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MTs in *Palaemonetes argentinus* as potential biomarkers of zinc contamination in freshwaters

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ABSTRACT

Aquatic invertebrates take up and accumulate essential and non-essential trace metals even when both are likely to be poisonous. In order to study the potential of the metallothioneins (MTs) as biomarkers of metal contamination in native shrimp *Palaemonetes argentinus*, organisms have been exposed at 0, 5, 50 and 500 $\mu g\,L^{-1}$ of zinc for 96 h. Moreover, accumulation and subcellular distribution of this essential metal were evaluated. A significant Zn accumulation was observed in different body sections. Higher Zn levels occurred in cephalothorax compared to abdomen, especially at the highest exposure concentration (500 μg Zn L^{-1}). A clear differential subcellular metal distribution between cephalothorax and abdomen was also observed. In cephalothorax Zn was similarly distributed between the soluble and insoluble fractions; while in abdomen, when total Zn increased, insoluble metal augmented more markedly than the soluble one. Cytosolic Zn levels increased more in cephalothorax than in abdomen of shrimps exposed to 500 μg Zn L^{-1} when compared to control. Finally, a significant induction of MTs was observed in cephalothorax at 500 μg Zn L^{-1}. A potential role for MTs as biomarkers in *P. argentinus* should be further studied to enhance the sensitivity of the response, although it is likely that MTs play a key role in metal detoxification since the increase of these proteins is linked to metal challenge.

1. Introduction

Environmental exposure to metal pollutants can provoke numerous toxic effects on individuals and may have community–wide consequences (Wallace et al., 2000). Aquatic invertebrates take up and accumulate essential and non-essential trace metals even when both have the potential to be poisonous (Phillips and Rainbow, 1994; Luoma and Rainbow, 2008). Differential tolerance to trace metals among populations or species is frequently related with intracellular metal storage within aquatic organisms (Goto and Wallace, 2009). In invertebrate, metal tolerance can be achieved by one or more possible physiological mechanisms: the reduction in metal uptake rates and/or enhancement of excretion rates, the incorporation of metals into insoluble deposits or granules, or the storage of accumulated metals in nontoxic physicochemical forms like bound to cytosolic compounds including metallothioneins (MTs) or metallothionein-like proteins (MTLP), among others (Rainbow, 2007). Although an increasing number of studies have explored intracellular partitioning of trace metals and their metabolism in marine or estuarine species, little attention has been paid to freshwater crustacean.

Metallothioneins, a family of low-molecular-weight cysteine-rich metal binding proteins, have been shown to occur in most zoological taxa, including crustaceans (Rainbow, 1998; Mouneyrac et al., 2001, 2002; Amiard et al., 2006; Nunez-Nogueira et al., 2010). A major function of MTs is homeostatic regulation of intracellular metals. They are involved in the detoxification of excess amounts of both essential and non-essential trace metals as well as transfer of essential metals like Zn (Roesijadi, 1996; Amiard and Cosson, 1997;

Abbreviations: dw, dry weight; ICP–MS, inductively coupled plasma–mass spectrometry; MTs, metallothioneins; MTLPs, metallothionein-like proteins; P1, insoluble fraction; QA, quality assurance; QC, quality control; S1, soluble fraction; S2, heat-denaturated supernatant; ww, wet weight.

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Lemoine et al., 2000; Pourang et al., 2004). The use of MTs as biomarkers of metal exposure is well documented in different aquatic species and is frequently employed in biomonitoring programs (Barka et al., 2001; Amiard et al., 2006; Romeó and Giambérini, 2013). However, in order to propose MTs use in a new bioindicator species, it is necessary to link the external exposure concentration of the pollutant with the internal level and the biomarker response.

In shrimps, MTs or MTLPs induced by metals have been reported in numerous species like *Litopenaeus vannamei* (Moksnes et al., 1995; Wu and Chen, 2005), *Penaeus indicus* (Nunez-Nogueira et al., 2006) *Mirocaris fortunata*,*Rimicaris exoculata* and *Palaemon elegans* (Gonzalez-Rey et al., 2007). By contrast, little work has been done in *P. argentinus* Brouwer et al. (2007) have described gene codifying for MTs in *P. pugio* (Genebank accession number AY935987), species of the same genus. MTLPs has been reported in *P. varians* (Gonzalez-Rey et al., 2007), *P. pugio* (Howard and Hacker, 1990) and more recently, in *P. argentinus* (Chiodi Boudet et al., 2013). Although this last report showed MT induction after acute cadmium exposure, the association of MT response with metal accumulation has not been analyzed in *P. argentinus* yet (Chiodi Boudet et al., 2013).

Decapods are a component of the aquatic community due to their density and their role in the energy transfer (Spivak, 1997; Collins et al., 2007). Biological characteristics of this group would allow their use as environmental stress indicators (Collins et al., 2004). Few publications have reported the sensitivity of *P. argentinus* to pollution in laboratory tests (Collins and Cappello, 2006; Galanti et al., 2013), and proposed that this species might be used as a bioindicator crustacean to provide information on environmental quality (Montagna and Collins, 2007). Moreover, *P. argentinus* is a species of ecologic interest because of its wide distribution in different country of South America such as Argentina, Uruguay, Southern Brazil and Paraguay (Morrone and Lopreto, 1995).

This study, therefore, investigates the accumulation and distribution of an essential metal like Zn between cytosolic and insoluble tissular fractions as well as the induction of MTs as part of the metabolic response to Zn in the native shrimp *P. argentinus* under laboratory conditions. Furthermore, the evaluation of the potential use of MTs as biomarkers of metal exposure has been discussed for future field biomonitoring with mentioned *P. argentinus.*

2. Materials and methods

2.1. Reagents and materials

All reagents were of analytical grade supplied by Sigma–Aldrich (Germany), Merck (Germany) and Sintorgan (Argentina). Ultra-pure water (Arium 611 UV system, Sartorius, Germany) was used to prepare standard solutions, dilutions, blanks and artificial freshwater used for metal exposure. Sub-boiling HNO_3 was obtained by distillation Figmay S.A. (Argentina). All glassware and aquaria used were left with sulfuric-nitric acids solution overnight and then washed with ultra-pure water to avoid metal contamination. Similar procedure was made using HCl or HNO_3 10% solutions to disposable materials such as sample tubes and laboratory materials.

AccuStandard® (USA) atomic absorption spectrometry standard solution (1000 mg L^{-1} in 1% nitric acid) was used as stock solution for calibration of metal quantification equipment.

2.2. Specimens

Adult freshwater shrimps, *Palaemonetes argentinus,* were collected on May 2012 from a reference site (La Calera, Suquía River - Córdoba, Argentina, Contardo-Jara et al., 2009; Monferrán et al., 2011) and immediately transported to the laboratory. Organisms were acclimated during two weeks in 40 L glass aquaria, with sediments and plants collected from the same place and filled with artificial freshwater (ultra-pure water containing 0.100 gL^{-1} sea salt, 0.200 gL^{-1} $CaCl_2$ and 0.103 gL^{-1} $NaHCO_3$), maintained at constant laboratory temperature (25 °C ± 1 °C) and under a light/dark regime of 12 h: 12 h photoperiod. All along this period, they were fed daily ad libitum with commercial food for fish (Vita Fish, Argentina) complemented until 54% proteins (Díaz et al., 2001).

2.3. Experimental exposure

Intermoult adults (body length >2.2 cm; Donatti, 1986) of *P. argentinus* (0.204 ± 0.013 g wet weight (ww); 2.876 ± 0.056 cm) were transferred to aquaria filled with artificial freshwater. During experimental exposure two shrimps within a 1 L of exposure media were considered for all treatments (Giri and Collins, 2003). The experimental design involved four experimental conditions: controls (not metal exposed, $\underline{n}$ = 28), shrimps exposed to 5 μg ZnL^{-1} (7.65×10^{-5} mM, n = 23), 50 μg ZnL^{-1} (7.65×10^{-4} mM, n = 23) and 500 μg ZnL^{-1} (7.65×10^{-3} mM, n = 23). A stock metal solution was prepared using $ZnSO_4 \cdot 7H_2O$ (99.9%) and specific aliquots were taken to provide nominal metal concentrations. Heavy metal concentrations tested were selected according to relevant environmental concentration and recommended by Argentinean Environmental Water Quality Guidelines (Niveles Guía Nacionales de Calidad de Agua Ambiente, 2003). Two days before the beginning of metal exposure, shrimps were transferred to exposure's aquaria. Zinc exposure was carried out for 96 h at similar temperature and photoperiod than during acclimation. Dissolved oxygen (mg O_2 L^{-1}), conductivity (μS cm^{-1}), temperature (°C) and pH were monitored daily with a multi-parameter probe (WTW Multiline F4, Set 3, Germany). The mean water quality parameters were pH 7.66 ± 0.06, conductivity 395 ± 3 μS cm^{-1}, water temperature 22 ± 1 °C and dissolved oxygen 7.06 ± 0.09 mg O_2 L^{-1} showing stable conditions along the whole test.

During metal exposure shrimps were fed with 3.5 mg of food (Vita fish complemented with proteins) per organism per day (0.066 μg Zn mg^{-1} food).

At the end of metal exposure, organisms were cryoanesthetized, washed three times with ultra-pure water, measured (rostrum–uropod length) and dissected under a binocular magnifying glass. Cephalothorax and abdomen sections of each organism were weighed. Cephalothorax section includes the antennal gland, major nervous system (cerebral ganglion), gills, secretory glands, hepatopancreas and gonads. The abdomen section includes most of the skeletical muscle, part of the nervous system (ventral nerve cord), midgut and hindgut (Felgenhauer, 1992). Additionally, exoskeleton was obtained from six organisms at each experimental condition and pooled to determine zinc levels in this tissue. Finally, samples were frozen with liquid nitrogen and kept at −80 °C until analysis.

Water samples from exposure aquaria were taken before starting metal exposure and at 96 h to evaluate changes in soluble metal concentration at the end of the experiment. Water samples were filtered at 0.45 μm (nitrate cellulose, Sartorius Stedim Biotech, Germany) and acidified to 2% with sub- boiling HNO_3.

2.4. Metal analysis in exposure media

Water sample were diluted using sub-boiling HNO_3 (2% final concentration). Zinc concentration measurement was carried out on an inductively coupled plasma–mass spectrometry (ICP–MS) (Agilent Technology 7500cx, USA) equipped with an auto-sampler

ASX-100 (CETAC Technologies, USA). The metal concentrations are expressed in μg Zn L^{-1}.

2.5. Metal accumulation in organism

Metal concentration was determined in cephalothorax (tissue weight 0.020 ± 0.006 g dry weight (dw)) and abdomen (0.021 ± 0.004 g dw) sections as well as in exoskeleton samples (0.016 ± 0.003 g dw), separately. Samples digestions were carried out according to Chappaz et al. (2012) with some modifications. Briefly, tissue samples were dried at 38 °C until constant weight and stored at −20 °C freezer until analysis. Biological samples were ground and homogenized with mortar. Then, tissues were digested with sub-boiling HNO_3 and H_2O_2 (30%, Merck, Germany) into Teflon tubes at 160 °C and evaporated near dryness. Afterward, samples were re-suspended in sub-boiling HNO_3 (2% final concentration) and maintained at 4 °C until metal determination on PerkinElmer 3110 flame atomic absorption spectrometer (USA). The metal accumulation in body sections (cephalothorax and abdomen) are expressed in μg Zn g^{-1} dw.

Concentrations of the element were determined by triplicate using atomic absorption spectroscopy. Quality assurance (QA) and quality control (QC) were done using spiked control samples. Variable amounts of Zn standard solution (AccuStandard®, USA), were added to 0.029–0.035 g of dried and homogenized shrimps (whole organisms) to provide nominal metal concentrations of 300 μg Zn g^{-1} dry weight (dw). The rest of the procedure was the same as used for non-spiked samples. The average of the assay recoveries were 104.0 ± 0.6%.

2.6. Metal compartmentation procedure and partial isolation of metallothioneins

For zinc compartmentation and MT analysis, individual samples (cephalothorax and abdomen of each individual) were homogenized at 4 °C in 20 mM Tris, 10^{-5}mM β-mercaptoethanol, 150 mM NaCl, solution adjusted to pH 8.6 (4 mL per g of soft tissue), and inhibitor protease cocktail (Sigma–Aldrich, Germany). The soluble (S1) and insoluble (P1) fractions were separated by centrifugation (25,000 ×*g* for 55 min at 4 °C). The cytosolic heat-stable compounds including MT (S2) were isolated by centrifugation of an aliquot of the soluble fraction (S1) (15,000 ×*g* for 10 min at 4 °C) after heat-treatment (75 °C for 15 min) (Mouneyrac et al., 1998).

2.7. Metallothionein analysis

In the heat-denaturated supernatant (S2), the amount of MTs was determined by differential pulse polarography (DPP), a technique based on SH-compound determination according to the Brdicka reaction (Brdicka, 1933) as described by Thompson and Cosson (1984) and Olafson and Olsson (1991). A MDE 150 Stand Polarographic (Radiometer, Denmark) Tracelab 50, controlled by the computer software Tracemaster 5 through a Polarographic analyser POL 150 was used. The temperature of the cell was maintained at 4 °C. The method of standard addition was used for calibration with rabbit liver MTs (Enzo Life Sciences, USA) in absence of a shrimp MT standard. Three measurements were performed for each sample. The MTs levels are expressed in mg MT g^{-1} ww.

2.8. Metal concentration in soluble and insoluble fractions

Zinc concentration was determined in soluble or cytosolic (S1) and insoluble (P1) fractions.

The soluble fraction S1 includes both the cytosolic metals and part of zinc which is initially bound (adsorbed) onto the exoskeleton (White and Rainbow, 1984). In order to evaluate the contribution of these external metals to the soluble fraction S1, desorption tests were carried out on extra *P. argentinus*. Accordingly, shrimps were exposed to 0, 5, 50 and 500 μg Zn L^{-1} as described in Section 2.3. After exposure, samples were agitated gently for 10 min in the Tris–NaCl buffer used for homogenization. Then the leachate and the samples were recovered and analyzed separately for zinc concentration.

Samples digestion was carried out according to Amiard et al. (1987). Briefly, the insoluble and soluble fractions were heated (75 °C, 12 h) with sub-boiling HNO_3 and H_2O_2 (30%, Merck, Germany) in plastic tubes. Zinc concentration was measured on an ICP–MS (Agilent Technology 7500cx, USA) equipped with an auto-sampler ASX-100 (CETAC Technologies, USA). Three measurements were performed for each sample. The metal levels in cytosolic and insoluble fractions are expressed in mg Zn g^{-1} ww. Quality assurance (QA) and quality control (QC) were done using spiked control samples. Procedure was similar than described before. The average of the assay recoveries were 94 ± 8%.

2.9. Statistical treatment

The normality (Shapiro Wilks test) and the homogeneity (Levene's test) of the data were checked. Then, analysis of variance (ANOVA) was used to assess a significant effect on all mean treatment at $p < 0.05$. A *T*-test with Bonferroni test adjustment was used to test for significant differences between the means of controls and other treatments ($p < 0.05$). Spearman correlation test was used to establish the association between different variables. Data were expressed as the average ± standard deviation (SD). Infostat (Version 2013p, Di Rienzo et al., 2013) was used for all statistical analysis.

3. Results and discussion

3.1. Metal accumulation

Some crustaceans, particularly decapods have a relatively constant total body concentration of essential metals, like Cu and Zn, over a wide range of dissolved metal availabilities (Nugegoda and Rainbow, 1989a,b; Rainbow, 1998). In different species of invertebrates a differential tissue accumulation of essential and non-essential metals were observed (Nunez-Nogueira and Rainbow, 2005; Shuhaimi-Othman et al., 2006; Cooper et al., 2013). Moreover, the concentration of metals accumulated in crustaceans varies widely among metals and among taxa (Rainbow, 1998) and is related to its speciation, function of tissues and other biotic variables (Pourang et al., 2004).

Metal accumulation has been reported in species of genus *Palaemonetes* (Nugegoda and Rainbow, 1989a; Rainbow et al., 2006; Rainbow and Smith, 2010) thought no information is known about metal accumulation in *P. argentinus*.

In the present study, the accumulation and distribution of Zn was investigated in *P. argentinus* when exposed to environmental relevant concentrations of this essential metal.

Analyses of zinc levels in water samples carried out at the beginning (0 h) and at the end (96 h) of metal exposure revealed a significantly decrease in metal concentration at 50 and 500 μg Zn L^{-1} conditions (Table 1). Surprisingly, measured Zn concentrations in the exposure media at control and 5 μg Zn L^{-1} conditions were higher than expected. These observed zinc levels could be due to desorption of the metal from the exoskeleton of shrimps favoured by the lower concentration in the medium.

The accumulation of metal in whole body of *P. argentinus* (Table 2) is in accordance with the observed metal decay in experimental media. However, this accumulation was only

Table 1
Zinc concentration in exposure media (μg Zn L^{-1}) at 0 h and 96 h. Mean ± S.D., $n = 4$.

Condition	Time	
	0 h	96 h
Control	23.2 ± 1.3	16.3 ± 7.6
5 μg Zn L^{-1}	11.2 ± 5.9	11.3 ± 4.5
50 μg Zn L^{-1}	49.0 ± 19.6	15.4 ± 6.3[a]
500 μg Zn L^{-1}	463.8 ± 31.7	229.0 ± 116.7[a]

[a] Significantly different between 0 h and 96 h for each condition ($p < 0.05$).

significant at 500 μg Zn L^{-1} when compared with control condition.

Body concentrations of Zn in the shrimps *P. elegans* and *Pandalus montagui* have been reported, with levels of essential metal equal to 80.6 μg Zn g^{-1} dw and 57.5 μg Zn g^{-1} dw respectively (Wong and Rainbow, 1986; Nugegoda and Rainbow, 1988). Zinc concentrations from control organisms of *P. argentinus* showed higher total body levels respect to species before mentioned (Table 2). However, according to Eisler (2010) interspecies variations in Zn content are considerable, even among closely related species. Metabolic rates, gill surface area, body surface area and filtration rates are examples of physiological factors that increase metal content per unit mass in smaller animals. In this case, the wide variation of Zn levels between species could be due to higher size of *P. elegans* and *P. montagui* in contrast to a smaller body size of *P. argentinus* (Nugegoda and Rainbow, 1989).

Zinc accumulation measured in cephalothorax and abdomen sections of *P. argentinus* can be seen in Fig. 1. At higher metal exposure levels, a significant increase of Zn concentration was observed in the cephalothorax. At 500 μg Zn L^{-1} (369.1 ± 49.8 μg Zn g^{-1} dw, $p < 0.0001$) and 50 μg Zn L^{-1} (216.7 ± 66.0 μg Zn g^{-1} dw, $p < 0.01$) the concentration of metal in this body section significantly increased when compared with others ones (control = 147.1 ± 21.5 μg Zn g^{-1}dw; 5 μg Zn L^{-1} = 144.7 ± 22.5 μg Zn g^{-1} dw; Fig. 1). By contrast, accumulation pattern in abdomen showed significantly increased levels of this essential metal only at the higher exposure concentration (500 μg Zn L^{-1} = 184.13 ± 38.25 μg Zn g^{-1} dw; $p < 0.01$, Fig. 1). In all conditions, cephalothorax showed a greater capacity to accumulate Zn than the abdomen ($p < 0.05$, Fig. 1).

These results are in agreement with the capacity of hepatopancreas to accumulate and detoxify heavy metals, including the essential ones (Amiard et al., 2006). Moreover, lower accumulation in the abdomen of *P. argentinus* could be associated with the lowest capacity of muscle to accumulate metals (Lindahl and Moksnes, 1993; Eisler, 1993; Yilmaz and Yilmaz, 2007). Although it was necessary to expose *P. argentinus* at 500 μg Zn L^{-1} to observe a significant response in abdomen, longer exposures could show significant accumulation in this body section at lower Zn concentrations.

In line with these results, Pourang et al. (2005) reported higher concentrations of Zn in hepatopancreas of *Penaeus semisulcatus* and *Penaeus merguiensis* respect to muscle. The presence of hepatopancreas in the cephalothorax section and the predominance of muscle in abdomen, allows us to suggest than *P. argentinus* showed a similar pattern of Zn accumulation than *Penaeus* species.

Metal values in cephalothorax and abdomen include the Zn contained in exoskeleton, then, the concentrations of metal in this structure were also measured (Fig. 2). In exoskeleton from organisms exposed to 500 μg Zn L^{-1}, metal levels were significantly higher (311.2 ± 36.6 μg Zn g^{-1} dw, $p < 0.05$) compared with others exposure conditions. No significantly differences were found among Control, 5 and 50 μg Zn L^{-1} exposed organisms. Considering that exoskeleton represents 23% of dry weight (unpublished data); the metal provided by exoskeleton to total body Zn of control *P. argentinus* is 8.8%. This percentage does not change the significance of accumulation results previously discussed.

Ability of decapod crustaceans to regulate the total body Zn concentration has been reported (White and Rainbow, 1982, 1984; Nugegoda and Rainbow, 1989). Different authors described a threshold of Zn regulation breakdown. This threshold could be specie-dependent: ca. 22 μg Zn L^{-1} for *P. montagui*, ca. 93 μg Zn L^{-1} *P. elegans* and ca.190 μg Zn L^{-1} *Palaemonetes varians* (Nugegoda and Rainbow, 1988, 1989, 1995; Eisler, 1993). While in the present study a significant increase in metal levels occurs in cephalothorax at 50 μg Zn L^{-1}, no significant accumulation was observed in whole body in this condition when compared to control organisms. Therefore, Zn regulation breakdown in *P. argentinus* would be taking place over 50 μg Zn L^{-1}.

3.2. Subcellular metal distribution and metallothionein induction

Metals accumulated in soft tissues are likely to be present in two phases: dissolved in the cytoplasm, mainly as complexes with metal-binding proteins like MTs, or incorporated into metal-rich granules (Mason and Jenkins, 1995). Intracellular partitioning of zinc and MTs levels have been measured in the present study in order to explain a possible physiological response to Zn in the native shrimp *P. argentinus*.

Zinc concentration in the cytosolic (S1) and insoluble (P1) fraction for both cephalothorax and abdomen sections can be seen

Table 2
Zinc concentration in whole body of *Palaemonetes argentinus* (μg Zn g^{-1} dw) after 96 h exposure. Mean ± S.D., $n = 10$.

Condition	μg Zn g^{-1} dw
Control	257.8 ± 42.8
5 μg Zn L^{-1}	222.2 ± 34.0
50 μg Zn L^{-1}	302.1 ± 94.4
500 μg Zn L^{-1}	541.7 ± 94.7[a]

[a] Significantly different with the control condition ($p < 0.05$).

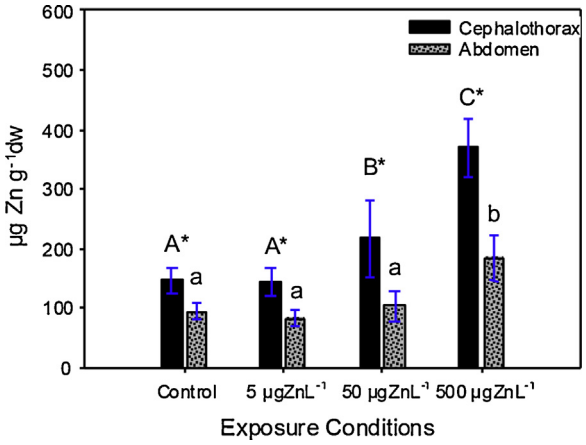

Fig. 1. Zinc accumulation (μg Zn g^{-1} dw) in cephalothorax and abdomen of *Palaemonetes argentinus* after 96 h exposure. The data represent average ± standard deviation (SD), $n = 10$. The same letter indicates no significant differences ($p > 0.05$) among treatments; capital letters for cephalothorax; lowercase for abdomen. * Represent statistically differences between cephalothorax and abdomen ($p < 0.05$).

in Table 3. Since the soluble fraction S1 includes both the cytosolic metals and part of zinc which is loosely bounded onto the exoskeleton (White and Rainbow, 1984), the percentage of Zn adsorbed onto the exoskeleton has been previously determined using desorption tests (Table 4). The percentage of body metal content desorbed varied according to the doses of exposure. In cephalothorax the percentage of desorption ranged from 8.8 to 24.7%, while in abdomen it varied from 6.4 to 33.0% (control and 500 μg Zn L^{-1}, respectively). According to these results, part of Zn body content was loosely bound and contributed to the metal estimated in the cytosolic fraction S1. Consequently, cytosolic metal concentrations have been calculated taking into account this decrease. Our results indicate the importance to consider the metal desorption from exoskeleton when analyzing Zn distribution among the soluble and insoluble fractions.

Analysing metal distribution, in cephalothorax a significant increase in Zn content in the cytosolic fraction at the highest exposure concentration was observed ($p < 0.001$); reaching levels three times higher at 500 μg L^{-1} than at control condition (Table 3). In abdomen, a similar pattern was observed. However, the cytosolic levels of Zn increased less than two times at higher exposure concentrations regarding to control organisms.

Insoluble levels of Zn were significantly greater in both cephalothorax and abdomen at 50 μg and 500 μg Zn L^{-1} compared to control organism. Moreover, insoluble metal rose significantly with concentration in abdomen of shrimps exposed at 50 μg and 500 μg Zn L^{-1}.

The similar concentration and distribution of Zn observed at control and 5 μg Zn L^{-1} conditions indicate a homogeneous availability of this essential metal in body sectors considered.

At 50 μg L^{-1} no significant differences were found in cephalothorax between cytosolic and insoluble fractions of Zn. However, the insoluble Zn was significantly higher than in control condition (Table 3). That could indicate that significant accumulation of Zn in cephalothorax of organisms, exposed at 50 μg L^{-1}, could be due to an increase in Zn granules. A similar distribution pattern was observed in abdomen. Nevertheless, as previously shown, no significant accumulation has been found in total zinc accumulation in abdomen at 50 μg L^{-1} condition when compared to control one (Fig. 1).

At 500 μg L^{-1} the Zn was detected in the same proportion in cytosolic and insoluble fractions in cephalothorax. In abdomen, concentrations of insoluble metal were greater than cytosolic ones indicating probably an increase in Zn deposits or granules. The significant accumulation of metal at 500 μg Zn L^{-1} (Fig. 1) in cephalothorax and abdomen, compared to control condition, could be due to an increase of metal both in soluble and insoluble fractions (Table 3). However, the differential accumulation

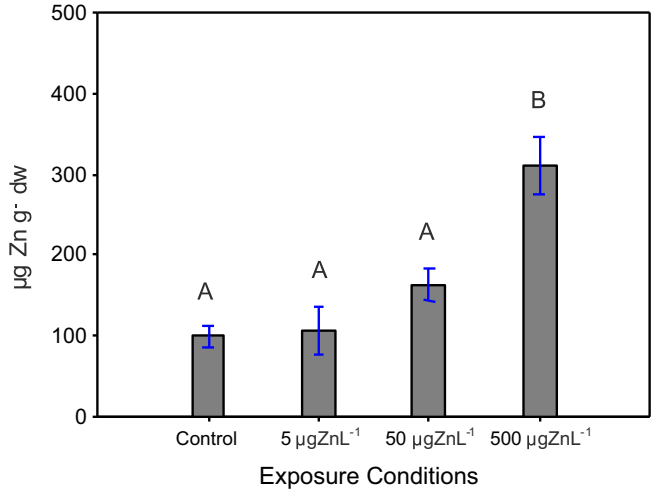

Fig. 2. Zinc concentration (μg Zn g^{-1} dw) in exoeskeleton of *Palaemonetes argentinus* after 96 h exposure. The data represent average ± standard deviation (SD), $n = 6$. The same letter indicates no significant differences ($p > 0.05$) among treatments.

Table 3
Zinc concentration (mg g^{-1} ww) in cephalothorax and abdomen from *Palaemonetes argentinus*, measured in the cytosolic (S1) and insoluble (P1) fraction and experimental conditions. Mean ± S.D.

Condition	*n*	Cephalothorax		Abdomen	
		Cytosolic	Insoluble	Cytosolic	Insoluble
Control	7	0.011 ± 0.001[a]	0.011 ± 0.003[a]	0.008 ± 0.003[a]	0.010 ± 0.002[a]
5 μg Zn L^{-1}	5	0.009 ± 0.002[a]	0.015 ± 0.005[a,b]	0.005 ± 0.001[a]	0.008 ± 0.002[a]
50 μg Zn L^{-1}	7	0.015 ± 0.004[a]	0.025 ± 0.008[b,c]	0.006 ± 0.003[a]	0.016 ± 0.006[b]
500 μg Zn L^{-1}	5	0.038 ± 0.006[b]	0.034 ± 0.005[c]	0.012 ± 0.002[b,d]	0.033 ± 0.003[c,d]

[a] Indicates no significant differences among treatments in the same fraction ($p > 0.05$).
[b] Indicates no significant differences among treatments in the same fraction ($p > 0.05$).
[c] Indicates no significant differences among treatments in the same fraction ($p > 0.05$).
[d] Significantly different between fractions for each body section ($p < 0.05$).

Table 4
Zinc desorption (%) in cephalothorax and abdomen from *Palaemonetes argentinus*. Mean ± S.D.

Condition	Cephalothorax	Abdomen
Control	8.7 ± 0.8	6.4 ± 0.8
5 μg Zn L^{-1}	11.1 ± 1.9	10.1 ± 0.8
50 μg Zn L^{-1}	16.6 ± 3.7	25.0 ± 4.2
500 μg Zn L^{-1}	24.7 ± 3.1	33.0 ± 2.4

between body sectors could be attributed to a significantly rise in cytosolic metal in cephalothorax respect to abdomen, without variation in insoluble fraction at 500 μg Zn L^{-1} exposure.

The distribution of Zn among cytosolic and insoluble fractions in the cephalothorax as well as in the abdomen of shrimps could be better observed in Fig. 3 (A and B, respectively). A clear variation in the essential metal distribution is observed between body's sectors analyzed. Whatever the dose of exposure, Zn remained approximately equally distributed between the cytosolic and insoluble fractions in cephalothorax (Fig. 3A). On the other hand, in abdomen a significant different pattern has been observed suggesting a different mechanism in this body sector and associated tissues like muscle. In abdomen, when total Zn increased, insoluble metal increased more markedly than soluble one (Fig. 3B).

Metallothioneins or MTLP have been found in different tissues of decapod crustaceans (Wong and Rainbow, 1986; Engel and Brouwer, 1993; Legras et al., 2000; Mouneyrac et al., 2001; Pourang et al., 2004) and in species of genus *Palaemonetes* (Howard and Hacker, 1990; Brouwer et al., 2007; Gonzalez-Rey et al., 2007, 2008). All of these species are from marine or estuarine environments and few records exist about MTLP metal induction in freshwater decapod crustaceans. MTs have been reported in freshwater species of crabs *Carcinus maenas* (Pedersen et al., 1998), *Potamonautes warren* (Schuwerack et al., 2009), crayfishs *Cambarus robustus* (Taylor et al., 1995), *Procambarus clarkii* (Torreblanca et al., 1996) and prawn *Macrobrachium rosenbergii* (Mahmood et al., 2009). Presence of MT in South American freshwater shrimps has not been reported except one report which evidences the MT presence in *P. argentinus* using a spectrometric method (Chiodi Boudet et al., 2013).

One of the important characteristics of MTs is inducibility, namely they can be synthesized in response to the presence of toxic metals or many other agents (Pourang et al., 2004). It is well known that the induction of MTs occurs in aquatic invertebrates after exposure to heavy metals such as Cd, Zn, and Cu (Roesijadi 1992; Canli et al., 1997; Geffard et al., 2002; Coyle et al., 2002; Mosleh et al., 2006). Moreover, Zn is the most potent inducer of MTs transcription (Geffard et al., 2002; Coyle et al., 2002; Pourang et al., 2004).

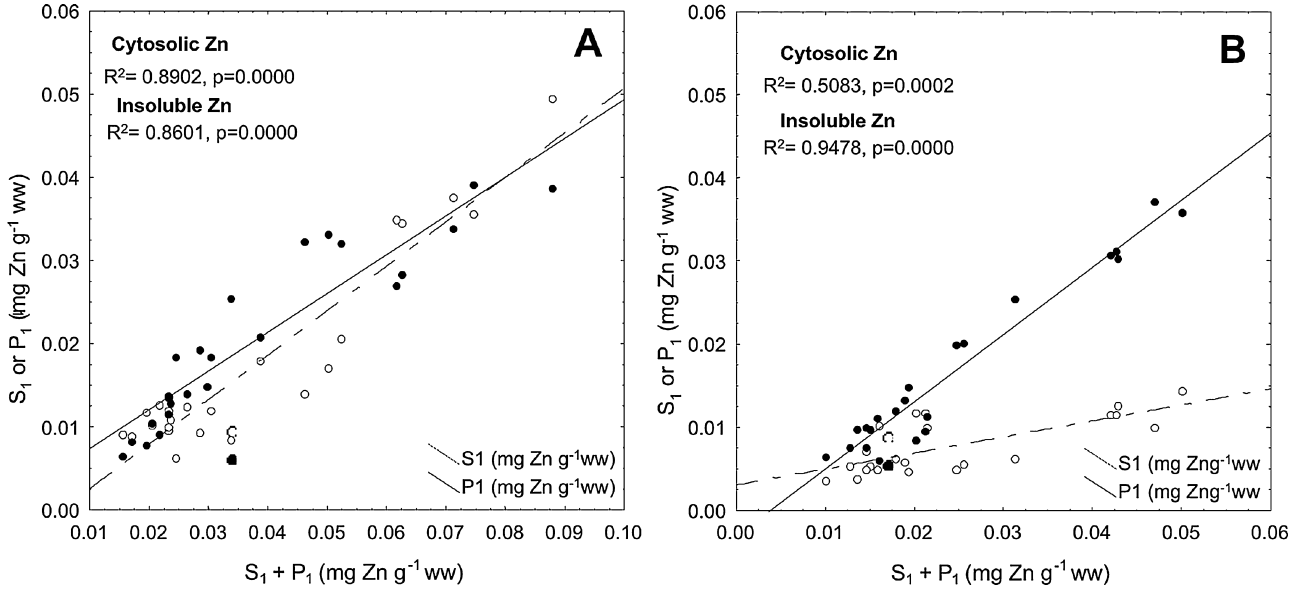

Fig. 3. Zinc distribution in cephalothorax (A) and abdomen (B) of *Palaemonetes argentinus* after metal exposure for 96 h to different concentrations of zinc. Total metal: S1 + P1; cytosolic S1 (○, dotted line) or insoluble P1 (●, continous line) metal. Metal concentrations in mg Zn g^{-1} wet weight.

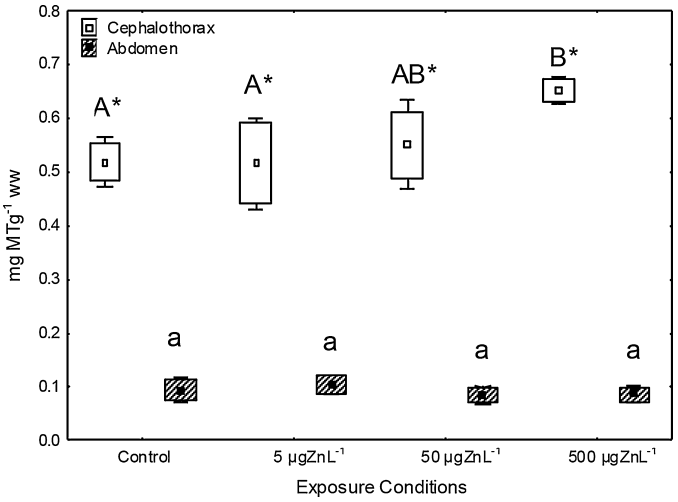

Fig. 4. Metallothioneins (MT) levels (mg MT g^{-1} ww) in cephalothorax and abdomen of *Palaemonetes argentinus* after exposure for 96 h to different concentrations of zinc. The data represent mean ± standard deviation (SD), $n = 46$. The same letter indicates no significant differences ($p > 0.05$) among treatments. * Represent statistically differences between cephalothorax and abdomen ($p < 0.05$).

Fig. 4 shows cephalothorax and abdomen concentrations of MT in *P. argentinus* exposed to zinc. Cephalothorax of organisms exposed to the higher (500 μg Zn L^{-1}) concentration of Zn showed a significant increase in MT levels (0.651 ± 0.025 mg MT g^{-1} ww) compared to cephalothorax from control and 5 μg Zn L^{-1} organisms (0.518 ± 0.045 mg MT g^{-1} ww and 0.516 ± 0.085 mg MT g^{-1} ww respectively, $p < 0.05$). Conversely, not induction of MT was observed in the abdomen of *P. argentinus* even at the maximum exposure concentration ($p > 0.05$). Moreover, MT concentrations were between 5 and 6.5 times higher in cephalothorax than in the abdomen, in all the exposure conditions ($p < 0.01$).

Because MT is a cytosolic heat-stable protein, it is logical to consider the relationship between MT concentration in a body sector and metal concentration in the cytosolic fraction.

A positive and significant correlation ($r^2 = 0.52$, $p < 0.01$) has been observed between cephalothorax MT concentration and cytosolic Zn. However, no significant correlation has been obtained between the same variables in abdomen ($p > 0.05$).

According to our results, MTs appear to be induced by Zn in cephalothorax at the higher metal concentration tested (Fig. 3), which is supported by this positive and significant correlation between MT and cytosolic metal concentrations. A similar result has been reported in *Pachygrapsus marmoratus* by Legras et al. (2000), were soluble Zn concentrations appeared as the major factors correlated with MT concentrations in the hepatopancreas. Similarly, a significant positive relationship has been observed between concentrations of Zn and MTs in *P. semisulcatus* (Pourang and Dennis, 2005). Geffard et al. (2002) have also found a similar response in *Mytilus galloprovincialis* after 96 h Zn exposure.

Some authors have suggested that the induction of MT is an intermediate step before insoluble deposits are formed (Nunez-Nogueira et al., 2010). In *L. vannamei* (Wu and Chen, 2005) and *P. indicus* exposed to Zn, the MTs appear to be the first detoxification strategy involved in non-essential metals exposure, while essential metals are regulated in cytoplasm by another process (e.g., insoluble deposits) (Nunez-Nogueira et al., 2006). Our results indicate a similar response in *P. argentinus* were a significant increase in insoluble Zn could be observed at lower exposure concentrations without a significant increase in MTs concentration. By contrast, at 500 μg Zn L^{-1} cytosolic Zn reached the maximum concentration accompanied by a significant increase in MTs concentration in cephalothorax suggesting the participation of this kind of protein in metal detoxification. In abdomen, insoluble metal increase more than soluble one and no MTs induction has been recorded showing a differential response.

Probably, at low and intermediate Zn concentration, the formation of granules involves pre-existing MTs. While at 500 μg Zn L^{-1} a new synthesis of MTs could be occurring to avoid toxic effect over organism. That is in concordance with the detoxifying function of hepatopancreas in decapod crustacean species and present in cephalothorax section of *P. argentinus* (Pourang and Dennis, 2005). According with that, in the crab *P. marmoratus* higher concentrations of soluble zinc and MT have been found in hepatopancreas respect to others tissues (Mouneyrac et al., 2001).

Our results show a fast response (96 h) in *P. argentinus* of MT induction at the higher concentration of Zn tested. It has been proved that longer period of exposure and higher metal concentrations induced MTs in crustaceans and aquatic or terrestrial invertebrates (Pan and Zhang, 2006; Canli et al., 1997). Longer exposure periods should be tested in *P. argentinus* in order to evaluate an improvement in the sensitivity of MTs response.

4. Conclusion

In summary, this study reports for the first time the zinc content in cephalothorax, abdomen and exoskeleton of *P. argentinus* exposed to environmental relevant concentrations of this essential metal. These results suggest that the crustacean manages to regulate the total body Zn concentration up to external levels of 50 μg Zn L^{-1}. Above this concentration, significant metal accumulation occurred in all the analysed body sectors.

This is also the first report of Zn subcellular distribution in a South American freshwater decapod crustacean species. Our results reveal a clear differential response between cephalothorax and abdomen to Zn exposure. Regardless of the concentration here tested, in cephalothorax, Zn was nearly equally distributed between cytosolic and insoluble fractions, being the cytosolic content significantly correlated with MTs induction. In abdomen, insoluble metal increased more than soluble one, while no induction of MTs was recorded in this body section.

A potential role for MTs as biomarkers in *P. argentinus* should be further studied to enhance the sensitivity of the response, although

it is likely that MTs play a key role in metal detoxification since the increase of these proteins is linked to metal challenge.

Argentinean Environmental Water Quality Guidelines indicate concentrations not higher than 50 μg Zn L^{-1} in superficial waters, depending of hardness, in order to protect the freshwater environments and their biota. Usually, limited information about toxicological effects of metals over native biota difficult an appropriate design of environmental guidelines, promoting the use of toxicological data from non-native species. Further studies would be required to rule out possible harmful effects in *P. argentinus* and to evaluate the appropriateness of the guidelines for the freshwater biota.

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