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Copper effects on key metabolic enzymes and mitochondrial membrane potential in gills of the estuarine crab *Neohelice granulata* at different salinities

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ABSTRACT

The estuarine crab *Neohelice granulata* was exposed (96 h) to a sublethal copper concentration under two different physiological conditions (hyperosmoregulating crabs: 2 ppt salinity, 1 mg Cu/L; isosmotic crabs: 30 ppt salinity, 5 mg Cu/L). After exposure, gills (anterior and posterior) were dissected and activities of enzymes involved in glycolysis (hexokinase, phosphofructokinase, pyruvate kinase, lactate dehydrogenase), Krebs cycle (citrate synthase), and mitochondrial electron transport chain (cytochrome c oxidase) were analyzed. Membrane potential of mitochondria isolated from anterior and posterior gill cells was also evaluated. In anterior gills of crabs acclimated to 2 ppt salinity, copper exposure inhibited hexokinase, phosphofructokinase, pyruvate kinase, and citrate synthase activity, increased lactate dehydrogenase activity, and reduced the mitochondrial membrane potential. In posterior gills, copper inhibited hexokinase and pyruvate kinase activity, and increased citrate synthase activity. In anterior gills of crabs acclimated to 30 ppt salinity, copper exposure inhibited phosphofructokinase and citrate synthase activity, and increased hexokinase activity. In posterior gills, copper inhibited phosphofructokinase and pyruvate kinase activity, and increased hexokinase and lactate dehydrogenase activity. Copper did not affect cytochrome c oxidase activity in either anterior or posterior gills of crabs acclimated to 2 and 30 ppt salinity. These findings indicate that exposure to a sublethal copper concentration affects the activity of enzymes involved in glycolysis and Krebs cycle, especially in anterior (respiratory) gills of hyperosmoregulating crabs. Changes observed indicate a switch from aerobic to anaerobic metabolism, characterizing a situation of functional hypoxia. In this case, reduced mitochondrial membrane potential would suggest a decrease in ATP production. Although gills of isosmotic crabs were also affected by copper exposure, changes observed suggest no impact in the overall tissue ATP production. Also, findings suggest that copper exposure would stimulate the pentose phosphate pathway to support the antioxidant system requirements. Although *N. granulata* is very tolerant to copper, acute exposure to this metal can disrupt the energy balance by affecting biochemical systems involved in carbohydrate metabolism.

1. Introduction

Aquatic organisms are subject to a series of environmental challenges such as salinity, temperature, and dissolved oxygen changes, as well as exposure to chemical contaminants. Coping with these challenges requires a series of physiological and biochemical adaptations to maintain the metabolic homeostasis. Regarding contaminants, copper is one of the most studied metals, especially because it is essential to aquatic animals, playing a key functional role in hemocyanin, the respiratory pigment in crustaceans. However, it is toxic to aquatic animals when at excessive concentrations in the water (White and Rainbow, 1982). Although dietborne copper uptake is a major route of copper accumulation in some crustaceans, dissolved copper uptake is more toxic and could be an important route in contaminated waters (Chang and Reinfelder, 2002; Lauer and Bianchini, 2010; Pinho and Bianchini, 2010). Waterborne copper toxicity is usually associated with the amount of metal bound to a biotic ligand like the fish gill membrane (Paquin et al., 2000, 2002; Arnold et al., 2005).

Metal bioavailability depends on the water chemistry. In fact, the amount of copper complexing [Cl^-, SO_4^{2-}, natural organic matter, $S_2O_3^{2-}$, sulfides, Br^-, and $B(OH)_4^-$] and competing (Na^+, Mg^{2+}, Ca^{2+}, K^+, e Sr^{2+}) agents augments at increasing salinities, offering a certain degree of protection against copper toxicity (Wright, 1995). However, evidence indicates that animal physiology is even more important than water chemistry to explain changes in copper toxicity across different water salinities. This would suggest that copper may display differential

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mechanisms of toxicity depending on the water salinity and species that are being tested (Grosell et al., 2007).

Among the different physiological and biochemical disturbances induced by copper, it was shown that exposure to this metal negatively affects glycolysis (Hansen et al., 1992; Satyaparameshwar et al., 2006; Carvalho and Fernandes, 2008), Krebs cycle (Balavenkatasubbaiah et al., 1984; Couture and Kumar, 2003), ionic and osmotic regulation (Stagg and Shuttleworth, 1982; Wilson and Taylor, 1993; Grosell et al., 2004a,b; Pinho et al., 2007), acid–base balance (Boitel and Truchot, 1990; Wilson and Taylor, 1993; Skaggs and Henry, 2002; Bielmyer et al., 2005; Blanchard and Grosell, 2006), ammonia excretion (Larsen et al., 1997; Grosell et al., 2003; Blanchard and Grosell, 2006), oxygen consumption (Katticaran and Salih, 1992; Santos et al., 2000; Manyin and Rowe, 2009), and growth (Santos et al., 2000; Manyin and Rowe, 2009). Most of these effects can be directly or indirectly associated with an insufficient production of energy to maintain cell metabolism and homeostasis. At extreme conditions, this situation can even lead to death.

Most of the information on physiological and biochemical effects of copper on aquatic animals is derived from studies on freshwater fish and crustaceans. Therefore studies in brackish and saltwater animals are needed for a better understanding of the toxic effects of copper across a wide range of water salinities. *Neohelice granulata* is a euryhaline crab inhabiting salt marshes from estuaries and lagoons along the Atlantic coast of South America. It tolerates water salinities ranging from 0 to 40 ppt salinity, with an isosmotic point close to 29 ppt salinity, hyperosmoregulating at lower salinities and hyposmoregulating at higher salinities. Biochemical and physiological mechanisms associated with the species ability to iono- and osmoregulate at different salinities were recently reviewed (Bianchini et al., 2008). Also, tolerance to copper was previously evaluated and shown to be very high and dependent on both acclimation salinity and presence of food in the water. In the absence of food, copper concentration inducing 50% mortality after 96 h of exposure (96-h LC_{50}) ranged from 0.028 to 91.2 mg Cu/L for water salinities ranging from 0.1% seawater to 30 ppt salinity. In the presence of food, it ranged from 0.422 to 120 mg Cu/L, respectively. The highest concentration of copper not inducing mortality after 96 h of exposure was reported as being 1 and 5 mg Cu/L in crabs acclimated to brackish and seawater, respectively (Bianchini et al., 2003).

Considering this background, the effects of sublethal concentrations of copper on the activity of enzymes associated with glycolysis (hexokinase, phosphofructokinase, pyruvate kinase, lactate dehydrogenase), Krebs cycle (citrate synthase), and electron transport chain (cytochrome c oxidase) were evaluated in gills (anterior and posterior) of *N. granulata* subjected to two different physiological conditions (hyperosmoregulating crabs: 2 ppt salinity; isosmotic crabs: 30 ppt salinity). The influence of copper exposure on the membrane potential of mitochondria isolated from gill cells was also considered in the present study. As a higher amount of energy would be necessary to cope with the extremely low salinity (2 ppt salinity) than with the salinity close to the isosmotic point (30 ppt salinity), we hypothesize that a more significant impact of copper exposure would be seen in gills of hyperosmoregulating crabs than in gills of isosmotic ones.

2. Material and methods

2.1. Crab collection and maintenance

Adult male crabs (*N. granulata*) were collected in salt marshes of the Patos Lagoon estuary (Southern Brazil) and acclimated to laboratory conditions for at least 2 weeks. Crabs were kept in 250-L tanks containing aerated salt water (2 or 30 ppt salinity), under fixed temperature (20 °C) and photoperiod (12 h L: 12 h D). Crabs were fed ground beef three times a week until satiation.

2.2. Copper exposure

Acclimated crabs (n = 10) were exposed (96 h) to copper in aquaria containing 2 L of water at the desired salinity. Copper (as $CuCl_2$) was added to water from a stock solution (1 g Cu/L). Copper concentrations tested were 1 and 5 mg Cu/L for salinities 2 and 30 ppt salinity, respectively. These concentrations were shown to be the highest non-lethal concentrations of copper to *N. granulata* under the same experimental conditions employed in the present study, at the respective water salinity tested. Furthermore, they are toxicologically equivalent since they have similar concentrations ($\sim 4.5 \times 10^{-6}$ mol/L) of free copper ion (Bianchini et al., 2003), the most toxic dissolved copper species. We are aware that copper concentrations in saltwater are much lower than those tested in the present study. However, it is important to stress that copper can be found accumulated at extremely high levels in tissues of estuarine and marine invertebrates, especially crustaceans and mollusks. For example, copper tissue burden was reported to range from 18 to 282 μg/g dry mass in several estuarine and marine invertebrates, including crustaceans (Boyden and Romeril, 1974). Even much higher levels (95.5–6480 μg/g dry mass) were reported for mollusks (Han and Hung, 1990). It is important to note that the estuarine crab *N. granulata* exposed in our laboratory to experimental conditions similar to those employed in the present study showed levels of copper accumulation in tissues, including gills, ranging from 200 to 900 μg/g dry mass (Ribeiro, 2006). Therefore, considering the premise that there is a correlation between the amount of copper accumulated in the target organ (gills) and its toxicity (Santore et al., 2001), we consider that the exposure conditions applied in the present study are suitable to evaluate the sublethal effects of acute waterborne copper exposure in the estuarine crab *N. granulata* acclimated to different water salinities.

Experimental media were completely renewed every 24 h and copper was added to the media 24 h prior crab introduction into the test chamber. A control group (no copper added to the water) was also tested in each experimental water salinity. Temperature (20 °C) and photoperiod (12 h L: 12 h D) were fixed. No food was provided to crabs during copper exposure. A first experiment was performed to collect samples for glycolysis and Krebs cycle enzyme assays. A second experiment was performed to collect samples for mitochondrial membrane potential and cytochrome c oxidase activity measurements.

After copper exposure, crabs were cryoanesthetized, and gills (anterior and posterior) were dissected and frozen (−80 °C) until enzyme analysis or pooled (2 crabs per pool) and homogenized for mitochondria isolation.

2.3. Glycolysis and Krebs cycle enzymes assays

Glycolytic and Krebs cycle enzymes were assayed according to Lallier and Walsh (1991) with slight modifications. Gills were homogenized for 5 min in ice-cold buffer (50 mM imidazole; 0.1 mM PMSF; pH 7.8) using a Potter-type homogenizer provided with a semi-micro plastic pestle. The homogenized obtained was centrifuged (10,000 *g*; 20 min; 4 °C) and the supernatant was used as enzyme source. Enzymes were assayed spectrophotometrically using a microplate reader (ELx808IU, BioTek Instruments, Inc, Winooski, VT, USA). Glycolytic enzymes (hexokinase, EC 2.7.1.1; phosphofructokinase-1, EC 2.7.1.11; pyruvate kinase, EC 2.7.1.40; lactate dehydrogenase, EC 1.1.1.27) assays were buffered with 50 mM imidazole (pH 7.4) and the NAD^+/NADH oxidation/reduction was followed at 340 nm. Krebs cycle enzyme (citrate synthase; EC 2.3.3.1) assay was buffered with 50 mM HEPES (pH 8.1), and DTNB reduction was followed at 405 nm. All assays were performed at 25 °C and specific conditions were used for each assay as follows (final concentrations): hexokinase (5 mM $MgCl_2$, 1 mM D-glucose, 0.16 mM NAD^+, 2 U/mL glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, and 1 mM ATP); phosphofructokinase-1 (10 mM $MgCl_2$, 50 mM KCl, 2 mM ATP, 0.12 mM NADH, 1 U/mL aldolase,