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Dietary copper effects in the estuarine crab, *Neohelice* (*Chasmagnathus*) *granulata*, maintained at two different salinities

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ABSTRACT

We analyzed the dietary copper effects in the estuarine crab *Neohelice* (*Chasmagnathus*) *granulata* and its interaction with water salinity. Crabs were maintained at 2‰ and 30‰ salinity for 5 weeks and they were fed with commercial food supplemented with the green alga *Scenedesmus vacuolatus* previously exposed to copper. No mortalities were observed, but crabs maintained at 2‰ salinity accumulated on average 40% more copper compared to animals maintained at 30‰ salinity. At 2‰ salinity, superoxide dismutase (SOD) activity and reduced glutathione (GSH) levels were increased at the first and second weeks, respectively, while lipid peroxidation and protein oxidation were evident after 4 weeks of copper exposure. At 30‰ salinity, all measured variables increased progressively but were significantly higher only at the end of the assay (5th week), except for protein oxidation that remained unchanged throughout the experiment. The hepatosomatic index (HSI) was significantly decreased in response to copper exposure, but only in crabs acclimated to 2‰. These findings have suggested that dietary copper exposure induces greater metal accumulation and larger oxidative stress responses in crabs maintained at 2‰ salinity.

1. Introduction

Copper is an essential nutrient to aquatic organisms, however, it is toxic at high concentrations (Pipe et al., 1999; Sabatini et al., 2009) and it may be persistent and bioaccumulates in the environment (Paez-Osuna et al., 1998). Copper is a potential hazard to human health because of its persistency, toxicity and bioaccumulation. Aquatic organisms take up and accumulate metals both directly from the water and from food sources. Metals can accumulate in aquatic organisms and are easily transferred through the food chain to the top consumers, including humans (Wallace et al., 2003; Fisk et al., 2005).

The degree of toxicity of each metal depends on its properties and concentration in the medium; the effects of metals on organisms vary depending on species, population density, and duration of metal exposure. (Gagneten and Vila, 2001; Chang and Reinfelder, 2002). The metal concentration found in an individual depends on the metal bioavailability in the medium, and also on the balance between metal absorption and excretion (Ying et al., 1993) and during exoskeleton molting. The remaining metal is usually transferred to higher trophic levels (Wang and Yu, 2002). Nevertheless, metal assimilation efficiency by the predator is affected by the metal biochemistry in the prey/food and the physiology of the predator (Tania and Wood, 2008).

Many trace metals play essential roles in metabolism, and all have the potential to cause ecotoxicological damage. Trace metals are important persistent pollutants in aquatic ecosystems worldwide and are especially prevalent in freshwater, estuarine and coastal marine ecosystems exposed to high degrees of urban pressure (De Mora et al., 2004; Hyun et al., 2006).

Heavy metals are commonly associated with the formation of reactive oxygen species (ROS), which lead to oxidative stress causing cell damage (Leonard et al., 2004). Oxidative stress is counteracted by defence systems such as the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) and non enzymatic antioxidant molecules such as reduced glutathione (GSH) and metallothionein (MT). Since the abundances of these enzymes and molecules are related to external stimuli that cause oxidative damage (Manduzio et al., 2005), some of these parameters could serve as indicators of toxic effects on aquatic organisms (Sabatini et al., 2009).

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To determine trace metal accumulation in aquatic biota, inductively coupled plasma atomic emission spectrometry (ICP-AES) using a microconcentric nebulizer (De Wit and Blust, 1998), inductively coupled plasma mass spectrometry (ICP-MS) (Marcos et al., 1998), and flame atomic absorption spectrometry (Welz et al., 1991) are commonly employed. However, these analytical techniques normally require large quantities of biological sample material, often more than 0.5 mg. Total reflection (TXRF) X-ray fluorescence (Barkács et al., 1999; Sabatini et al., 2009) is a relatively economical micro-analytical method allowing simultaneous multielement analyses with detection limits in the low picogram range (Klockenkämper, 1997).

Metals generally enter food chains through photosynthetic organism, accumulating and transferring pollutants to consumers, including humans (Moreno Sanchez and Devars, 1999). *Scenedesmus sp.* is among the most widely distributed green microalgae; these algae are found in most aquatic environments in the world. Species of the genus *Scenedesmus* are often used in toxicity tests due to their sensitivity to different contaminants, their relatively short life cycle, and the ease to handle them in laboratory cultures (Sabatini et al., 2009).

The estuarine crab *Neohelice granulata* (formerly *Chasmagnathus granulatus*) inhabits salt marshes from Brazil to Argentina (Boschi, 1964) and has been employed in toxicological studies (Chaufan et al., 2006), including a few that examined the effects of heavy metals on physiological parameters (Rodríguez et al., 2001; Medesani et al., 2004). Salinity is one of the most variable parameters in the estuarine environments where *N. granulata* lives. Changes in salinity will also alter the concentration of inorganic complexing agents, particularly of chloride, and affect the bioavailability and consequent uptake of trace metals by euryhaline invertebrates. In many cases, salinity-related effects on metal uptake can be explained by changes in chemical speciation. Additionally, Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} ions can compete with copper ions for binding sites at the organism–water interface (Di Toro et al., 2001; Santore et al., 2001). Therefore, the salinity and specific ionic composition of the water greatly influences copper toxicity (Di Toro et al., 2001; Santore et al., 2001; De Schamphelaere and Janssen, 2002).

Neohelice granulata is an important member of the estuarine food chain due to its high abundance and its multiple roles as a scavenger, predator and prey. Given *N. granulata's* ecological role and the extensive knowledge about their biology and maintenance conditions in captivity, *N. granulata* is an excellent model for ecotoxicological studies.

This study was carried out to analyze the effects of dietary copper on oxidative damage in the estuarine crab *N. granulata* and its interaction with water salinity. Copper had important oxidative effects, particularly in crabs acclimated to the more dilute salinity.

2. Materials and methods

2.1. Organisms

BAFC CA4 strain of *Scenedesmus vacuolatus* (Chlorophyceae, Chlorophyta) was originally isolated by Emerson R. Y, as *Chlorella vulgaris*, and is currently kept in the Culture Collection of the Laboratory of Phycology, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires.

Adult male crabs (*N. granulata*) at stage C (Drach and Tchernigovtzeff, 1967), 28–32 mm carapace width, were collected in a mud–sand flat at Faro San Antonio beach (36° 18′S, 56° 48′W), near the Southern edge of the Río de la Plata estuary, Argentina, where pollution is considered to be minimal (Colombo et al., 2003).

2.2. Algae cultures

For algae bioassays copper solutions were prepared by using analytical grade copper chloride ($CuCl_2.2H_2O$). A 100 mg L^{-1} stock solution was prepared with deionised water. Solutions of varying concentrations were prepared by diluting the stock solution with Bold's basal medium (BBM) (Bischoff and Bold, 1963). The control contained 6.2 µM Cu (already present in the BBM media), and 108 µM copper were prepared by adding copper as $CuCl_2.2H_2O$ (Sabatini et al., 2009).

Water of 2‰ and 30‰ salinities were prepared by adding HW-Marinemix salts to dechlorinated water. Copper content in water was measured by Total X-Ray Reflection Fluorescence Analysis (TXRF). The analysis did not show the presence of metal in the water.

Bioassays were carried out in 250 mL flasks containing 140 mL of medium with an initial cell density of 30,000 cells mL^{-1}. An exponential growing phase culture was used as inoculum. Suspension cultures were maintained at 24 ± 1 °C, with continuous cool-white fluorescent light illumination (80 µM photons m^{-2} s^{-1}) and agitation in an orbital shaker. After 7 days, the cultures from both groups (control and treated) were divided into aliquots containing, on average, 20×10^6 cells each, which were added to the food of each crab. The number of cells from the different cultures was determined by cell counting in Neubauer's chamber.

Total copper contents were determined in *S. vacuolatus* in both groups (control and treated) by TXRF (Sabatini et al., 2009). Copper content in treated algae was 1 µg Cu/10^6 cells and in control were 0.06 µg Cu/10^6 cells.

2.3. Experimental design

Individual crabs were placed in a 2 L glass container, a total of 120 crabs was used for this study. Water was changed twice a week after animals were fed. Laboratory conditions were 20 ± 2 °C and 12:12 light/dark photoperiod. After 15 days of acclimation to two different salinities (2‰ and 30‰), crabs from both salinities were randomly sorted into two groups, control and treated. The control group was fed twice a week with rabbit food supplemented with control algae (12 µg Cu/g food). The treated group was fed twice a week with rabbit food supplemented with algae previously treated with copper (300 µg Cu/g food). The food was ground, mixed with the algae and repelleted with a commercial pasta maker. Crabs were fed twice a week at a total ration of 1% bodyweight, and approximate mean weekly doses of Cu were 6 µg Cu/g crab/week in treated groups and 0.12 µg Cu/g crab/week in control groups at both assayed salinities. Animals were fed and processed at the same time of the day according to Maciel et al. (2004). After 1, 2, 3, 4, and 5 weeks of treatment, 6 crabs of each group were collected at random. Before dissection, they were rapidly killed by destroying their nervous system with a spike. Then, the body was weighed, and the hepatopancreas was carefully removed, weighed and homogenized (1:5 w/v) in 0.154 M KCl. The hepatosomatic index (HSI) was calculated from the formula (Maddock and Burton, 1998)

$$\mathrm{HSI} = \frac{\mathrm{DHW} \times 100}{\mathrm{DBW}}$$

where DHW = dry hepatopancreas weight and DBW = dry body weight.

2.4. Hepatopancreas copper content

To determine copper content, *N. granulata* hepatopancreas were homogenized in 0.134 M KCl (1:5, w/v) and digested in a microwave oven with nitric acid and hydrogen peroxide (2:1, v/v). A known amount of gallium was added to the dissolution, as an internal standard. Aliquots (10 µL) were placed in a quartz reflector for analysis by Total X-Ray Reflection Fluorescence Analysis (TXRF) (Prange and Schwenke, 1992). This analysis was performed with a disperse energy spectrometer equipped with a total reflection module with double reflector and collimator, a Si (Li) detector (CANBERRA model SL30170). An X-ray tube with a molybdenum anode was used as intracellular

excitation source. Spectrum evaluation and quantitative analysis were performed using the QXAS software package from IAEA, using least square regression analysis and calibration curves within the range of 1–20 ppm. Results are expressed as µg of copper per mg proteins.

2.5. Protein content

Total soluble protein content was measured by the method of Bradford (1976), using bovine serum albumin as standard. Results were expressed as µg protein per mL.

2.6. Lipid peroxidation

Lipid peroxidation was determined measuring thiobarbituric acid reactive substances (TBARS) in the hepatopancreas of *N. granulata* using a modified form of Beuge and Aust procedure (1978). Briefly, 11,000 ×*g* supernatant from total homogenate was mixed with thiobarbituric acid (TBA) solution following incubation at 95–100 °C for 15 min. After cooling, the reaction mixture was centrifuged and supernatant absorbance was determined at 535 nm. TBARS concentration was estimated using the extinction coefficient of MDA–thiobarbituric acid complex (156 mM^{-1} cm^{-1}). Results were expressed as nmol MDA per mg proteins.

2.7. Reduced glutathione (GSH) content

GSH levels were measured in hepatopancreas of *N. granulata* following the Anderson procedure (1985), with some modifications. Briefly, 100 µL supernatant from the 11,000 ×*g* sample was acidified with 50 µL of 10% sulfosalicylic acid. After centrifugation at 8000 ×*g* for 10 min, supernatant (acid-soluble GSH) aliquots were mixed with 6 mM 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) in 0.143 M buffer sodium sulfate (pH 7.5), (containing 6.3 mM EDTA). Absorbance at 412 nm was measured after 30 min incubation at room temperature. GSH content was determined by standard curve generated with a known GSH amount. Results were expressed as nmol thiols (GSH equivalents) per mg proteins.

2.8. Protein oxidation

Carbonyl content was quantified as described by Resnick y Packer (1994). Supernatant from the 11,000 ×*g* hepatopancreas was incubated 15 min at room temperature with 10% streptomycin sulfate to eliminate DNA debris. Two samples of extracted proteins were placed in two glass tubes. To one tube 10 mM DNPH in 2.5 M HCl was added, while to the other tube 2.5 M HCl was added. Tubes were left for 1 h of incubation at room temperature in the dark. Then, 20% TCA (w/v) was added in both tubes, left in ice for 15 min and centrifuged at 6000 ×*g* to collect the protein precipitates. The precipitates were dissolved in 6 M guanidine hydrochloride and were left for 10 min at 37 °C. Carbonyl content was calculated from the peak absorbance (355–390 nm) using an absorption coefficient of 22,000 M^{-1} cm^{-1}. Results were expressed as nmol carbonyl per mg proteins.

2.9. Antioxidant enzyme activities

Crab hepatopancreatic tissues were homogenized in 0.134 M KCl (1:5, w/v) containing 0.5 mM PMSF and 0.2 mM benzamidine. The homogenate was centrifuged at 11,000 ×*g* for 20 min. Determinations were carried out in 11,000 ×*g* supernatants from total homogenate.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured using the procedure of Beauchamp and Fridovich (1971). The standard assay mixture contained 10, 12.5, and 15 µL enzymatic sample, 0.1 mM EDTA, 13 mM DL-methionine, 75 µM nitroblue tetrazolium (NBT) and 20 µM riboflavin, in 50 mM phosphate buffer (pH 7.9), to a final volume of 3 ml. Samples were exposed for 15 min to intense cool-white light, and then kept in the dark until absorbance was measured at 560 nm. Results were expressed as enzymatic units per mg proteins. A SOD unit was defined as the enzyme amount necessary to inhibit 50% the reaction rate.

2.10. Statistical analyses

Results from different treatments were compared statistically by one-way analysis of variance (ANOVA) followed by a Dunnett's *post hoc* test. The suppositions of normality and homogeneity of variances were tested with Lillieford and Bartlett tests, respectively (Sokal and Rohlf, 1999). Graph Pad Prism 3 software was used for statistical analysis.

3. Results

3.1. Copper uptake

Fig. 1a and b show differences in copper uptake in hepatopancreas from crabs maintained at two different salinities for various times (1–5 weeks). Treated crabs (fed with algae exposed to copper) maintained at 2‰ salinity (Fig. 1a) accumulated on average 40% more copper than the animals maintained at 30‰ (Fig. 1b). At 2‰ salinity, copper accumulation was significantly higher ($p<0.01$) from the first week of treatment, whereas in the animals maintained at the higher salinity copper accumulation progressively increased but it was not significantly higher until the fifth week ($p<0.01$).

The presence of copper in control crabs was due to copper present in the *S. vacuolatus* culture medium, which is necessary for normal algae growth.

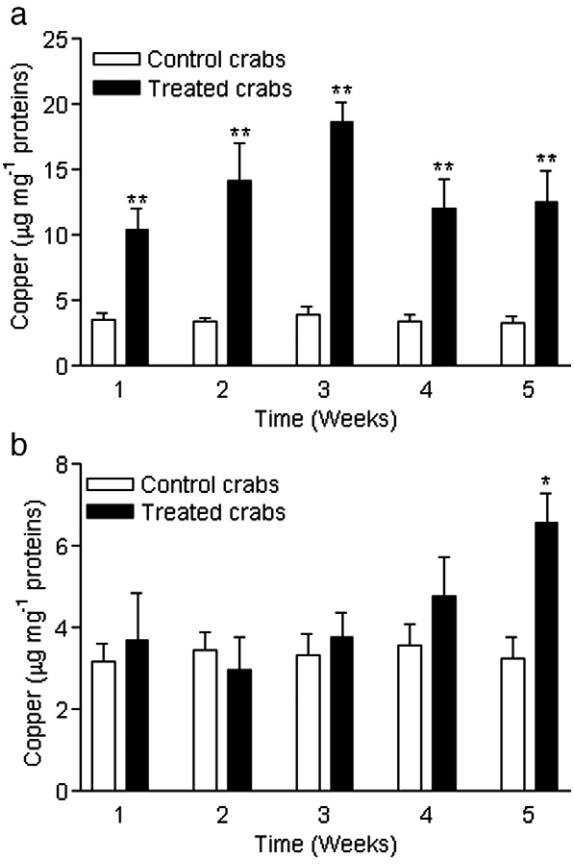

Fig. 1. a, b. Copper content (µg copper mg^{-1} proteins) in *N. granulata* during 5 weeks at 2‰ (a) and 30‰ (b) salinity. Data are expressed as means ± S.D. ($n=6$). Significant differences between control and copper treatments are indicated by asterisks: *$p<0.05$ and **$p<0.001$.

3.2. Hepatosomatic index

We investigated if exposure to copper induced an alteration of the hepatosomatic index (HSI). At 30‰ salinity, HSI was not affected by copper (Fig. 2b), but at 2‰ salinity copper induced a progressive decrease in HSI index, which was significantly lower ($p<0.05$) at the end of the assay (Fig. 2a). The HSI of treated crabs at the fifth week of exposure was 30% lower compared to control crabs.

3.3. Lipid peroxidation and protein oxidation

To analyze oxidative damage by copper in *N. granulata*, we analyzed lipid peroxidation and protein oxidation. Oxidative damage is seen as an increase in lipid peroxide formation in the hepatopancreas. Lipid peroxidation measured in the form of MDA content showed a progressive increase for both salinities in crabs fed with algae exposed to copper. In the 2‰ salinity group, treated crabs showed a significant damage in lipids caused by copper from the fourth week of treatment ($p<0.01$) (Fig. 3a). At 30‰ salinity, an increase in TBARS levels was observed in treated crabs starting at the fifth week ($p<0.01$) (Fig. 3b).

Protein oxidation caused by copper was evident at the fourth and fifth weeks at 2‰ salinity (Fig. 4a), as significant differences ($p<0.05$) were detected between treated and control crabs. At 30‰ salinity (Fig. 4b), crabs did not show much variation in protein damage compared with control crabs ($p>0.05$).

3.4. Antioxidant defenses

SOD activity in hepatopancreas showed different responses depending on the salinity. At 2‰ salinity (Fig. 5a), a significant increase ($p<0.05$) in SOD activity was only observed in treated crabs during the first and second week of assay. On the other hand, in crabs acclimated to 30‰ salinity (Fig. 5b), SOD activity showed a progressive increase that was significantly higher ($p<0.05$) at the end of the assay.

GSH showed increased values in treated compared to control crabs in both salinities (Fig. 6a, b), which were significantly higher during the second and third week at 2‰ salinity ($p<0.05$), and at the fifth week of copper exposure at 30‰ ($p<0.05$).

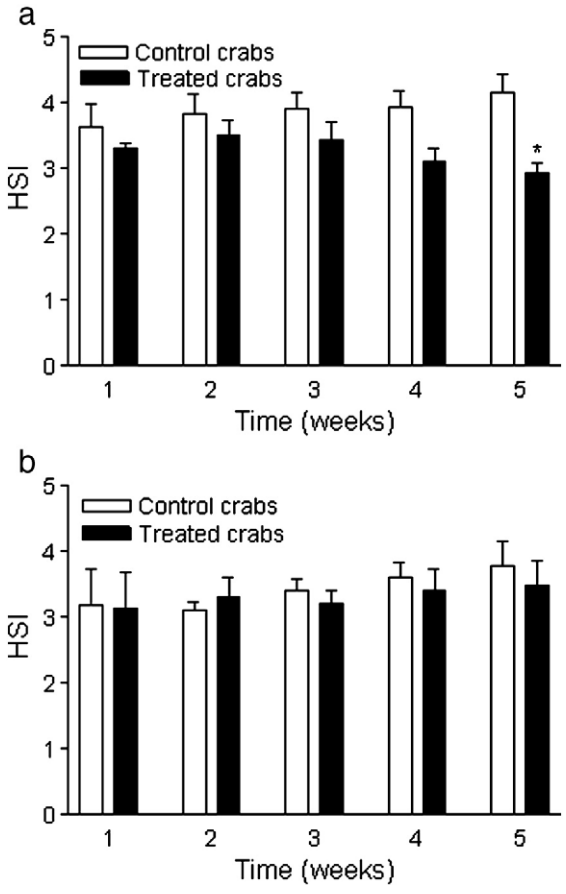

Fig. 2. a, b. Hepatosomatic index (HSI) in *N. granulata* during 5 weeks at 2‰ (a) and 30‰ (b) salinity. Data are expressed as means ± S.D. ($n=6$). Significant differences between control and copper treatments are indicated by asterisks: *$p<0.05$.

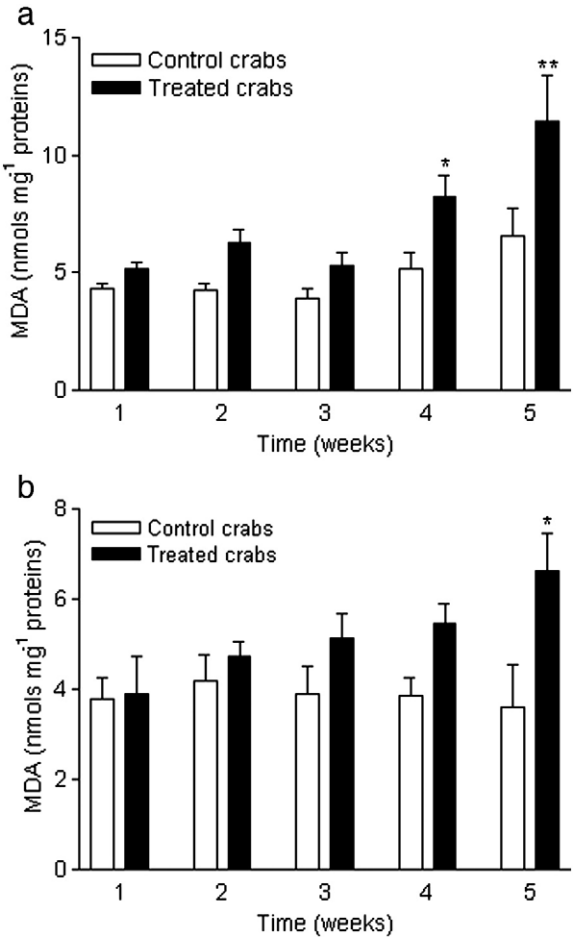

Fig. 3. a, b. Malondialdehyde (MDA) content (nmol mg^{-1} proteins) in *N. granulata* during 5 weeks at 2‰ (a) and 30‰ (b) salinity. Data are expressed as means ± S.D. ($n=6$). Significant differences between control and copper treatments are indicated by asterisks: *$p<0.05$ and **$p<0.001$.

4. Discussion

Our results demonstrate that *N. granulata* accumulates large amounts of copper from their diet, and that crabs maintained at the lowest salinity accumulated on average 40% more copper compared to crabs maintained at 30‰ salinity during the five-week experiment. Concordantly, copper toxicity in *N. granulata* was enhanced at lower salinity (2‰). This is in agreement with previous studies using other metals in several other crabs (Zaunders and Rojas, 1996; Rodríguez et al., 2001), where heavy metal toxicity was reported to increase as the salinity decreased. Increasing salinity may decrease metal bioavailability through its influence on metal speciation (Verslycke et al., 2003), Cu–Cl complexes will increase as the salinity increases and this will decrease bioaccumulation and toxicity. Additionally, it is possible that cooper enters the blood via sodium transporters in the digestive tract, and that the higher NaCl concentration presumably present in the gut lumen of seawater crabs has a protective effect

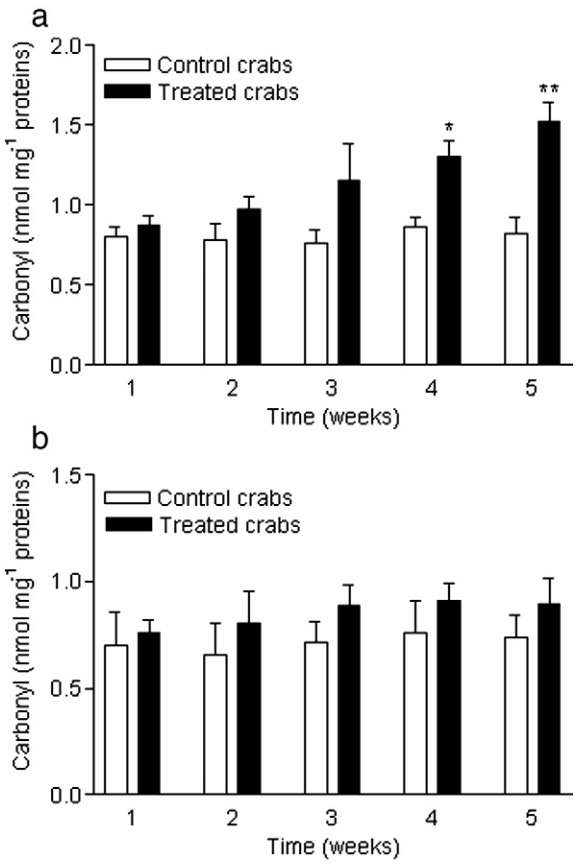

Fig. 4. a, b. Carbonyl content (nmol mg^{-1} proteins) in *N. granulata* during 5 weeks at 2‰ (a) and 30‰ (b) salinity. Data are expressed as means ± S.D. ($n = 6$). Significant differences between control and copper treatments are indicated by asterisks: *$p < 0.05$ and **$p < 0.001$.

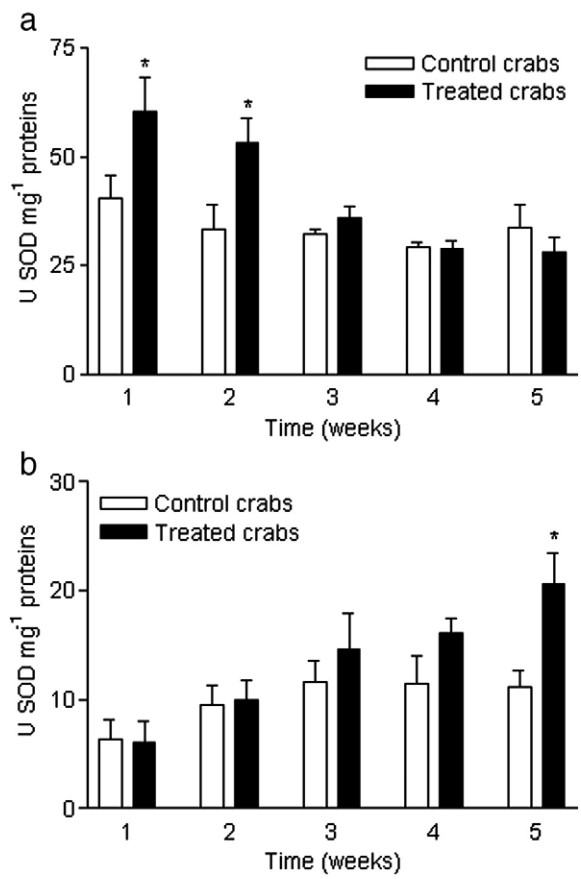

Fig. 5. a, b. Superoxide dismutase activity (U SOD mg^{-1} proteins) in *N. granulata* during 5 weeks at 2‰ (a) and 30‰ (b) salinity. Data are expressed as means ± S.D. ($n = 6$). Significant differences between control and copper treatments are indicated by asterisks: *$p < 0.05$.

on dietary copper accumulation, because of a simple competition effect between Na and Cu for the same transporter(s). Some naturally occurring cations, including Na, compete with metals such as Cu for binding sites in the organism; this competition may lead to reduced metal uptake (Niyogi and Wood, 2003). This phenomenon has already been described in trout gills (Grosell and Wood, 2002), but not much information about the molecular mechanism for dietary copper uptake, nor about its interaction with salinity, is available for any aquatic animal. Furthermore, to our knowledge, the mechanisms responsible for salt, water and nutrients transport in the digestive tract of crustaceans are also not known.

The hepatosomatic index (HSI) was decreased at the end of the experiment (5th week) in *N. granulata* exposed to dietary copper maintained at 2‰ salinity. A reduction of HSI was previously observed in *N. granulata* exposed to hexachlorobenzene (Chaufan et al., 2006) and in *Carcinus maenas* exposed to different metals, including copper (Elumalai et al., 2005). One explanation for the reductions in HSI is due to a reduction in food intake. Baker et al. (1998) observed in *Chelon labrusus*, that animals exposed to dietary copper suffered from mal-absorption which resulted in a decline of energy intake and food conversion. That decline in energy intake was reflected in lower values for hepatosomatic index compared to controls. One cause of reduced food intake is poor food palatability arising from the oxidation of lipids (Cowey et al., 1984).

Increased generation of free radicals could lead to lipid peroxidation and protein oxidation. The higher levels in lipid peroxides and oxidative proteins observed of crabs fed with algae exposed to copper indicate that the crab's defence system was overwhelmed, and therefore the animals experienced severe oxidative stress. It is important to note that significant lipid peroxidation increase of only in the last week both at 2 and 30‰ salinities, and protein oxidation in the 2‰ group was also observed in the last week. This suggests that *N. granulata* anti-oxidative defences are adequate in the short-term, but they are not entirely sufficient to prevent oxidative damage to cellular membranes during chronic exposure to copper. Higher levels in lipid peroxides were also observed by Brouwer and Brouwer (1998) in the blue crab *Callinectes sapidus* exposed to copper.

Toxicants cause distortions to cell organelles, which may induce an increase (Venugopal et al., 1997) or an inhibition (Chaufan et al., 2006) in the activities of different antioxidant enzymes. The effect of toxicants on enzymatic activity is one of the most important biochemical parameters which are affected under pro-oxidant conditions. Free radical scavengers with antioxidant properties in animal cells compensate the damaging effects caused by unstable free radicals. Antioxidant enzymes such as SOD and CAT constitute the major defensive system against ROS (Sies, 1993). The changes in SOD activity in response to copper at both salinities suggest this enzyme is important to compensate the oxyradicals generated by copper in crabs. In the long-term, SOD activity decreased in copper-exposed crabs at low salinity, reaching values similar to control crabs. Also in the long-term oxidative damage was observed in copper-exposed crabs. Thus, the inability to keep an increased SOD activity in the long-term might at least partially explain oxidative damage in crabs at low salinity. Different amino acids within the same peptide differ in their susceptibility to attack, and the various forms of ROS also differ in their potential reactivity. Oxidation of iron–sulphur centres by O^{2-} is a common reaction that inactivates enzymatic function (Stadtman, 1986; Green and Reed, 1998). The increased SOD levels could combat free radical generation during copper exposure, indicating activation of the enzymatic antioxidant defensive system. SOD increase was also

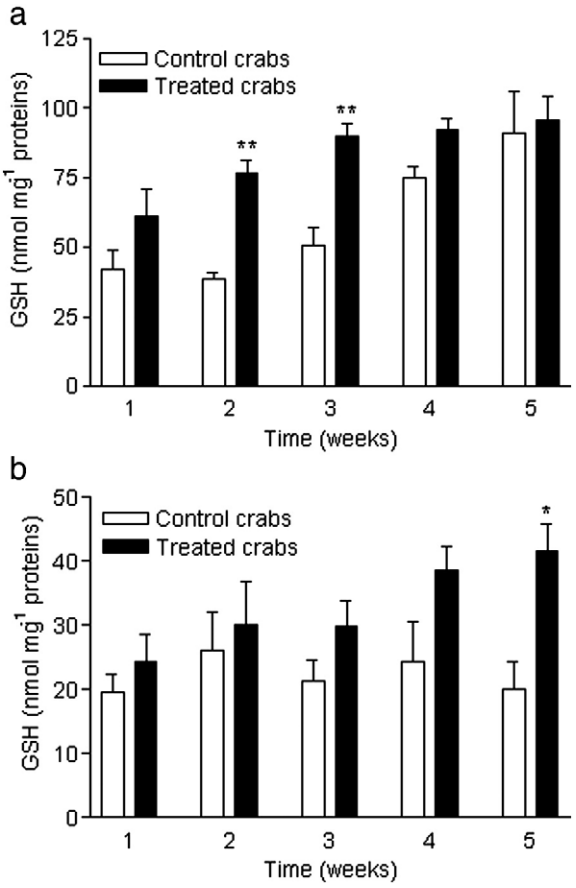

Fig. 6. a, b. Reduced glutathione (GSH) content (nmol mg^{-1} proteins) in *N. granulata* during 5 weeks at 2‰ (a) and 30‰ (b) salinity. Data are expressed as means ± S.D. ($n = 6$). Significant differences between control and copper treatments are indicated by asterisks: *$p<0.05$ and **$p<0.001$.

observed by Venugopal et al. (1997) in the field crab *Barytelphusa guerini* exposed to cadmium. Increase in SOD activity in hepatopancreas indicates this organ is important to counteract copper induced oxidative stress.

Glutathione (GSH) is one of the intracellular chelators of copper in mammals and also in invertebrates (Freedman et al., 1989; Brouwer and Brouwer-Hoexum, 1991). GSH is a very important modulator of cellular homeostasis, including detoxification of copper by inhibiting free radical formation from Cu (I) and H_2O_2 through chelation (Hanna et al., 1992). GSH reacts with $O_2^{\bullet}$, peroxy radicals ($ROO^{\bullet}$) and singlet oxygen ($^1O_2$) followed by the formation of GSSH and other disulfides (Meister, 1988). In this study, the significant increase in GSH content in treated crabs suggests that GSH may help counteract oxidative damage caused by copper in *N. granulata* by binding copper ions with its SH group, giving rise to metal–SG complex (Rabestein et al., 1985). GSH content in control crabs acclimated to 2‰ salinity displayed an increasing tendency. It is possible that, even though dietary copper in controls was low, after 4 weeks of ingestion, copper accumulated to high enough levels in the crab hepatopancreas, which could have triggered the upregulation of GSH.

The results in this study indicate that water salinity affects dietary copper uptake in *N. granulata*. Also, enzymatic and non enzymatic antioxidant defence systems in the hepatopancreas of *N. granulata* were activated during exposure to copper. Nonetheless, oxidative damage was still induced to proteins and lipids in crab hepatopancreas. Greater metal accumulation and higher oxidative stress responses were observed in crabs maintained at 2‰ salinity. We were able to identify and quantify copper accumulation by using the TXRF technique in *N. granulata* fed with *S. vacuolatus* exposed to copper. This finding may have great implications on the choice of copper concentration and salinities for future dietary toxicity experiments.

Acknowledgments

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