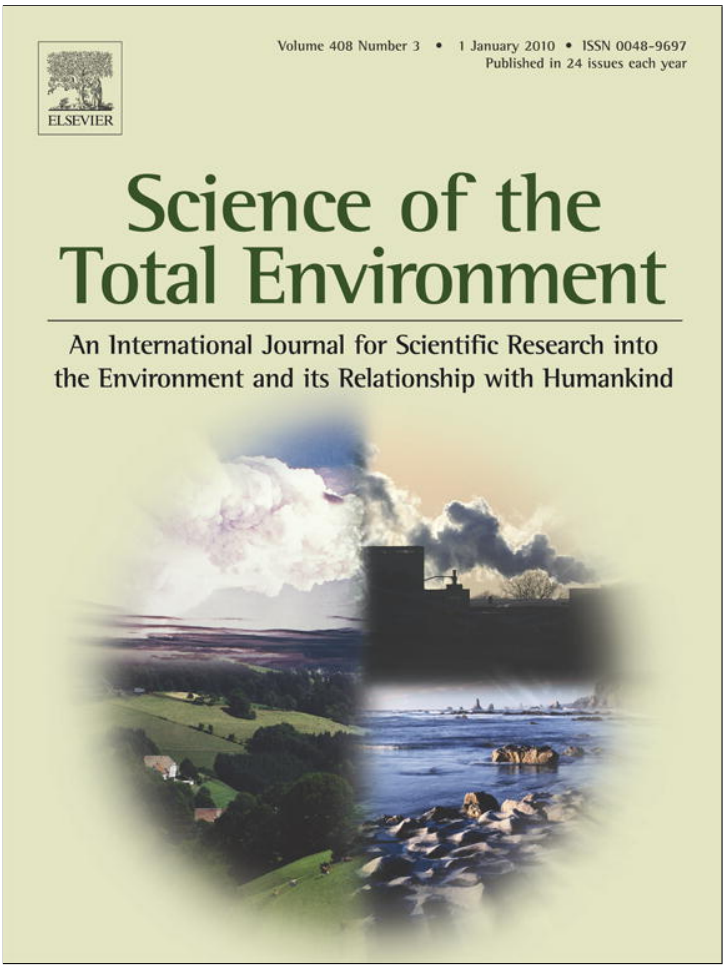

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Effects of zinc on molting and body weight of the estuarine crab *Neohelice granulata* (Brachyura: Varunidae)

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ABSTRACT

The semiterrestrial burrowing crab *Neohelice granulata* is one of the main inhabitants of the supratidal and intertidal zones of brackish salt marshes, estuaries and coastal lagoons from South America's Atlantic littoral. A large population of this species spreads out Mar Chiquita coastal lagoon (in Argentina) and its corresponding wetlands, and is considered as a key species within this system.
Since high values of dissolved heavy metals (including Zn) have been recently reported within Mar Chiquita coastal lagoon, with levels unusually higher than those from other coastal systems within Argentina, it has been explored that the existence of a risk of environmental conditions endanger these populations. So, juveniles of this estuarine crab were experimentally exposed to increasing concentrations of dissolved Zn (i.e., 0, 0.5 and 1 mg Zn^{2+} L^{-1}) during six months, the time involved between two successive molts; in addition, both the size and weight reached after each molt were also studied in this assay. It can be concluded that zinc can be toxic to crabs only at high concentrations. Considering that levels up to 1 mg Zn L^{-1} were recently reported in Mar Chiquita coastal lagoon waters, the potential occurrence of mean chronic effects on the crab population within the coastal lagoon is discussed.

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1. Introduction

Estuaries and coastal zones, together with their corresponding wetlands, usually act as sinks of land materials — including pollutants — from agricultural, industrial and urban origins which are therefore transferred to the sea (Marcovecchio, 2000). Aquatic environments are increasingly being contaminated with different kinds of organic and inorganic pollutants. Many of these dangerous compounds, such as heavy metals, can readily accumulate within crustacean tissues at much higher concentrations than those in water column and sediment (Rainbow, 2007) and both, essential and nonessential metals, can become toxic. Recent studies have focused on the evaluation of specific endpoints in invertebrates to assess their potential endocrine disruption. Laboratory studies on crustaceans have demonstrated that several heavy metals and organometallic compounds negatively affect several hormonal-controlled processes, such as molting, reproduction, growth and pigmentary responses (Reddy et al., 1997; Fingerman et al., 1998; López Greco et al., 2000; Rodríguez et al., 2007). These are remarkable points considering that the protection of aquatic habitats from damage due to contaminants requires an understanding of both the sensitivity of invertebrates to contaminants and their ecological requirements.

Crustacean growth and development are achieved by ecdysis (namely the periodic shedding of the rigid exoskeleton) which is part of their molting cycle. Each ecdysis is followed by a post-molt uptake of water and, as consequence, a rapid increase in body size during the short soft-skinned period (Hartnoll, 2001). Molting is a very important physiological process for crustaceans because it not only allows growth and development of these animals, bearing a rigid and confining exoskeleton, but it is also tied with metamorphosis during the early stages of the life cycle, on the one hand, and with reproduction during the adult stage, on the other hand. Crustacean molting is regulated by a multihormonal system called ecdysteroids (Chang et al., 1993) and associated with environmental cues, including light and temperature. It is closely linked to reproduction, growth and development, and it therefore stands as an interesting endpoint for evaluating invertebrate-specific endocrine toxicity (Verslycke et al., 2007). Change in weight is another commonly accepted sensitive endpoint in ecotoxicity testing (Odendaal and Reinecke, 1999). It represents a sublethal effect but could have a long-term impact on populations. These are several facts which

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sustain the present study, considering that prior to death or overt sickness, organisms may respond to stress by changing molecular, physiological or behavioural responses; the ability to recognise and measure these changes, defined as biomarkers, may provide an early warning of later, much more serious consequences (Livingstone, 1993).

The semiterrestrial burrowing crab *Neohelice granulata* (Brachyura, Varunidae) is a eurihaline species widely distributed along the Atlantic coast of South America, from Rio de Janeiro (Brazil) to Patagonia (Argentina), and it is one of the main inhabitants of both the supratidal and intertidal zones of brackish salt marshes, estuaries and coastal lagoons within the southeast of South America. The large populations of this species distributed along wide salinity gradients have an important ecological role in salt marshes, considering every stage of the crab's life cycle becomes a relevant food component for many fish, shellfish and bird species (Spivak et al., 1994).

A large population of *N. granulata* inhabits Mar Chiquita coastal lagoon and its corresponding wetlands. This is a shallow, unique coastal lagoon in Buenos Aires province (Argentina), located between 37°3′–37°43′S, and 57°15′–57°30′W (Fig. 1), which has been declared as *Biosphere Reserve* by Man and Biosphere Reserve Program from UNESCO. This coastal lagoon provides refuge and food for numerous native and migratory species (e.g. crustaceans, fishes, birds) and constitutes an estuarine environment with a very particular behavior (Marcovecchio et al., 2006). It has a very important input of inland water through streams and channels coming from the Tandilia orographic system–where a very important farming activity is developed–, and consequently several anthropic impacts can occur there. So, high concentrations of heavy metals, specially zinc, both in pore water and dissolved in water column have been recently reported within this coastal lagoon (Beltrame et al., 2008), with values higher than 1000 μg L^{-1}, which are in the same order than those recognized as marine water quality criterion for the protection of human health for recreational use in the marine environment (1250 μg L^{-1}; Chongprasith et al., 1999).

Considering the importance of molting and body weight in the life of crustaceans, it is necessary to develop screening assays that could be used to identify the income of pollutants capable of interfering

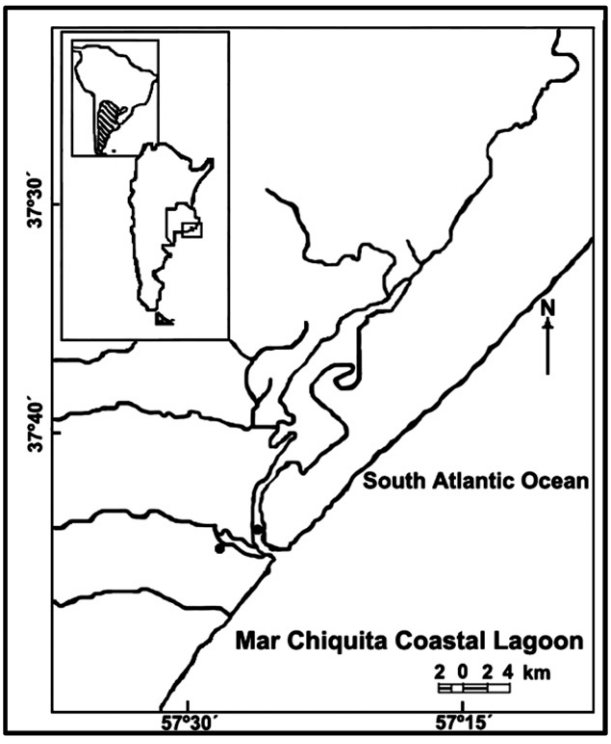

Fig. 1. Location of the sampling site at Mar Chiquita coastal lagoon.

with their molting process. So, and keeping in mind the hypothesis that the considered populations of crabs could be endangered due to high Zn levels within Mar Chiquita coastal lagoon, the main purpose of the present study is to evaluate the effects of this metal on the intermolt period (i.e. the time between two successive molts), together with body size and weight variations reached after each molt in juveniles of the crustacean *N. granulata*, one of the most important key species that inhabits this coastal lagoon. In addition, this kind of study related to the effect of zinc on the molt of this crab population has at present not been carried out yet.

2. Materials and methods

The obtained crabs have been exposed to increasing concentrations of $ZnCl_2$ considering – on the one hand – that this chemical form has been reported as the most important fraction within Mar Chiquita coastal lagoon (Marcovecchio and Ferrer, 1999), and – on the other one – the recognized fact that most of the inorganic Zn is complexed by chloride within estuaries (Rainbow and Black, 2005). $ZnCl_2$ was obtained from Biopack® (Argentina). Stock solutions were prepared using deionised water and stored in a dark refrigerator. The Zn^{2+} concentration in the stock solution was 1 g L^{-1}. Water used for treatment was collected in the same area where organisms were caught, and its purification included filtration through cotton, filter paper, Millipore HA filters (0.45 μm mesh), and storage with activated charcoal in order to remove dissolved organic matter and trace metals (Kremling, 1999; Marsh and Rodríguez-Reinoso, 2006). Then, another filtration through a 0.45 μm mesh filter was carried out to remove activated charcoal. In order to obtain the desired salinity for both the acclimation period and bioassays, either distilled or hypersaline water was added.

Specimens of *N. granulata* were handpicked during low tide at the "cangrejales" covered by *Spartina densiflora,* in Mar Chiquita coastal lagoon. Sluggish crabs or those lacking one or more appendixes were discarded. Crabs were transported to the laboratory in thermally isolated boxes with *in situ* collected water. Immature juveniles (Gavio et al., 1994) with carapace width (maximum distance between the two prominent lateral spines) between 6.5 and 10 mm and initial average weight of 236 ± 71 mg in intermolting stage were selected (Drach and Tchernigovtzeff, 1967).

Once in the laboratory, crabs were kept in closed glass aquariums, covered with natural water from the lagoon, with constant aeration, acclimated in a culture chamber and under the same conditions than those utilized within the toxicity bioassays. Since changes in salinity and pH can affect metal speciation, and consequently modify both their availability and toxicity, the experimental conditions (salinity and pH) of the toxicity test were an average of those found within the environment (Marcovecchio et al., 2006). During the acclimation period (2 weeks) animals were fed *ad libitum* with rabbit pellet food twice a week, and the water of aquariums was renewed after each feeding, as reported as adequate in previous studies (Rodríguez Moreno et al., 2003).

A chronic toxicity experiment was performed to evaluate the potential ability of zinc to interfere with crab molting and body weight variation within the selected species. The experimental exposure period for this test was 6 months. Toxicity bioassays were carried out according to the procedures outlined by FAO (Ward and Parrish, 1982; Reish and Oshida, 1987) as well as the American Public Health Association (APHA, 1992).

Juveniles were individually placed in 500 ml glass recipients containing 300 ml of the selected test concentration, with constant aeration, at a salinity of 20 ± 1 psu, photoperiod: 12 h light/12 h dark cycle (fluorescent light), room temperature at 25 ± 1 °C and pH 7–8.2 upH. Each crab was randomly distributed among the different test concentrations: 0 (control) — 0.5–1 mg Zn^{2+} L^{-1}. These concentrations were based on previous environmental researches on *N. granulata*

(Beltrame et al., 2008) within the studied environment. Crabs were fed *ad libitum* twice a week; water of the aquariums was renewed after each feeding and exposure solutions were renewed every 72 h, but water quality (temperature, salinity, pH, dissolved oxygen, ammonia, and particulated organic matter) was daily controlled. The initial number of crabs was 95 (35 in each experimental zinc exposure and 25 in the control group).

The aquariums were daily examined for newly molted juveniles; the date of molting and death were recorded. Toxicological endpoints include time (days) to achieve the molt (intermolt period: IMP), and body wet weight and size changes during each IMP. The size change measurement (maximum distance between the two prominent lateral spines) was carried out at the beginning of the assay and two days after each molt, using a caliper to the nearest 0.1 mm. At the same time, the body weight was also measured: crabs were dried on absorbent paper and weighed to the nearest 0.0001 g using a digital balance.

The size change at each molt was calculated for each specimen using the formula

$$(Cw_a - Cw_b)^{*}100^{*}Cw_b^{-1}$$

where Cw_b and Cw_a are the carapace width before and after the molt, respectively.

The body weight change at each molt was calculated for each specimen using the formula

$$(Ww_a - Ww_b)^{*}100^{*}Ww_b^{-1}$$

where Ww_b and Ww_a are the wet weight before and after the molt, respectively.

Size increase, body weight change and IMP data from the sublethal experiments were statistically analysed by means of one-way ANOVA, after testing normality and homosedasticity. This was followed by multiple pairwise comparisons, using the Holm–Sidak method. Overall significance level = 0.05 (Sigmastat Software 3.0®).

3. Results

3.1. Effect of zinc on juveniles of N. granulata survival

Percentages of surviving crabs and their initial size and number in each treatment are shown in Table 1. After a 6 month time-period, survival of juveniles among concentrations varied from 52% (control) and 46% (0.5 mg Zn^{2+} L^{-1}) to 63% (1 mg Zn^{2+} L^{-1}). No significant differences among treatments ($p<0.01$) were recorded.

3.2. Effect of zinc on juveniles of N. granulata intermolt period (IMP)

The long-term effect of zinc on the IMP is shown in Fig. 2. In the control groups, the first IMP (incomplete) lasted 42.4 ± 18.3 days on average, whereas the second IMP took about 55.9 ± 18.4 days. Only one crab reached a third molt with an IMP of 38 days. With the lowest Zn^{2+} assayed concentration (0.5 mg Zn^{2+} L^{-1}), the elapsed time for the first IMP was about 49.9 ± 27.3 days, and for the second IMP was 70.4 ± 10.1 days. Only one crab reached a third molt with an IMP of 64 days. According to the assay, the time in to achieve the first molt was significantly longer at the highest exposure concentration (1 mg Zn^{2+} L^{-1}) compared to the other treatments (control and 0.5 mg Zn^{2+} L^{-1}). The first IMP took 62.5 ± 28.4 days, and the second IMP lasted 66.2 ± 15.9 days. A third molt with an IMP of 53 days occurred in only one crab. The second IMP appeared to be longer in both Zn^{2+} treatments (0.5 and 1 mg Zn^{2+} L^{-1}), though these differences were not statistically relevant ($p<0.01$). Only the first IMP was significantly longer in *N. granulata* exposed to 1 mg Zn^{2+} L^{-1}; and the third one appeared to be longer as well in this Zn^{2+} treatment.

Table 1
Number at start, inicial carapace width and percentage of survival crabs *N. granulata* of each treatment.

	Control	0.5 mg $Zn^{2+}.L^{-1}$	1 mg $Zn^{2+}.L^{-1}$
Number at start	25	35	35
Initial CW (mm)	8.02 ± 0.91	7.99 ± 1.26	8.04 ± 1.15
Survival (%)	52	46	63

Means ± standard deviation are indicated.
CW: maximun carapace width.

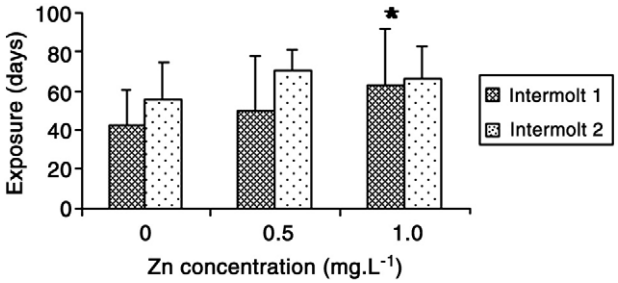

Fig. 2. Effect of zinc on first intermolt period (incomplete) and second intermolt period during six months bioassay with juveniles of *N. granulata*. * significantly different from control (Holm–Sidak method, $p<0.05$).

3.3. Effect of zinc on juveniles of N. granulata size changes

The effects on juveniles of *N. granulata* size changes when exposed to Zn^{2+}, during a six month bioassay, are shown in Fig. 3. The first size changes were 7.7 ± 4.3%, 9.1 ± 6.5% and 8.1 ± 5.2% for the control, 0.5 mg Zn^{2+} L^{-1} and 1 mg Zn^{2+} L^{-1}, respectively. The second size changes were 4.7 ± 4.1, 1.9 ± 5.3 and 0.8 ± 4.3 for the control, 0.5 mg Zn^{2+} L^{-1} and 1 mg Zn^{2+} L^{-1} respectively. Non-significant effects ($p<0.01$) were recorded on juveniles of *N. granulata* size changes exposed to Zn^{2+} for all molts. Nevertheless, in the second size change, results showed a reduced increment on size as far as concentration of Zn^{2+} increased. It is worth mentioning that in some cases where juveniles were under Zn^{2+}, a reduction in the size was observed. The percentages of individuals that reduced their size during the first and second change registered were: 0% for control, 0 and 33.3% for 0.5 mg Zn^{2+} L^{-1}, and 3.8 and 37.5% for 1 mg Zn^{2+} L^{-1}.

3.4. Effect of zinc on juveniles of N. granulata wet weight changes

After each molt, all organisms were weighted. Fig. 4 shows the effect of exposure to zinc on wet weight changes from *N. granulata* juvenile crabs. There was a significant decrease in wet weight change at the highest exposure concentration compared to both control animals and those under the lowest exposure zinc concentration. For the control group organisms, the first wet weight change average was

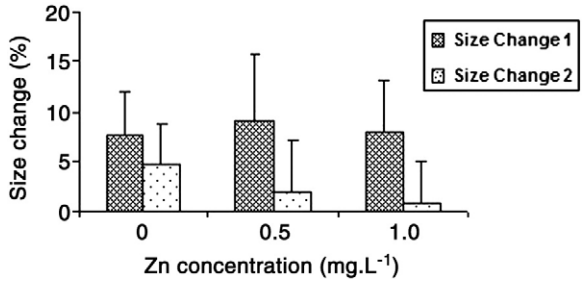

Fig. 3. Effect of zinc on first and second size change registered during six months bioassay with juveniles of *N. granulata*.

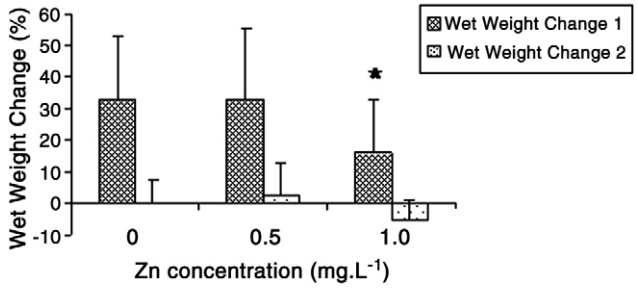

Fig. 4. Effect of zinc on first and second wet weight change registered during six months bioassay with juveniles of *N. granulata*. * significantly different from control (Holm–Sidak method, $p<0.05$).

$32.8 \pm 19.7\%$, and $32.9 \pm 22.4\%$ at the lowest exposure zinc concentration. The first wet weight change average in 1 mg Zn^{2+} L^{-1} treatment was $16.1 \pm 16.9\%$, denoting a subtle change compared to the both other treatments. This change included a minor increase in wet weight and, in some cases, a reduction in the crab weight between the two molts. The second wet weight change average of control organisms was $0.3 \pm 7.0\%$, while $2.7 \pm 9.9\%$ in 0.5 mg Zn^{2+} L^{-1} treatment, and $-5.2 \pm 6.5\%$ in 1 mg Zn^{2+} L^{-1} treatment. These changes were not significantly different ($p<0.01$). However, a decrease in the wet weight at the highest zinc concentration treatment was detected; this change was negative, suggesting a decrease in the wet weight of crabs.

4. Discussion

Even though the process of growth through molting of *N. granulata* has been described after experimental studies (López and Rodriguez, 1998; Luppi et al., 2002), its disruption by chemicals through specific mechanisms has at present not been properly studied, and only few results have at present been obtained (i.e. Snyder and Mulder, 2001; de Souza et al., 2008). In addition, studies on the effect of zinc on molting of this crab species have neither been carried out, and consequently its potential effects on crab's populational growth and recruitment are not well known.

In the present research, the potential chronic effects of zinc on crab molting and growth were evaluated by exposing *N. granulata* juveniles to sublethal concentrations of zinc (0, 0.5, and 1 mg Zn^{2+} L^{-1}) during a six month assay; the survival, intermolt period, size change and body wet weight change were recorded, considering they could be potential tasks for this metal effect on crustaceans (Wang and Rainbow, 2008). Zinc did not significantly affect size changes at the tested levels, even though, in some cases, a surprising decrease in size could be observed; these results were different to those opportunely reported by Marsden and Rainbow (2004) for several Decapoda species like *Palaemon elegans* or *P. longirostris*, which showed a strong relationship between increasing size and decreasing Zn concentrations. Physiological processes in crustaceans are mostly coordinated by hormones, and it has been largely reported that changes in hormone levels use to occur after exposure to environmental stressors (Fingerman et al., 1998). Molting is crucial to normal crustacean growth, development and reproduction, and it has been demonstrated to be a sensitive endpoint to evaluate the chemically induced endocrine disruption (Verslycke et al., 2007). Molting is regulated by a multihormonal system, under immediate control of the steroid hormones called ecdysteroids (Chang et al., 1993) and associated with environmental cues, including light and temperature. Studies made on crustaceans with heavy metals and organometalic compounds have shown to negatively affect several hormonal-controlled processes, such as molting, reproduction, glucose level and pigmentary responses (Fingerman et al., 1998). In this sense, Sarabia et al. (2008) demonstrated the deleterious effect of sublethal levels of Zn on demographical parameters of *Artemia parthenogenetica*, including reproduction and growth rates.

On the other hand, results from the present study have shown that 1 mg Zn^{2+} L^{-1} concentration significantly delayed the intermolt period of *N. granulata*. The IMPs on both experimental 0.5 mg L^{-1} and control treatments, were shorter than on 1 mg L^{-1} exposure, presumably due to the impact of this toxic on the hormone-regulated process. Studies made on decapod crustaceans showed different effects on growth and molting due to zinc and other heavy metals exposure. So, Kogan et al. (2000) observed that cadmium did not produce effects on the growth of juvenile females of *Chasmagnathus granulata*, while Rodríguez Moreno et al. (2003) have reported an inhibition of molting in adults of the same crab species exposed to cadmium from the beginning of premolt. Their results suggested that cadmium could be hindering the normal peaking of ecdysteroids essential for molting, and – considering eyestalk-ablated crabs were used for these experiments – these authors have presumed the occurrence of a direct effect of cadmium on the Y-organ, affecting cytoplasmatic calcium concentration and/or other actions. Moreover, López Greco et al. (2001) observed that cadmium and copper produced significant effects on molting time of *C. granulata* compared to control, but their effects were opposite: copper produced a precocious molting, while cadmium caused a retardation of this process. These findings were included within the toxicological scenario as described by Monserrat et al. (2007) for different estuarine organisms, and were also in agreement with the effects of other pollutants (i.e. the insecticide methoprene) on molting of *Neomysis integer* (Crustacea:Mysidacea), according to the report by Ghekiere et al. (2006).

The mean body weight of juvenile crabs unexposed to Zn has – as expected – increased over the six months of observation. An increment was also observed on the control and 0.5 mg Zn^{2+} L^{-1} exposure groups after the first change in body weight. There was a significantly lower mean body weight of the 1 mg Zn^{2+} L^{-1} exposed *N. granulata* in comparison to the control ones, suggesting that this concentration of Zn^{2+} has produced a strong negative effect. Besides, no differences were observed in the second weight change; the mean values recorded for the changes in the 1 mg Zn^{2+} L^{-1} group were mostly lower than those of the control crabs and 0.5 mg Zn^{2+} L^{-1} groups. The well-known ability of *N. granulata* to resist this type of metal contamination (i.e. LC_{50}96 h 50 mg L^{-1} in Bahía Blanca population according to Ferrer et al., 2006; and, LC_{50}96 h 11.41 mg L^{-1} in Mar Chiquita population after Beltrame et al., 2008), may provide a clue to the fact that crabs exposed to 0.5 mg Zn^{2+} L^{-1} did not have a significantly lower body weight than those exposed to 1 mg Zn^{2+} L^{-1}. It has been recognized that metals inhibit the chemoreception in aquatic animals (Santos et al., 2000), and within this scenario crabs exposed to increasing metals concentrations could be suffering a significant reduction on their food detection ability, leading them to a decrease within their body weight. Furthermore, it was demonstrated that metal effects on the nervous system could induce impairment of prey capture and manipulation, as well as locomotory activity (Bryan et al., 1995). Pestana et al. (2007) showed that an increase in Zn and Cd concentrations at sublethal levels resulted in significant reductions of the feeding rate of *Atyaephyra desmarestii* (Decapoda) and *Echinogammarus meridionalis* (Amphipoda). This kind of responses have potential repercussions on other life history traits such as growth, reproduction and, ultimately on survival of organisms (Maltby, 1999), with the consequent potential impact on the corresponding crabs populations. The observed effect of lower body weight on the studied crabs could have also occurred because they would presumably need and consume most of their energy in order to resist the contaminant, either by avoidance and/or detoxification, and thereby would have scarce energy available for growth. Barata et al. (2004) suggested that several pollutants, especially heavy metals, are able to inhibit food intake on small crustaceans, such as cladocerans and copepods. Thus, such pollutants could be diminishing/lowering the

growth rate merely by reducing the energy acquisition and/or by increasing the energy demand associated with the stress caused by their presence.

On the other hand, also the role of hepatopancreas –which is a vital and major organ of decapod that combines many of the functions of the liver, pancreas, intestine and other organs within vertebrates– must be considered to fully understand the results of the present study. The adequate functioning of hepatopancreas is important to health, growth and survival of decapods, and – in fact – this organ has been used as a monitor one for the overall assessment of the health and well-being of the intact organism (Gomiero et al., 2006). Hepatopancreas primary role is the synthesis and secretion of digestive enzymes, final digestion of the ingested food and subsequent uptake of nutrients (Caceci et al., 1988). Nevertheless, Ferrer (2001) observed that several metals, including Zn, accumulated in this organ in *C. granulata*. If metals accumulate in and affect the organ, weight and growth of crabs would presumably be negatively impacted, as the results of this study have reflected. Furthermore, these results were similar to those by Santos et al. (2000) who observed that chronic exposure to sublethal concentrations of copper and zinc, both alone or combined, have significantly reduced the total length and weight (wet and dry) of the shrimp *Farfantepenaeus paulensis*.

From an ecotoxicological point of view, the scenario where *N. granulata* shows a delay in the intermolt period and/or a minor increase in the body weight due to zinc exposure could produce serious ecological problems, not only at the individual level (i.e. delay to reach the sexual maturity size; dysfunction within vital activities or life cycle), but also at the populational one (more dangerously exposed to some illness, parasites and predators, leading to cohorts or even population size decrease). Moreover, it must be highlighted that this crab has been recognized not only as a key species but also as an ecosystem engineer one within Mar Chiquita coastal lagoon, and consequently dysfunctions on its growth processes could also affect organisms from other tropic levels within the system, or other indirectly related species, thus conducting to ecosystem alterations. Finally, considering the hypothesis of this study that populations of *N. granulata* from Mar Chiquita coastal lagoon could be endangered due to the high dissolved Zn recorded levels (Beltrame et al., 2008), the obtained results allowed to sustain that no critical effects were observed within the studied populations. Nevertheless, the occurrence of sublethal effects should be highlighted due to the potentiality of deleterious effects on the evaluated populations. Consequently, the health *status* of the crab population at Mar Chiquita coastal lagoon must be carefully monitored, in order to prevent critical ecological disruptions within this environment.

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