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# Integrative study at the biochemical level in a intertidal crab from Mar Chiquita coastal lagoon (Buenos Aires, Argentina) upon acute exposure to high zinc

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**Abstract** In Mar Chiquita coastal lagoon (Argentina) acute high increases in Zinc (Zn) occur reaching 1.22 mg x $L^{-1}$, but integrative studies on possible effects at the biochemical-physiological level on conspicuous invertebrate species inhabiting this area are lacking. We determined body and vital organs mass, hyper/ hypo-regulatory capacity; $Na^+$-$K^+$ATPase activity alkaline phosphatases (AP) activities in chela muscle, maltase and sucrase activities in the hepatopancreas and glycemia of adult males of the euryhaline crab *Neohelice granulata* upon exposure during 96 h to 1.22 mg x $L^{-1}$ Zn in hyper- and hypo-osmotic media. Body, anterior and posterior gills, hepatopancreas and chela muscle mass and hyper and hypo-regulatory capacities were not affected suggesting maintenance of body and physiological condition. The fact that upon Zn exposure in hypo-osmotic media $Na^+$-$K^+$ATPase activity decreased in posterior gills while increased in anterior gills along with increased glycemia suggests the occurrence of biochemical compensatory adjustments. Since no changes occurred in maltase and sucrase activities in the hepatopancreas or AP in chela muscle in hypo-osmotic media, other key metabolic parameters appeared not to be comprised. The fact that in hyper-osmotic media Zn exposure no effects were found suggests the occurrence of specific effects of Zn along upon a hypo-osmotic challenge.

**Key words**:: estuaries; glucose homeostasis; euryhaline crabs; osmotic adaptation, heavy metal.

**Resumen: Estudio integrativo a nivel bioquímico en un cangrejo intermareal de la laguna de Mar Chiquita (Buenos Aires, Argentina) luego de la exposición a elevadas concentraciones de zinc.** En la laguna costera de Mar Chiquita (Argentina) ocurren incrementos agudos en la concentración de Zn (hasta 1,22 mg x $L^{-1}$). Sin embargo, faltan estudios integrativos sobre posibles efectos a nivel bioquímico-fisiológico en especies conspicuas de invertebrados. Determinamos la masa corporal y de órganos vitales, la capacidad híper/hipo-regulatoria; actividad $Na^+$-$K^+$ ATPasa en branquias anteriores y posteriores; glicemia, actividades de maltasa y sacarasa en hepatopáncreas y de fosfatasas alcalinas (AP) en músculo de la quela en el cangrejo eurihalino *Neohelice granulata* expuesto 96 h a 1,22 mg x $L^{-1}$ Zn, en medios hípo e hiperosmóticos. La masa corporal y de órganos vitales y la capacidad

híper/hiporregulatoria no fueron afectadas, sugiriendo el mantenimiento de la condición física y fisiológica. Sin embargo, luego de la exposición a Zn en medio hipo-osmótico, disminuyó la actividad de $Na^+$-$K^+$ ATPasa en branquias posteriores mientras aumentó en branquias anteriores lo cual, junto con el incremento en glicemia sugieren la existencia de ajustes compensatorios. Otros parámetros bioquímicos-fisiológicos (actividades de maltasa y sacarasa en hepatopáncreas o AP en músculo de la quela) no fueron afectados. En medio hiper-osmótico no se afectaron ninguno de los parámetros estudiados sugiriendo la existencia de respuestas específicas en relación al desafío osmótico concomitante.

**Palabras claves:** estuarios; homeostasis de glucosa ; cangrejos eurihalinos; adaptación osmótica, metales pesados

## Introduction

Estuaries and coastal lagoons are being impacted by anthropogenic wastes from surrounding areas and a concern exist since contaminants such as heavy metals can represent a deleterious risk for key organisms in these habitats (i.e. euryhaline crabs) (Jakismska *et al.* 2011, Khan *et al*. 2014). A broad variability exists concerning the vulnerability of different crabs to metal exposure (Rainbow, 2007). This variability appears to be related to intrinsic characteristics of a particular species along with the differential features of the particular habitat. This fact clearly points out the need to evaluate the potential impact of heavy metals in representative species for each particular habitat. In this context, several Argentinian estuaries are being impacted by pollutants, derived mainly from sewage discharge and agricultural or industrial activities (Kroppio *et al*. 2014). In the wet Pampa region (Buenos Aires, Argentina) an accumulative effect of Zinc (Zn) has been noticed to occur in the unique Argentinean coastal lagoon, Mar Chiquita (an UNESCO Man and the Biosphere Reserve), during the last decades (Marcovecchio *et al*. 2004, Beltrame *et al*. 2008, 2009, Kroppio *et al*. 2014). Thus, markedly high Zn concentrations have been found (up to 1.22 mg $L^{-1}$), which are far above from the quality criteria concentration for marine and estuarine environments (Beltrame *et al*. 2008). However, integrative studies on possible effects at the biochemical-physiological level of the peak concentration of Zn reported to be reached in this coastal lagoon on conspicuous invertebrate species inhabiting this area (i.e. euryhaline crabs) are lacking. Intertidal estuarine crabs have to cope with a variety of challenges such as abrupt changes in environmental salinity, therefore requiring strategies at different levels for controlling movements of water and ions between the individuals and their medium (Anger 2001; Kirschner 2004; McNamara & Faria 2012; Romano. Zeng 2012). Hyper/hypo-osmoregulator crabs are able to maintain the osmotic concentration of the hemolymph within a stable range, above or below that of the external medium in low and high salinities, respectively (Péqueux 1995; Lucu & Towle 2003; Freire *et al.* 2008; McNamara QFaria 2012; Romano . Zeng 2012). Flexibility in osmoregulatory behavior is essential for survival and successful occupancy of estuarine habitats (McNamara & Faria 2012; Romano. Zeng 2012). Biochemical adaptation to environmental salinity is a complex process involving the participation of different enzymes and transport systems in branchial and extrabranchial tissues such as hepatopancreas and muscle (Jahn et al. 2006; Pinoni and López Mañanes 2004, 2008, 2009; Martins *et al.* 2011; Athamena *et al.* 2011; Michiels *et al.* 2013).

*Neohelice* (*Chasmagnathus*) *granulata* is a euryhaline burrowing crab considered as an emergent animal model for biochemical physiological and ecological research (Spivak 2010). This crab is distributed on intertidal areas of the southwestern Atlantic from southern Brazil to the northern Argentinean Patagonia (Boschi 1964; Botto & Irigoyen 1979; Spivak 1997; Iribarne *et al*. 2003; Luppi *et al*. 2013). In Mar Chiquita coastal lagoon (Argentina) *N. granulata* is one of the dominant euryhaline crabs inhabiting the whole interidal area. where it plays an essential ecological role affecting different physico-chemical and biological aspects (Iribarne *et al*., 2003; Mendez-Casariego *et al.* 2011). *N. granulata* burrowing activities modifies water, inorganic and organic matter availability of sediments, and consequently affects the structure of food webs (Botto *et al*. 2005, 2006). *N. granulata* from Mar Chiquita coastal lagoon exhibits genetic and morphological differences from their congeners from Brazil probably associated with local adaptation to different environmental conditions (Ituarte *et al*. 2012). Previous works of our laboratory show that *N. granulata* from the mudflat of Mar Chiquita coastal lagoon has a high osmoregulatory capacity behaving as hyper/hypo-regulator since it exhibits hemolymph osmolalities values higher and below from those of the corresponding external medium upon

acclimation to low and high salinity, respectively (López Mañanes *et al*. 2000, Schleich *et al*. 2001; Pinoni & López Mañanes 2009; Asaro *et al*. 2011; Pinoni *et al*. 2005, 2013). The ability and maintenance of osmoregulatory capacity in males of *N. granulata* is crucial for survival and is in accordance with the successful occupancy of the intertidal area of Mar Chiquita coastal lagoon (Pinoni *et al*. 2013; Luppi *et al*. 2013). Males individuals dominates in mudflat (Spivak *et al*. 1994; Luppi *et al*. 2013) thus they are more potentially exposed to peaked Zn concentration in this area. Like in other euryhaline osmoregulating crabs (Mc Namara *et al.* 2012), branchial $Na^+/K^+$-ATPase activity in posterior gills is a key component at the biochemical level of osmoregulatory responses in *N. granulata* from Mar Chiquita coastal lagoon (Schleich *et al*. 2001; González *et al*. 2012). However, we have shown that $Na^+/K^+$-ATPase activity in anterior gills also appears to have a role in osmoregulatory responses (López Mañanes *et al*. 2000, 2002; Schleich *et al*. 2001; González *et al.* 2012). Interpopulation differences exist in biochemical (i.e., ion-transport systems in gills) and physiological adaptations to salinity (Luquet *et al*. 1992, 2005; López Mañanes *et al*. 2000; Schleich *et al*. 2001; Genovese *et al*. 2005; Pinoni *et al.* 2013), suggesting the influence of habitat. Osmoregulatory capacity, which is defined as the difference between the osmotic pressures of the hemolymph and that of the external medium is commonly used to evaluate osmoregulatory performance (Charmantier *et al*. 1989; Lignot *et al.* 2000). Comparisons of osmoregulatory capacity values and of $Na^+K^+$ATPase activity in anterior and posterior gills of individual non-exposed and individuals exposed to a particular environmental factor (i.e. Zn) allow to evaluate a possible differential performance (i.e., lower value indicates a lower osmoregulatory performance) and possible adjustments at the biochemical level to maintain this performance.

Body and vital organs mass allow to estimate the healthy body condition (del Valle & Lopez Mañanes 2008, 2011). In decapod crustaceans, the maintenance of adequate levels of glucose in the hemolymph is essential for supporting several key functions and in the response to various environmental stresses (Verri *et al.* 2001; Lorenzon 2005), and glycemia is then commonly used as index of metabolic status. Maltase and sucrase activities in the hepatopancreas constitute important components of the digestive battery in *N. granulata* that are involved in the maintenance of glucose homeostasis (Asaro *et al*. 2011; Pinoni *et al.* 2011). We have shown that biochemical osmotic adaptation of *N. granulata* from Mar Chiquita coastal lagoon also involves the differential modulation of alkaline phosphatase activities in the chela muscle and key digestive enzymes in the hepatopancreas (Pinoni *et al.* 2005; Pinoni 2009; Pinoni & López Mañanes 2009, 2011; Asaro *et al*. 2011; González *et al.* 2012; Pinoni *et al*. 2013). Field studies in Mar Chiquita coastal lagoon have shown that adult males of *N. granulata* exhibit Zn accumulation in the hepatopancreas, values being similar or even higher than those found in related species in other ecosystems (Beltrame *et al.* 2011), however and strikingly, studies on the possible influence of high Zn on key biochemical and physiological parameters are lacking.

The aim of this work was to determine the effect of acute exposure to 1.22 mg $L^{-1}$ Zn, the highest concentration at which *N granulata* are exposed in Mar Chiquita coastal lagoon) on key biochemical-physiological parameters. In this context, we compared body condition; hyper and hypo-regulatory capacity, $Na^+$-$K^+$ATPase activity in anterior and posterior gills; AP activities in chela muscle; glycemia; and the activity of maltase and sucrase in the hepatopancreas of males individuals of *N. granulata* from the mudflat of Mar Chiquita coastal lagoon non-exposed and exposed to Zn in hypo and hyper-osmotic media under hyper and hypo-regulating conditions, respectively.

## Material and methods

*Animal collection and maintenance:* The crabs (n=40) were caught of Mar Chiquita coastal lagoon (Buenos Aires, Province Argentina) (37º32' 37º45'S; 57°19' 57°26'W). Only intermolt adult male crabs with a carapace width greater than 2.5 cm were collected. Animals were transported to the laboratory in lagoon water on the day of collection. During the acclimation period crabs were kept in aquaria for 10 days in artificial seawater upon hyper-osmotic (37 psu, 997 mOsm. $L^{-1}$, composition: 400mM NaCl, 28.2 mM $Na_2SO_4$, 9mM KCl, 23 mM $NaHCO_3$, 0.82mM KBr, $7.10^{-5}$mM $H_2BO_2$, 53mM $MgCl_2.6H_2O$, 10mM $CaCl_2.6H_2O$) or hypo-osmotic conditions (10 psu, 300 mOsm. $L^{-1}$, composition: 114mM NaCl, 8mM $Na_2SO_4$, 2.6mM KCl, 6.6mM $NaHCO_3$, 0.2mM KBr, $2.10^{-5}$mM $H_2BO_2$, 15mM $MgCl_2.6H_2O$, 2.9 mM $CaCl_2.6H_2O$) (López Mañanes *et al.* 2000, Schleich *et al.* 2001, Pinoni & Lopez Mañanes 2009, Asaro *et al*. 2011, Pinoni *et al.* 2013). For all the experiments, salinity was measured in practical salinity units (psu). The aquaria contained 36 L of water, continuously aerated and filtered. A regime of 12-h light/12-h dark

was applied, and the temperature was kept at 22±2 ºC. Aquaria was shielded by a black plastic to reduce disturbance. Crabs were fed three times a week with commercial food (Cichlind T.E.N., Wardley, USA) (48% carbohydrates, 40% protein, 3% fat, 4% fiber) (about 0.07 g/individual), but they were starved 48 h prior to experiments. After the acclimation period, the crabs were individually placed in separate aquaria containing 1 l of exposure medium of similar composition used for acclimation and with the adequate salinity (10 or 37 psu) and osmolality (300 or 997 mOsm, respectivley) to perform a semi-static assay for 96 h in the absence (control group) or in the presence of Zn (final concentration 1.22 mg $L^{-1}$). Zinc was added as Zn $Cl_2$. Every 24 h, experimental media was totally renewed. Temperature, pH, salinity and osmolality of the exposure medium were controlled through the experimental period. Crabs (n=5 for each condition) (hyper and hypo-osmotic media and not exposed or exposed to Zn) were sacrificed immediately at the beginning of the semi-static assay (t=0) and after 96 h.. In control group body and vital organs mass, hyper/ hypo-regulatory capacity; Na+-K+ATPase activity alkaline phosphatases (AP) activities in chela muscle, maltase and sucrase activities in the hepatopancreas and glycemia were similar at $t_o$ and after 96 h, indicating that semi-static assay did not affect any of the parameters determined (not shown). No mortality occurred upon exposure of crabs to Zn. All individuals maintained the ability to respond to tactile stimuli (index of perception and response capacity to environmental cues) remain reactive and has no external signals of impairment.

*Sampling procedures:* Crabs were weighed and cryoanesthesized by putting them on ice for about 20 min. A sample of hemolymph was withdrawn for assaying the concentration of glucose and osmolality. Both chelae were cut off and carapaces were removed.

Hemolymph (about 500 µL) was sampled from the infrabranchial sinus by mean of a syringe previously rinsed with sodium citrate buffer 10% w/v pH 7.4, at the base of the cheliped, and transferred to an iced centrifuge tube. Serum was separated by centrifugation at 10000 × g (Beckman, Microfuge, B) for 30 s. Osmolality was measured by an automatic crioscopic osmometer (Osmomat 030 D, GONOTEC). The results are expressed as osmoregulatory capacity (defined as the difference between the hemolymph osmolality and the corresponding medium (Charmantier & Anger 2011). To determine the concentration of glucose in hemolymph (glycemia) an adequate aliquot of serum hemolymph (20 µl) was incubated with 1.5 mL of the glycemia reagent (Wiener-Lab AA Kit). After 5 min at 37 °C, the amount of glucose was determined by reading the absorbance at 505 nm of the colored quinonimine complex (Pinoni *et al*. 2011, 2013). Osmolality and the concentration of glucose in hemolymph were measured immediately after hemolymph extraction.

The hepatopancreas, chela muscle, anterior (1–5) and posterior (6–8) gills (López Mañanes *et al.* 2000) were immediately excised, weighed and homogenized. Wet mass was measured to the nearest 0.01 g. The hepatopancreas was homogenized in 0.1 M Tris/HCl pH 7.4 (4 mL $g^{-1}$ of tissue) (CAT homogenizer ×120, tool T10) and centrifuged at 10000 x g for 15 min (Sorval, rotor SS34, refrigerated). The gills were mixed with homogenizing medium (0.25 M sucrose/0.5 mM EGTA–Tris, pH 7.4) (4 ml $g^{-1}$ of gill tissue) and homogenized on ice with 20 strokes in a motor-driven hand-operated Teflon-glass homogenizer. The homogenate was centrifuged at 10000 xg (Beckman, Microfuge, B) for 30 s. The supernatant was separated into 200 µl aliquots and stored at –20 ºC until use. The chela muscles were mixed with homogenizing medium (0.25 M sucrose/0.5 mM EGTA–Tris, pH 7.4) (8 mL $g^{-1}$ of muscle tissue) and homogenized with CAT homogenizer ×120, tool T10 on ice. The muscles from both chelae of one individual were pooled and used for each preparation of homogenate. The homogenate was fractionated into 400 µL aliquots and stored at −20 °C. Glycerol (1.3% v/v) was added to samples before freezing (Ljungström *et al*. 1984). The freezing procedure did not alter the activity values, activities being stable at least for up to eight months of freezing. However, enzyme assays were performed no longer than a week from the sampling procedure and always by using samples without any previous thawing.

Maltase and sucrase (EC 3.2.1) activities were assayed by measuring the glucose released from the hydrolysis of the corresponding substrate (maltose and sucrose, respectively) (Asaro *et al.* 2011; Pinoni *et al.* 2011, 2013). The reaction was initiated by adding an aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot) to a reaction mixture containing 28mM of the corresponding substrate (sucrose or maltose) in 0.1 M maleate–NaOH buffer (pH 5.2) at 37 °C. After incubation for 10 min, the reaction was stopped by addition of 1.5 mL of the combined enzyme color glucose reagent solution (10 kU/L glucose oxidase, 1 kU peroxidase, 0.5 mmol/L 4-aminophenazone, 100 mmol/L phosphate buffer pH 7.0, 12 mmol/L

hydroxybenzoate) (Wiener-Lab AA Kit cod. 1400107). After 5 min at 37 °C, the amount of released glucose was determined by reading the absorbance at 505 nm of the colored quinonimine. The disaccharidase activities were expressed as µg glucose x $min^{-1}$ x mg $protein^{-1}$. Levamisole-insensitive AP activity (EC 3.1.3.1) was determined by measuring pNPP hydrolysis in a reaction medium containing 4 mMm MgSO4 in 0.1 M Tris–HCl buffer (pH 7.7) in the presence of 16 mM levamisole. Levamisole-sensitive activity was determined as the difference between the pNPP hydrolysis in a reaction medium containing 4 mM MgSO4 in 100 mM Tris–HCl buffer (pH 8.5) in the absence (total AP activity) and in the presence of 16 mM levamisole (Pinoni *et al*. 2005, 2011; Pinoni 2009). An aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot) was added to the reaction mixture and pre-incubated for 5 min at 37 °C. The reaction was initiated by the addition of pNPP (final concentration 9.5 mM). Incubation was carried out at 37 °C for 10 min. The reaction was stopped by addition of 2 mL of 0.1 MKOH. The amount of released pNP was determined by reading the absorbance at 410 nm (Pinoni *et al.* 2005, Pinoni 2009).

Total ($Mg^{2+}$–$Na^{+}$–$K^{+}$) ATPase activity was determined by measuring ATP hydrolysis in a reaction medium containing 100 mM NaCl, 30 mM KCl, 10 mM $MgCl_2$ and 0.5 mM EGTA in 20 mM imidazole buffer (pH 7.4). Residual ($Mg^{2+}$–$Na^{+}$) ATPase activity was assayed in the same medium but without KCl and in the presence of 1 mM ouabain. $Na^{+}$-$K^{+}$ ATPase activity was determined as the difference between the two assays. An aliquot of the corresponding sample (linearity zone on activity vs. protein concentration plot) was added to the reaction mixture and pre-incubated for 5 min at 30 °C. The reaction was initiated by the addition of ATP (final concentration 13 mM). Incubation was carried out at 30 °C for 15 min. The reaction was stopped by the addition of 2 ml of cooled Bonting's reagent as described above for ouabain-insensitive $Na^{+}$ATPase activity (Schleich *et al*. 2001; López Mañanes *et al*. 2002; Pinoni & López Mañanes 2009).

Protein was assayed according to Bradford (1976). Bovine serum albumin was used as standard. Results are presented as mg x g $tissue^{-1}$.

*Statistical analysis:* Statistical analyses were performed using the Sigma Stat statistical package for Windows operating system, which automatically performs a previous test for equal variance (Levene test) and normality (Kolmogorov-Smirnov test). If analyzed data failed to pass these tests a non-parametric test was performed. A confidence level of 95% was considered significant. For each osmoregulatory condition (hyper or hyporegulation), a t-test was used to estimate differences in osmoregulatory capacity between individuals not exposed (control) or exposed to zinc. To assess the existence of differences in body mass, hemolymphatic glucose concentration, and enzyme activities ($Na^{+}$-$K^{+}$ATPase, phosphatase, sucrase and maltase) between individuals exposed or not exposed to Zn, a parametric (ANOVA) or non-parametric (Kruskal-Wallis) test was used. The existence of differences in organ mass was evaluated by ANCOVA test using body mass as covariance.

## Results

No changes in body mass or in the mass of anterior and posterior gills, muscle or hepatopancreas occurred upon exposure for 96 h to Zn under of crabs acclimated to hypo or hyper-osmotic external medium ($P$> 0.05) (Table I).

Individuals exposed to Zn exhibited similar hyper- and hypo-osmoregulatory capacity than those of non-exposed crabs ($P$>0.05) (Figure 1). Compared to the corresponding value in non-exposed crabs, $Na^{+}$-$K^{+}$ ATPase activity in anterior gills was about 150% higher (*ANOVA* $F$= 8.75 $P$=0.008) while it was lower in posterior gills (about 38%) (*Kruskall-Wallis* $H$=4.88, $P$=0.028) after Zn exposure in hypo-osmotic external medium (Figure 2). $Na^{+}$-$K^{+}$ ATPase activity in anterior and posterior gills ($P$>0.05) (Figure 2) was similar to the corresponding value of non-exposed crabs ($P$>0.05) after Zn exposure in hyper-osmotic medium (Figure 2).

Crabs acclimated to hypo-osmotic medium exhibited a higher concentration of glucose in hemolymph (about 160%) ($F$=7.29, $P$=0.031) after exposure to Zn compared to that of non-exposed crabs (Figure 3). No differences in the concentration of glucose in the hemolymph occurred in crabs exposed to Zn under hyper-osmotic conditions ($P$>0.05) (Figure 3).

In crabs exposed to zinc in both hypo and hyper-osmotic media, maltase and sucrase activities in the hepatopancreas (Figure 4 a, b) and total, levamisol-insensitive and levamisole-sensitive AP activities in the chela muscle (Figure 5) were similar to the corresponding values in non exposed-crabs ($P$>0.05).

**Table I**. Body mass (g) and mass (g) of anterior (1-5) and posterior (6-8) gills, chela muscle and hepatopancreas of individuals of *Neohelice granulata* non-exposed and exposed to zinc. **(**Data are means ± S.E of five individuals for each condition).

| | Hypo-osmotic medium | | Hyper-osmotic medium | |
|---|---|---|---|---|
| | - Zinc | +Zinc | - Zinc | +Zinc |
| Body mass | 22.01±2.08 | 22.67±1.67 | 26.46±1.92 | 27.36±2.36 |
| Anterior gills | 0.20±0.01 | 0.30±0.03 | 0.31± 0.03 | 0.3±0.02 |
| Posterior gills | 0.13±0.02 | 0.19±0.02 | 0.17±0.01 | 0.13±0.02 |
| Hepatopancreas | 0.49±0.07 | 0.71±0.07 | 0.77±0.07 | 0.94±0.07 |
| Chela muscle | 0.69±0.07 | 1.14±0.19 | 1.78±0.24 | 1.65±0.29 |

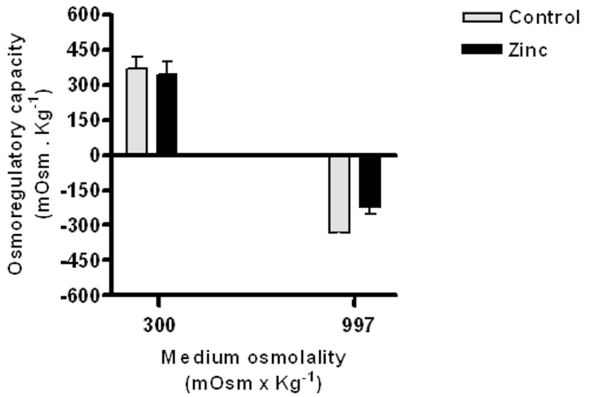


**Figure 1.** Osmoregulatory capacity (mOsmol . $Kg^{-1}$) of individuals of *Neohelice granulata* non-exposed (control) and exposed to zinc under hypo-osmotic and hyper-osmotic conditions. (Data are means ± S.E of five individuals for each condition)

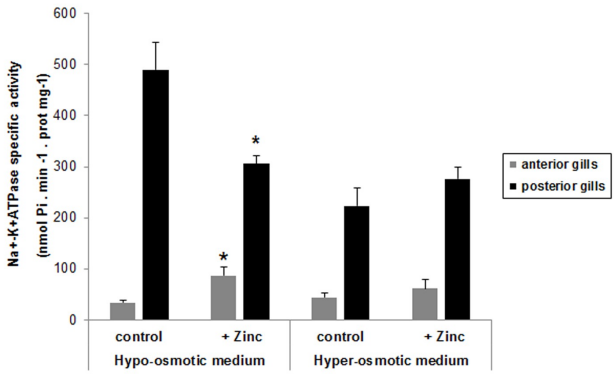


**Figure 2.** $Na^{+}$-$K^{+}$ ATPase activity in anterior (1-5) and posterior (6-8) gills of individuals of *Neohelice granulata* non-exposed (control) and exposed to zinc under hypo-osmotic and hyper-osmotic conditions. (Data are means ± S.E of five individuals for each condition.). (*) Different from the corresponding activity in the absence of zinc (*ANOVA F*= 8.75 *p*=0.008, *Kruskall-Wallis H*=4.88, *P*=0.028).

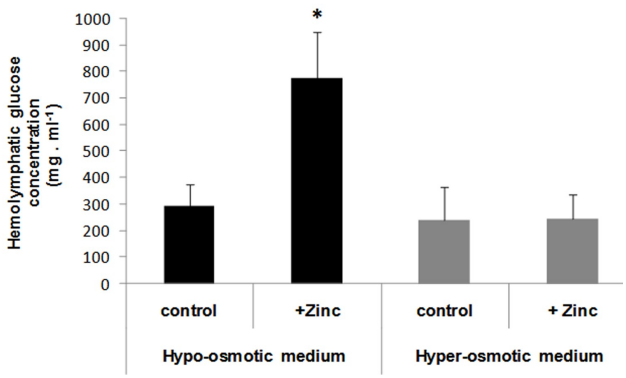


**Figure 3.** Hemolymph glucose concentration of individuals of *Neohelice granulata* non-exposed (control) and exposed to zinc under hypo-osmotic and hyper-osmotic conditions. (Data are means ± S.E of five individuals for each condition.) *Different from the corresponding hemolymph glucose concentration in the absence of zinc (*F*=7.29, *P*=0.031)

## Discussion

In Mar Chiquita coastal lagoon (Argentina**),** adult male of the euryhaline crabs of *Neohelice granulata* face acute high Zn levels **(**up to 1.22 mg $L^{-1}$) and the accumulation of this metal in the hepatopancreas has been shown, being proposed as a potential bioindicator of heavy metals in this estuarine systems (Beltrame *et al*. 2008; 2011). In this context, we evaluated the potential effect of exposure to high Zn level (1.22 mg $L^{-1}$, the highest level reported for Mar Chiquita coastal lagoon) in hyper and hypo-osmotic media under hypo- and hyper-regulating conditions, respectively, on body condition and key biochemical-physiological parameters of adult males of *N. granulata* from the mudflat of Mar Chiquita coastal lagoon. The fact that body mass and those of key metabolic tissues of this crab such as anterior and posterior gills, hepatopancreas and chela muscle (Pinoni & López Mañanes 2009; Pinoni *et al*. 2005, 2011, 2013)

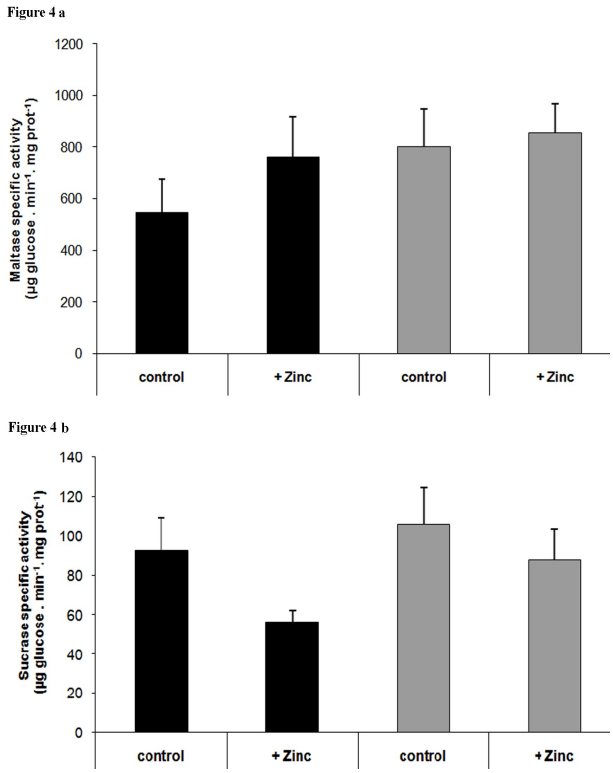


**Figure 4**. Maltase (a) and sucrase (b) specific activity in hepatopancreas of individuals of *Neohelice granulata* non-exposed and exposed to zinc under hypo-osmotic and hyper-osmotic conditions. (Data are means ± S.E of five individuals for each condition).

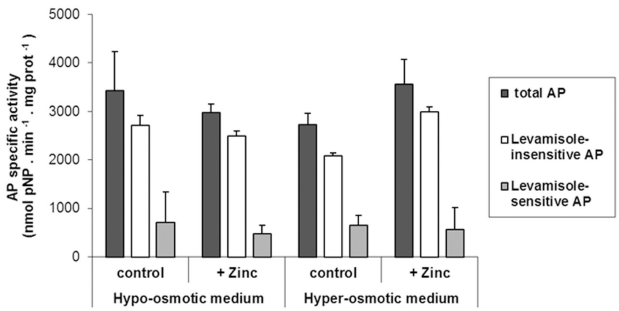


**Figure 5**. Total, levamisole-insensitive and levamisole-sensitive alkaline phosphatase activity (AP) in chela muscle of individuals of *Neohelice granulata* non-exposed (control) and exposed to zinc under hypo-osmotic and hyper-osmotic conditions. (Data are the means ± S.E of five individuals for each condition).

(Table I) and hyper and hypo-regulatory capacity (Figure 1) were not affected suggests the maintenance of body and key physiological functions such as osmoregulation. Osmoregulatory capacity appears to be a reliable tool to evaluate the physiological condition and the effect of stressors in crustaceans (Charmantier *et al*. 1989; Lignot *et al*. 2000; Ardiansyah *et al*. 2012). In several crustaceans, exposure to heavy metals affects osmoregulatory capacity (Lington *et al*. 2000, Wu & Chen 2004, Silvestre *et al*. 2005, Ardiansyah *et al.* 2012) and negatively the activity of $Na^+$-$K^+$ATPase in gills (Lignot *et al*. 2000, Kang *et al*. 2012). On the other hand, in the freshwater amphipod *Gammarus fossarum* (Issartel 2010) an enhancement in branchial $Na^+$-$K^+$ ATPase expression was found in response to Cd**.** Since adaptation of organisms to environmental stress involves genetically fixed traits in natural populations, it was proposed that the selection for resistance to a particular heavy metal may lead to locally adapted organisms with an elevated tolerance to metal toxicity (Haap & Köhler 2009)**.** The ability for differentially adjust the activity of enzymes involved in the maintenance of key physiological process (i.e. $Na^+$-$K^+$ATPase in gills) could be one of this traits that could lead to locally adapted organisms in relation to tolerance to environmental potential stressors In this context, interpopulation differences in biochemical and physiological adaptations (i.e. modulation of $Na^+$-$K^+$ATPase in anterior and posterior gills) to salinity exist in individuals of *N. granulata* from different geographical areas (Pinoni *et al*., 2013). Hyper and hypo-regulation constitutes key physiological processes for adult males of *N. granulata* from Mar Chiquita coastal lagoon (López Mañanes *et al*. 2000; Schleich *et al*. 2001; Pinoni & López Mañanes, 2009; Asaro *et al*. 2011; Pinoni *et al*. 2013). The maintenance of both hyper and hypo-regulatory capacity (Figure 1) suggests that adult males of *N. granulata* would be able to withstand osmoregulatory capacity under acute high level of Zn. $Na^+$-$K^+$ ATPase activity in posterior gills of *N. granulata* from Mar Chiquita coastal lagoon is a central component of the hyper-regulatory responses at the biochemical level (López Mañanes *et al*. 2000, Schleich *et al*. 2001; González *et al*., 2012). $Na^+$-$K^+$ ATPase activity in posterior gills has a key role by in hyper-regulation process by supporting the activities of different enzymes and transporters involved in the active uptake of sodium from the external media to the hemolymph (Mc Namara *et al.* 2012; Romano. Zeng 2012). However, $Na^+$-$K^+$ ATPase activity in anterior gills of *N. granulata* from Mar Chiquita coastal lagoon also appears to be involved in biochemical adaptation to low salinity (López Mañanes *et al*. 2000, Schleich *et al*. 2001) as described in other euryhaline crabs from this area (López Mañanes *et al*. 2002). In this context, a decrease in $Na^+$-$K^+$ATPase activity in posterior gills by exposure to any potential stressor (i.e. Zn) could potentially lead to an impairment of hyper-regulatory capacity. However, this appeared not to be the case for *N.*

*granulata* from Mar Chiquita coastal lagoon, since, in spite of the decrease in $Na^+$-$K^+$ATPase activity in posterior gills under Zn exposure, hyper-regulatory capacity was not affected (Figure 1). Based on what we described above about the role of $Na^+$-$K^+$ATPase activity in anterior gills in responses to low salinity, the enhanced activity in anterior gills of *N. granulata* upon Zn exposure in hypo-osmotic media (Figure 2) suggests the occurrence of biochemical compensatory adjustments (i.e. to compensate for the decrease in $Na^+$-$K^+$ATPase activity in posterior gills) likely to maintain hyper-regulatory capacity (Figure 1). Further experimental approach such as working with individual gills (López Mañanes *et al*. 2002, Schleich *et al*. 2001) and/or *in vitro* assays of anterior and posterior gills are needed to test this hypothesis. Based on *in vitro* experiments in the freshwater amphipod *G. fossarum* (Issartel 2010) and results in the field in the crab *Callinectes danae* (Harris & Santos 2000) it has been suggested that an enhancement in branchial $Na^+$-$K^+$ ATPase constitutes a physiological response to heavy metals contamination allowing a more efficient ion exchange between external medium and hemolymph.

In crustaceans, the maintenance of adequate concentrations of hemolymphatic glucose is essential for a good morphological and physiological performance **(**Dutra *et al*. 2008**).** The enhanced glycemia of individuals of *N. granulata* upon exposure to Zn in in hypo-osmotic media (Figure 3) suggests that availability of glucose from the hemolymph would not be a constraint and further support the occurrence of biochemical compensatory adjustments. As we pointed out above, anterior gills have an important role in biochemical osmotic adaptation in *N. granulata* from Mar Chiquita coastal lagoon and also in the metabolism of carbohydrates being a site of fate of dietary glucose and glycogen storage and mobilization (López Mañanes *et al*. 2000; Schleich *et al*. 2001; Artillo *et al*. 2008; Pinoni 2009; Pinoni *et al*. 2011, 2013). Whether, the concomitant enhancement in glucose in hemolymph after Zn exposure in hypo-osmotic media (that is under hyper-regulatory conditions) is related to the increase in $Na^+$-$K^+$ ATPase activity in anterior gills (Figure 2) which could lead to a major requirement of energy metabolites (i.e. glucose) remains to be investigated. Glucose uptake is known to occur in anterior and posterior gills of *N. granulata* from other geographical areas which can be modified by external and internal conditions (Kucharski *et al*. 2002; Valle *et al*. 2009). Digestion of glycogenic substrates by carbohydrases in the hepatopancreas and the posterior absorption of digested products is one of the main sources of hemolymphatic glucose in the hemolymph (Verri *et al*. 2001, Pavasovic *et al*. 2004, 2007). Since the level of activity of key digestive enzymes (i.e. maltase and sucrase) involved in the degradation of glycogenic substrates in the hepatopancreas (Asaro *et al*. 2011, Pinoni *et al*. 2011) was not affected by Zn (Figure 4 a, b) suggests that the ability for digestion and utilization for key energy substrates would not be compromised and that the level of these digestive enzymes would be enough to support the increase in hemolymphatic glucose induced by Zn exposure in hypo-osmotic media. However, since we did not determine the glycogen reserves in the chela muscle (the main storage tissue of this reserve in *N. granulata* from Mar Chiquita coastal lagoon (Pinoni *et al*. 2011, 2013), the occurrence of a mobilization of glucose as a result of glycogen degradation in this tissue cannot be discarded. On the other hand, we cannot discard that 1.22 mg $L^{-1}$ Zn may be not a so high concentration or that the 96 h exposure was not so long to induce other metabolic effects besides changes in glycemia.

Alkaline phosphatase (AP), an enzyme found in almost all animal cells which is involved in various metabolic processes (Mazorra *et al*. 2002, Jiang *et al*. 2012), is generally used to detect alterations of metabolism induced by metal exposure (SenthilKumar *et al*. 2007, Jiang *et al*. 2012). We have shown that biochemical osmotic adaptation of *N. granulata* from Mar Chiquita lagoon is a complex process involving also the differential modulation of AP activities in the chela muscle (Pinoni *et al*., 2005; Pinoni & López Mañanes 2009). Since AP activities in chela muscle appeared not to be affected by Zn exposure in hypo-osmotic media (Figure 5), components involved in biochemical adjustments underlying hyper-regulation (Pinoni *et al*. 2005) would not be altered which could further support the maintenance of physical condition and survival.

On the other hand, the fact that Zn exposure in hyper-osmotic media did not affect the various studied parameters suggests the occurrence of specific responses in relation to the concomitant type of osmotic challenge. Hyper and hypo-osmoregulation appear to require different mechanisms (McNamara & Faria 2012, Romano & Zeng 2012). Thus, since $Na^+$-$K^+$ ATPase activity in anterior and posterior gills was not affected by Zn exposure in hyper-osmotic media (Figure 2**)** would suggest that other biochemical components of the hypo-regulatory machine of *N. granulata* from Mar Chiquita coastal lagoon (i.e. gill V-ATPase, González *et al*. 2012) could be adjusted to keep hypo-regulatory capacity. However, further

investigation is needed to test this hypothesis. Furthermore, whether the lack of effect of Zn exposure in hyper-osmotic media on $Na^+$-$K^+$ ATPase activity in anterior or posterior gills of *N. granulata* is due to a differential permeability to the metal under distinct osmotic conditions of the external medium (Rainbow & Black 2002, 2005) cannot be discarded. It has been proposed that in hyper-osmotic media a decrease in Zn availability could occur due to a major complexity of this metal with Cl ion (Rainbow 1997, Rainbow & Black 2002). However, we have previously shown that hyper and hypo-regulation in *N. granulata* from Mar Chiquita coastal lagoon appears to imply quite different underlying digestive and metabolic adjustments at the biochemical level (Pinoni *et al*. 2013). Thus, it is likely that other metabolites (i.e lipids metabolites, amino acids) could be being modulated in response to Zn.

In conclusion, the results of the present investigation suggest that adult males of *N. granulata* from the mudflat of Mar Chiquita coastal lagoon is able to support body condition and key physiological processes upon high Zn level faced in the natural ambient and the occurrence of differential adjustments at the biochemical level in relation to the concomitant osmotic challenge. Future studies should be focused to establish the effect of longer exposures to have a better understanding of the potential deleterious effect of high Zn encountered in the field and possible plastic responses underlying survival since the significant importance of adults of *N. granulata* in the community structure of Mar Chiquita coastal lagoon.

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