, $H_{0.16}$=25.48, $p\leq0.01$ for all tests).

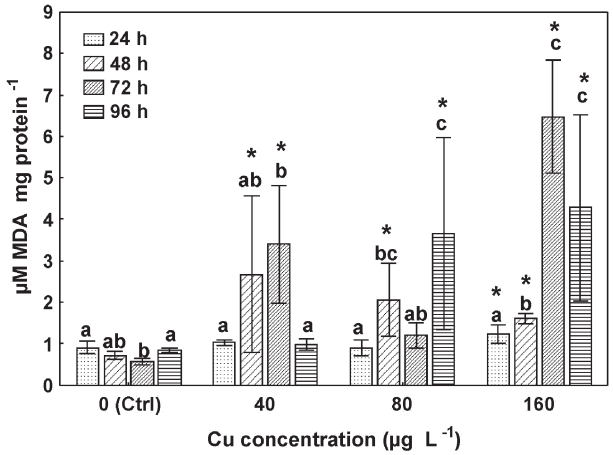

FIG. 3. – Lipid peroxidation of Zoea I of *Lithodes santolla* exposed to copper. Mean ± S.D. Columns of each treatment sharing different letters are significantly different. Asterisks denote significant differences from the corresponding control (Kruskal-Wallis, $p<0.05$). (S.D. = standard deviation).

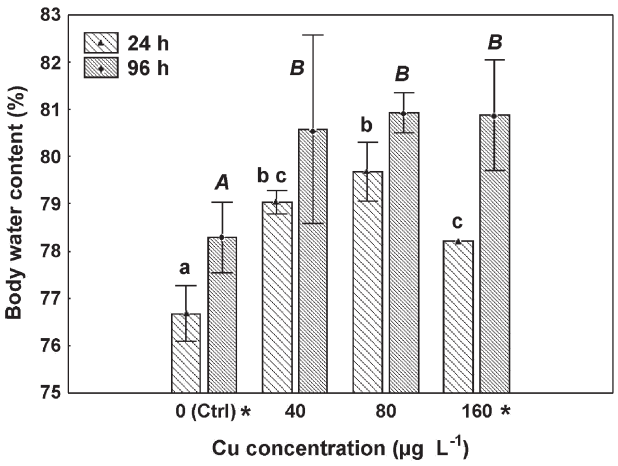

FIG. 4. – Body water content at 24 and 96 h of Zoea I of *Lithodes santolla* exposed to copper. Mean ± S.D. Columns of each treatment and time sharing same letter are not significantly different (ANOVA, $p>0.05$). Asterisks denote significant differences among exposure time. (S.D.= standard deviation).

Regarding the exposure time, the body water content of treatments was significantly higher than the corresponding control group (Fig. 4, ANOVA, F=18.6, p=0.004 and F=5.22, p=0.023 for 24 and 96 h respectively). Similarly, for both the control and treatment groups a time-dependent response was observed, and was significant in the control and 160 µg L^{-1} treatment.

DISCUSSION

Copper toxicity for *L. santolla* was in the same order of magnitude as in other crustacean larvae

(Munshi *et al*., 1996; Ramachandran *et al*., 1997; Scelzo, 1997; López Greco *et al*., 2001; Ferrer *et al*., 2003). Copper was one order of magnitude more toxic than other metals assayed on the same species and larval stage, resulting in the following relative scale of acute lethal toxicity: Cu>Pb>Cd>Zn (Amin *et al*., 2003). This tendency has also been observed in the local species *Exosphaeroma gigas*, in which copper was more toxic than Cd and Zn (Giarratano *et al*., 2007).

Oxygen consumption is considered to give a good indication of the overall metabolic state of an animal and is a useful indicator of sublethal physiological effects of heavy metal poisoning. Decreases in oxygen consumption have been attributed to ultrastructural damage to gill epithelium, while increases reflect an extra metabolic demand of the copper-exposed animals (Vosloo *et al.*, 2001). In others cases, as Knops *et al.* (2001) also found, significant changes may not happen, although slight increases were detected in the present study. Knops *et al.* (2001) described three possible explanations for an unchanged metabolic rate under stress: the first is that the additional costs associated with stress are masked by other toxicant effects; the second is that this energy demand is too small compared with whole-metabolic costs, and the third is that at least during the exposure period there is no additional cost due to chemical stress.

On the other hand, the decrease in the ammonia excretion rate during copper exposure could be attributed either to decreased protein and amino acid catabolism or to an inability to clear ammonia excess from the body. The most consistent response to copper exposure is impaired ammonia excretion rather than the decrease in its production (Wilson and Taylor, 1993; Blanchard and Grosell, 2006); this could be an explanation for the present results, since no significant changes in oxygen consumption were detected.

As was established by Mayzaud and Conover (1988), a low O:N ratio corresponds to a period when protein is heavily used, while the highest values are closely related to the depletion of lipid reserves. Accordingly, in the present study the increase in O:N ratio at the higher concentration was related to the decrease in ammonia excretion, rather than the utilisation of lipid reserves. As stated by Cheng *et al*. (2009), it is important to take into account that the O:N ratio as a stress index requires additional measures related to metabolic effects. In fact, variation in O:N ratio could be due to a change in one parameter or both parameters varying in a different proportion.

In molluscs and other crustacean species it has been shown that oxyradical production has a polluted-mediated mechanism of toxicity, and lipid peroxidation has been observed in individuals exposed to copper (Brouwner and Brouwner, 1998; Correia *et al*., 2002; Barata *et al*., 2005). Copper may act as a catalyst for the Fenton reaction, facilitating the conversion of superoxide anion and hydrogen peroxide to hydroxyradical, a species frequently proposed to initiate lipid peroxidation (Stohs and Bagchi, 1995). In particular, lipid peroxidation is considered to be the major mechanism by which oxyradicals can cause tissue damage, leading to impaired cellular function and alterations in physicochemical properties of cell membranes, which in turn disrupt vital functions (Rikans and Hornbrook, 1997). The observed increase in LPO may cause cellular injuries that possibly account for the observed decrease in ammonia excretion; however this needs to be evaluated further.

Water uptake is a natural mechanism which helps crustaceans in the moult process. In fact, previous studies established the mean duration of *Lithodes santolla* zoeae I as 5.2-5.5 days (Comoglio and Vinuesa, 1991; Amin *et al*., 2003), so increments in the body water content at 96 h observed in the present study could be related, in part, to this event. Furthermore, the main mode of toxic action of copper is regarded as affecting the membrane permeability (Ringwood *et al*., 1999; Viarengo *et al*., 2000). In accordance with these previous studies, our results have shown a relative water content increase in all treatments in comparison with the control group.

As has been demonstrated, the highest concentration employed in the physiological and biochemical studies (160 μg L^{-1}), which represents about 50% of estimated 96-h LC_{50}, had evident effects on the analysed parameters. Furthermore, in addition to the information given previously, the values of copper in water reported for the coastal zone of Ushuaia bay (up to 60 μg L^{-1}, Amin *et al*., 1997) exceed the one established by the United States Environmental Protection Agency (USEPA) (1999) for ambient water quality criteria. This reported value is also higher than those reported for other coastal zones of Argentina (i.e. 12 μg L^{-1} for Río de la Plata, Villar *et al*., 1999; and 4.65 μg L^{-1} for Bahía Blanca Estuary, Ferrer *et al*., 2003). It is therefore important to further the studies related to other aspects, such as

moult, bioaccumulation and/or antioxidant defence responses of such a high heavy metal presence in the coastal zone of the Beagle Channel.

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