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Effects of Zinc Exposure on Oxygen Consumption and Gill Na^+,K^+-ATPase of the Estuarine Crab *Chasmagnathus granulata* Dana, 1851 (Decapoda-Grapsidae)

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The presence of heavy metals in water can affect the physiology of estuarine crustaceans, leading to a lack of ability to cope with environmental salinity variations (Crespo 1984). In some of these animals, anterior gills are primarily involved in gas exchange, while posterior gills are implicated in iono and osmoregulation (Péqueux 1995). Water contamination with heavy metals could cause structural modifications in the anterior gills changing the capacity to exchange gases. Under this situation, we can expect the aerobic metabolism to be modified. On the other hand, the Na^+,K^+-ATPase from posterior gills is considered the primarily mediator of the NaCl transport throughout the branchial epithelium, and plays a major role in the hemolymphatic iono and osmoregulation (Péqueux 1995). Thus, alterations in enzyme activity induced by heavy metals could lead to and iono and/or osmoregulatory imbalance in these animals. In fact, it has been demonstrated that gill Na^+,K^+-ATPase from crustaceans is sensible to several heavy metals (Haya et al. 1983; Hansen et al. 1992; Péqueux et al. 1996). However, the effect of these metals on the maximum Na^+,K^+-ATPase activity in the two functionally different types of gills remains still unknown.

The crab *Chasmagnathus granulata* inhabits estuarine and coastal areas from Southern Brazil to Argentina. In the Patos Lagoon estuary, it is frequently exposed to large salinity fluctuations and has been considered as a good hyper-osmoregulator when exposed to low salinities (Bromberg et al. 1995). However, as mentioned above, crab exposure to heavy metals could affect its aerobic metabolism and osmoregulatory capacity. In this case, it is important to note the enrichment of the Patos Lagoon estuary with zinc, which has been considered as the major available metal for biota in this environment (Baisch et al. 1988). Thus, the effects of crab exposure to sub-lethal concentrations of zinc on the oxygen consumption and the Na^+,K^+-ATPase activity in anterior and posterior gills were studied. The *in vitro* enzyme sensibility to zinc was also measured.

MATERIALS AND METHODS

Adult male crabs (7,83±3,46 g) in stage C or early D of the intermolt cycle were captured in salt marshes of the Patos Lagoon estuary around the city of Rio

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Grande/RS (Southern Brazil). In the laboratory, they were maintained in 250 L tanks, with diluted sea water (salinity 2), and under controlled temperature (21.6 ±1.9°C) and photoperiod (12L:12D). Crabs were acclimated to these conditions for 30 days. During this period, they were fed regularly with ground beef

Acclimated crabs were directly contaminated with zinc in a semi-static bioassays for 96 hr, in order to estimate LC_{50}(96 hr). Every 24 hr, experimental media were totally renewed and the survival monitored. The nominal zinc concentrations tested were: 0.5; 1; 10; 20; and 40 mg $Zn.L^{-1}$. They were obtained from a stock solution of $ZnSO_4$. In each zinc concentration, twenty crabs were tested and the death criterion adopted was absence of response to mechanical stimuli. Using probit analysis, the LC_{50}(96 hr) was estimated as 15.7 mg $Zn.L^{-1}$. Based on this result, five concentrations (0.05; 0.1; 0.2; 0.4; and 0.8 mg $Zn.L^{-1}$) were selected in order to evaluate the sub-lethal effects of zinc on the crab oxygen consumption and gill $N^{a}+, K^{+}$-ATPase activity.

The oxygen consumption of previously contaminated crabs was measured employing a flux constant respirometer adapted to an oxymeter, following the method described by Lomholt and Johansen (1979).

In order to study the effect of *in vivo* exposure to sub-lethal concentrations of zinc on the maximum Na^{+}, K^{+}-ATPase activity, crabs were contaminated as described above, cryoanesthetized, and sacrificed by removing the exoskeleton. Gills were then dissected and isolated in anterior (3^{rd}; 4^{th}; 5^{th}) and posterior (6^{th}; 7^{th}; 8^{th}) pairs. They were then rinsed in the homogenizing medium (250 m*M* sucrose and 5 m*M* EDTA) at 0-4°C. Homogenization was performed with a glass-Teflon Potter and the microsomal fraction (10,000 g pellets) were obtained by differential centrifugation (Péqueux and Chapelle 1982). Gill Na^{+}, K^{+}-ATPase activity was measured using a modification of the method described by Morris and Edwards (1995). It was determined as the difference between phosphate liberated from ATP in the presence of K^{+}(medium A) and in the absence of $K^{+}$with 1 mM ouabain (medium B). For each assay, 100 µL of the microsomal fraction were added and mixed to 2.5 mL of assay media containing the following final concentrations: (medium A) 77 m*M* NaC1; 19 mM KCl; 6 mM $MgCl_2$; 3 m*M* ATP and buffer Tris-HCl 0.1 *M* at pH 7.6 and (medium B) 96 m*M* NaCl; 6 m*M* $MgCl_2$; 3 m*M* ATP; 1 m*M* ouabain and buffer Tris-HCl 0.1 *M* at pH 7.6. The reaction started with the addition of the microsomal fraction and was incubated at 30°C for 60 min. The reaction was stopped adding 0.2 mL tricloroacetic acid (50%) to the reaction medium. Phosphate concentration in the reaction medium was determined using a reaction kit (Doles Ltda, Belo Horizonte-MG, Brazil) which is based on a modification of the method described by Fiske and Subbarow (1925). Protein concentration in the microsomal fraction was measured using a reaction kit (Doles Ltda, Belo Horizonte-MG, Brazil) based on the method described by Bradford (1976). Enzyme specific activity was expressed as µmoles $P_i$released per milligram protein per hour.

The *in vitro* effect of zinc on the maximum Na^+, K^+-ATPase activity was determined as described in the *in vivo* study, but using gills of non previously contaminated crabs as enzyme source. In this case, zinc contamination was performed adding different quantities of the $ZnSO_4$ stock solution until to attain the desired final concentration (0.05; 0.08; 0.10; 0.15; 0.20; and 0.40 mg $Zn.L^{-1}$).

All chemicals employed, except reaction kits, were purchased from Sigma Co. (St. Louis, USA) and Merck (Darmstadt, Germany). Data were analyzed using one-way ANOVA followed by Newman-Keuls test (α=0.05).

RESULTS AND DISCUSSION

Among heavy metals, zinc is considered as presenting relatively low toxicity to aquatic invertebrates. For these animals, the LC_{50}(96 hr) has been estimated varying between 0.5 and 15 $mg.L^{-1}$ and the cause of death has been related to a progressive decrease in the animal osmoregulatory ability (Crespo 1984). In the present study, the LC_{50}(96 hr) was estimated as 15.7 $mg.L^{-1}$, which suggested that *Chasmagnathus granulata* is comparatively less sensible to this metal than most other invertebrates studied. This statement is based on both the LC_{50}(96 hr) value estimated, and the fact that our estimation was performed employing crabs acclimated to very low salinity, condition in which they are strongly hyperosmoregulating (Bromberg et al. 1995).

Despite of the comparatively high tolerance of *C. granulata* to zinc, *in vivo* contamination with concentrations higher than 0.1 $mg.L^{-1}$ significantly increased the crab oxygen consumption (Fig. 1). In general, the oxygen consumption of mollusks and crustaceans is reduced when they are acutely exposed to heavy metals (Spicer and Weber 1991). However, as pointed out by these authors, the susceptibility of the respiratory processes during the metal exposition could be modified by intrinsic factors (sex, life stage, etc.) and/or extrinsic ones (hypoxia, saline stress, etc.). So, *C. granulata* response to zinc exposure in diluted media could have been modified and/or intensified due to the interaction between these factors. Nevertheless, the increase in the oxygen consumption rate of *C. granulata* observed when it was exposed to zinc seems to be related to other processes than those involved in hemolymph osmoregulation, This assumption is based on the fact that the both *in vivo* and *in vitro* zinc exposure inhibited the gill Na^+, K^+-ATPase in the posterior gills of *C. granulata* (Figs. 2 and 3). In such case, energy consumption by enzymatic activity is expected also to be reduced.

Several crustaceans present ability to regulate the internal concentrations of essential metals, but some of them, as zinc for example, could become potentially toxic in higher concentrations. In order to compensate the toxic levels of metals, marine and estuarine invertebrates present physiological and biochemical processes of detoxification. They include the production of physiologically inert granules and the synthesis of metalothioneins (Rainbow 1985). Thus, as suggested by Depledge (1984) for the crab *Carcinus maenas* contaminated with mercury, the respiratory

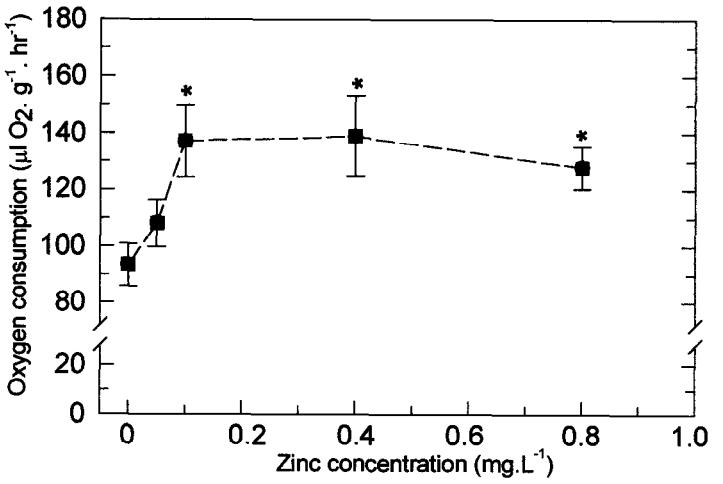

Figure 1. Oxygen consumption of *Chasmagnathus granulata* acclimated to salinity 2 after 96 hr of exposure to sub-lethal concentrations of zinc as $ZnSO_4$. Data are means ± SE (N=6). * Indicates means significantly different from control condition ($P<0.05$).

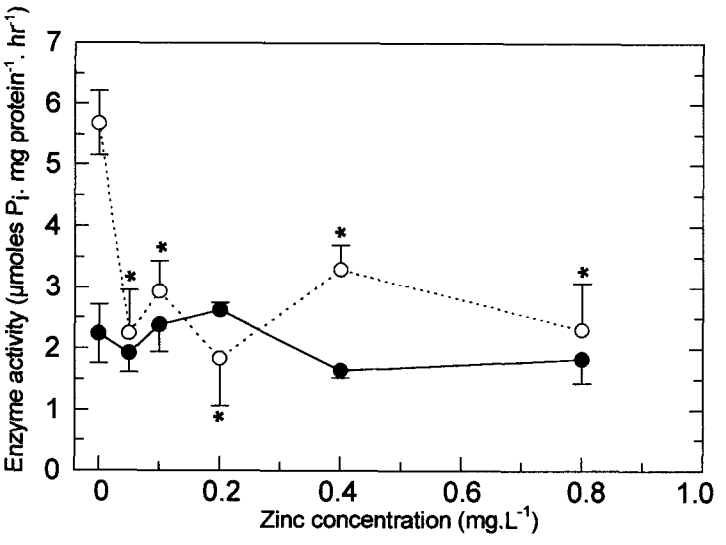

Figure 2. Maximum Na^+, K^+-ATPase activity in gills of *Chasmagnathus granulata* acclimated to salinity 2 after 96 hr of *in vivo* exposure to zinc as $ZnSO_4$. (•) Anterior gills; (o) Posterior gills. Data are means ± SE (N=6). * Indicates means significantly different from control condition ($P<0.05$).

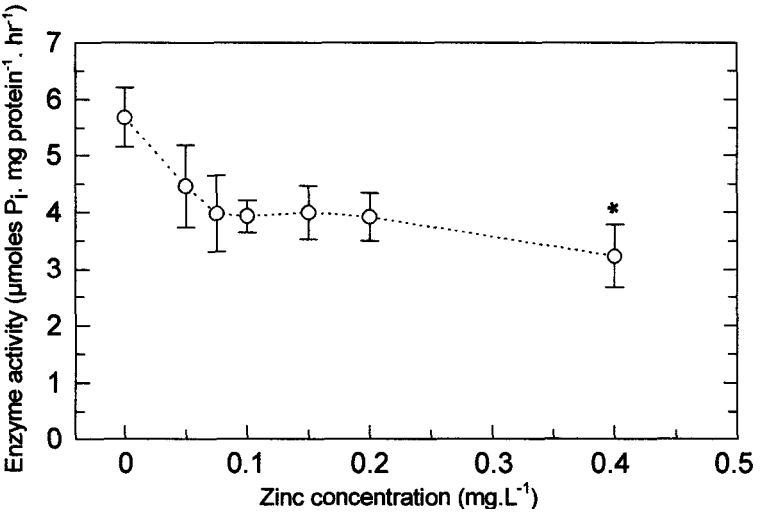

Figure 3. Maximum Na^+, K^+-ATPase activity in posterior gills of *Chasmagnathus granulata* acclimated to salinity 2 after 1 hr of *in vitro* exposure to zinc as $ZnSO_4$. Data are means ± SE (N=6). * Indicates means significantly different from control condition ($P<0.05$).

response registered in this study could be, at least in part, related to an increased energy consumption by the processes involved in mobilization, detoxification and excretion of zinc.

Regarding the enzyme activity, it was demonstrated, in some species of decapod crustaceans, that several heavy metals inhibit both *in vivo* and *in vitro* activity of the Na^+, K^+-ATPase. In the lobster *Homarus americanus* and the crabs *C. maenas* and *Eriocheir sinensis*, a significantly reduction in the gill enzyme activity was observed when they were exposed to zinc (Haya et al. 1983), copper (Hansen et al. 1992) and mercury choride (Péqueux et al. 1996). In the present study, *C. granulata exposure* to sub-lethal concentrations of zinc did not affect the maximum Na^+, K^+-ATPase activity in the anterior gills. However, it was significantly reduced in posterior gills from crabs exposed to concentrations as low as 0.05 $mg.L^{-1}$ (Fig. 2). It is interesting to note that the maximum enzymatic activity registered after zinc exposure is similar to that reported for the posterior gills of *C. granulata* acclimated to salinity 30 (Castilho 1996), a condition near to the isosmotic point (Bromberg et al. 1995). Thus, the enzymatic inhibition induced by zinc could be leading to a decrease in the NaCl transport across the posterior gills and to a long-time disruption of the hemolymphatic osmotic balance in the crab. In the mysid *Praunus flexuosus*, McLusky and Hagerman (1987) reported that salinity changes, below or above the isosmotic point reduced the mean survival time during exposure to zinc. Thus, our results from enzymatic study are in accordance with the idea that the cause of death induced by zinc is, at least in part, related to a progressive

decrease in crab osmoregulatory capacity. This hypothesis is based on both, the lack of zinc effect on Na^+, K^+-ATPase activity from anterior gills, and the reduction of about 50% observed of the enzyme activity in the posterior gills (Fig. 2).

Considering that *in vivo* zinc exposure did not significantly affect the maximum Na^+, K^+-ATPase activity in the anterior gills, only posterior gills were tested *in vitro*. In this case, a concentration-related inhibition induced by zinc was observed (Fig. 3). A significantly enzymatic inhibition (43%) was registered at the higher concentration tested (0.4 $mg.L^{-1}$). This inhibition was very similar to that obtained after 96 hr of *in vivo* exposure to the same concentration (42%). Further, a similar level of inhibition was attained after 96 hr of *in vivo* exposure to any zinc concentration tested (Fig. 2). Haya et al. (1983) reported similar inhibition (45%) of the gill Na^+, K^+-ATPase in the lobster *Homarus americanus* acclimated to sea water and exposed to very high zinc concentration (25 $mg.L^{-1}$). Thus, these results could indicate that a restrict number of active sites are present in the enzyme molecule and the total interaction between these sites and zinc lead to an inhibition of approximately 50% of the enzyme activity. They also suggest that these active sites are quickly occupied (less than 1 hr).

The fact that several washes and centrifugations were realized to perform the enzymatic assay, suggest that the enzyme inhibition observed could be irreversible. Further, it is also interesting to note that the maximum enzymatic activity registered in posterior gills after *in vivo* exposure to any zinc concentration tested was very similar to that presented by non contaminated anterior gills. Thus, this result suggests that the difference in activity between non contaminated anterior and posterior gills are probably due to the presence of zinc-sensible catalytic sites in the latter, which are absent or inaccessible in the first.

Finally, after 1 hr of *in vitro* exposure to zinc concentrations lower than 0.04 $mg.L^{-1}$, we registered a lower inhibitory effect on the enzymatic activity than that observed after 96 hr of *in vivo* exposure to same concentration of metal (Figs. 2 and 3). This result suggest the involvement *in vivo* of bioconcentration processes, which are frequently reported in the literature related to crustaceans (for example: Rainbow 1985; Bianchini and Gilles 1996).

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