Strategies for cadmium detoxification in the white shrimp *Palaemon argentinus* from clean and polluted field locations

Leila Chiodi Boudet [a, b, *], Julieta Mendieta [c, d], María Belén Romero [a, b], Arantxa Dolagaratz Carricavur [a, b], Paula Polizzi [a, b], Jorge E. Marcovecchio [e], Marcela Gerpe [a, b]

[a] Laboratorio de Toxicología Ambiental, Departamento de Ciencias Marinas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Funes 3350, 7600, Mar del Plata, Argentina
[b] Instituto de Investigaciones Marinas y Costeras, Consejo Nacional de Investigaciones Científicas y Técnicas (IIMyC-CONICET), Argentina
[c] Instituto de Investigaciones Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Funes 3250, 7600, Mar del Plata, Argentina
[d] Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC), Argentina
[e] Área de Oceanografía Química, Instituto Argentino de Oceanografía (IADO-CONICET/UNS), Bahía Blanca, Argentina

HIGHLIGHTS

- Tolerant and non-tolerant shrimp showed different strategies to detoxify Cd.
- MT synthesis was the main mechanism in Cd detoxification in non-tolerant shrimp.
- The MRG formation was responsible of Cd resistance in the tolerant population.

ARTICLE INFO

ABSTRACT

In this study, we investigated the metal handling capacity of non-tolerant and tolerant populations of *Palaemon argentinus* to cadmium (Cd), through evaluating of the main mechanisms of metal detoxification, metallothioneins (MT) and metal-rich granules (MRG), to probe that the presence of MRG in the second population is responsible of that condition. The tolerant population were exposed to 3.06 and 12.26 μg $Cd \cdot L^{-1}$, while the non-tolerant shrimp were exposed to 3.06 μg $Cd \cdot L^{-1}$. Each experiment involved the exposure during 3, 7, 10 and 15 days and, the depuration during 7, 14, 21 and 28 days, for which shrimp were transferred to clean water. The range values of MT concentrations for non-tolerant shrimp were: 12.24–23.91 μg g (w.w), while for tolerant shrimp were: 8.75–16.85 μg g (w.w); MRG levels were: 0.12–0.57 μg g (w.w) and 0.3–2.1 μg g (w.w), respectively. The results showed different strategies for Cd detoxification: the induction of MT was the main pathway in the non-tolerant population, while the formation of Cd-MRG was the main mechanism for tolerant shrimp. These differences could be related to the environmental history and the health status of each populations.

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1. Introduction

Cadmium (Cd) is a nonessential toxic metal, water-soluble that has a high capacity to bioaccumulate in aquatic invertebrates. Cadmium forms stable complexes with proteins (Bridges et al., 2006), by displacement of the essential metals at structural and catalytic positions, resulting in loss of function, and subsequently cytotoxicity due to the disruption of cell signaling and Ca^{2+}- homeostasis (Janssens et al., 2009). Cadmium is known to be genotoxic (Pavlaki et al., 2016), and it has been demonstrated to inhibit the repair of DNA and to cause lipid peroxidation (Bertin and Averbeck, 2006).

Aquatic invertebrates possess diverse strategies in the handling and storage of metals that allow them to cope with elevated concentrations in the environment (Rainbow, 2002, 2007). Once

assimilated, metals usually go through a series of metabolic processes and are subsequently incorporated into various cellular components (Bednarska et al., 2017; Wang and Rainbow, 2005). For aquatic invertebrates, trace elements are potentially stored in two different detoxified forms: metallothioneins (MT) and various types of metal-rich granules (MRG) (Goto and Wallace, 2007; Bednarska et al., 2017). Metallotioneins, a family of low molecular weight (6−7 kDa), cysteine-rich metal-binding proteins have been shown to occur in most zoological taxa (Binz and Kägi, 1999). It is generally thought that these proteins play a primary role in the homeostasis of essential metals such as zinc (Zn) and copper (Cu), as well as being involved in the detoxification of nonessential metals, such as Cd (Viarengo et al., 2000). They have been widely used as biomarkers of metal exposure in aquatic organisms, as their synthesis is induced as a result of exposure to metal contaminants (Romero-Isart and Vašák, 2002). Additionally, there is increasing evidence that MTs may also have an antioxidant role, acting as scavengers of free radicals and reactive oxygen species (Cavaletto et al., 2002; Correia et al., 2002a; Viarengo et al., 2000).

Numerous metals, among them Cd, Cu, and Zn, can also be sequestered as electrondense granules, the occurrence, form, and function of which have been the subject of several reviews (Ahearn et al., 2004; Marigomez et al., 2002; Rainbow, 2007; Vivjer et al., 2004). Like MT, MRGs serve as biomarkers of trace-element toxicity (Correia et al., 2002b), and they are present in most macro-invertebrate phyla (Ahearn et al., 2004; Corrêa et al., 2002; Khan et al., 2010). They occur intra- or extracellularly and are associated with organs that have digestive, storage or excretory functions (Nassiri et al., 2000). In some cases, the relative enhancement of one of these fractions can reflect preferential storage in one particular detoxified form (MT or MRG), with increasing metal contamination (Mouneyrac et al., 2002). Therefore, they may be used to assess the metal handling capacities of aquatic organisms chronically exposed to trace metals and to elucidate the mechanisms involved in population - or species-specific tolerance to trace elements (Goto and Wallace, 2009).

Palaemon (= *Palaemonetes*) *argentinus* (Crustacea: Decapoda: Caridea) is a freshwater shrimp of ecologic interest because of its abundance in the north and centre of Argentina, in Uruguay, and in southern Brazil (Morrone and Lopreto, 1995). Due to its abundance, it plays a very important role in trophic chains, sustaining a great variety of fish and birds (Azevedo et al., 2004). Being a detritivorous species, it plays a key role in the nutrient cycle and consequently in the energy balance in their habitats (Reinsel et al., 2001). Several authors reported their sensitivity to toxicants both in laboratory tests (Bertrand et al., 2015, 2016; Chiodi Boudet et al., 2015; Collins and Capello, 2006; Lavarías and García, 2015) and in the field through active monitoring (Bertrand et al., 2018). Therefore, this species can be used as a bioindicator to provide information on environmental quality (Montagna and Collins, 2007). In a previous study, *P. argentinus* from contaminated sites were more tolerant to Cd than shrimp from uncontaminated areas. In addition, short-term exposure (96 h) of non-tolerant shrimp showed a fast induction of MT synthesis while tolerant shrimp did not induce MT nor oxidative stress (Chiodi Boudet et al., 2013). This contrasting pattern between populations allows to hypothesize the presence of MRG in the tolerant population as the main mechanism to cope Cd concentrations. Therefore, the goal of this study was to evaluate the mechanisms and strategies involved in Cd detoxification in both tolerant and non-tolerant shrimp during subchronic and depuration assays. The depuration assay allows to evaluate their capacity of recovery after the restitution of the water quality, with the elimination of the stressor.

2. Materials and methods

2.1. Specimens

Freshwater shrimp, *Palaemon argentinus*, were collected with a hand net and immediately transferred to the laboratory. They were obtained from Los Padres lagoon (37°57′ S, 57°44′ W) and Nahuel Ruca lagoon (37° 37′S - 57° 25′W), both located in one of the most productive agricultural area of Buenos Aires Province, Argentina. Los Padres lagoon (LP) is surrounded by horticultural fields and their sediments have high metal concentrations (0.7 μg Cd·g^{-1}; 1 μg Hg·g^{-1}; 72 μg Cr·g^{-1}; 15.3 μg As·g^{-1}, 119 μg Zn·g^{-1}; Chiodi Boudet et al., 2008), that exceed the levels safe for the biota (CCME, 2002). Nahuel Ruca lagoon (NR) is located within a reserve (Program for Man and Biosphere, MAB, UNESCO) (Martínez, 2001) and their sediments has low metal concentrations and are below the limit established as safe for biota (Chiodi Boudet et al., 2010). Previous studies demonstrated that shrimp from LP lagoon have a higher tolerance to Cd relative to those from NR lagoon, evidenced by LC$_{50}$-96h values, reflecting the environmental history (Chiodi Boudet et al., 2013).

Organisms were acclimated for three days in 140 L glass aquaria filled with aerated freshwater (pH = 8.30 ± 0.05, water hardness = 235 mg CaCO$_3$ · L^{-1}), maintained at constant laboratory temperature (17.0 ± 0.9 °C) and under a 12h: 12h light: dark photoperiod. During acclimation, those aquaria that had more than 2% mortality were not used for the experiments (Khan et al., 1988). All along this period, they were fed daily *ad libitum* with commercial food for fish (Tetramin, Germany).

2.2. Subchronic exposure and depuration assay

Two days before the onset of each experiment, adult shrimps (body length >2.2 cm; Donatti, 1986) of *P. argentinus* were randomly distributed in sixteen 20-L glass aquaria filled with aerated freshwater. The density was five individuals per liter along all the experiment (100 individuals per aquaria). The Cd concentrations were selected according to previous studies and LC$_{50}$-96h values for Cd of each shrimp population (LC$_{50}$: 24.50 and 12.26 μg Cd·L^{-1} for LP and NR population, respectively) (Chiodi Boudet et al., 2013). The tolerant population (LP) were exposed to 3.06 and 12.26 μg Cd·L^{-1}, while the non-tolerant (NR) were only exposed to 3.06 μg Cd·L^{-1}, because 12.26 μg Cd·L^{-1} is lethal for 50% of exposed population at 96h.

Each experiment involved the exposure during 3, 7, 10 and 15 days and, the depuration during 7, 14, 21 and 28 days, for which shrimp exposed for 15 days were transferred to clean water. Each exposure and depuration condition had its corresponding control. All along the experiments, shrimp were fed before the renewal of the medium (every 48h - 50% of total volume); retiring uneaten food immediately. All other conditions were kept the same as those used for acclimation. The shrimp were checked daily and dead individuals were removed and counted, being the absence of response to gentle mechanical stimulus the criterion for death.

At the end of each treatment, shrimp were cryoanesthetized and hepatopancreas were dissected carefully. The hepatopancreas is the main organ involved in the accumulation and detoxification of metals (Núñez Nogueira et al., 2006) and was, therefore, the object of interest for this study. Finally, samples were frozen with liquid nitrogen and kept at −80 °C until analysis.

2.3. Reagents and metal analysis

All reagents were of analytical grade supplied by Sigma-Aldrich

and Merck (Germany). Double distilled water (ddH$_2$O) was used to prepare standard solutions, dilutions, and blanks. All glassware and aquaria used were left with nitric acid solution overnight and then washed with ddH$_2$O to avoid metal contamination. A stock metal solution was prepared using CdCl$_2$ (99.9%) and specific aliquots were taken to provide nominal metal concentrations.

Water samples from exposure aquaria were taken before starting metal exposure to check the nominal concentrations. Samples were acidified to 2% with HNO$_3$ and measured by Anodic Stripping Voltammetry (ASV) following the technique described by Andrade et al. (2006).

2.4. Metallothionein assay

MT concentrations were measured in pooled samples of hepatopancreas (5–6 individuals $\approx$ approx. weight 30 μg) according to the methodology described by Viarengo et al. (1997). Application of this methodology yields a partially purified metalloprotein fraction by acidic ethanol/chloroform fractionation of the tissue homogenate. During extract preparation, MT is denatured by low pH and high ionic strength. MT concentration in extracts is measured by spectrophotometry employing the Ellman's −SH reagent (5, 5-dithio-bis 2-nitrobenzoic acid; DTNB). According to Viarengo et al. (1997), procedures performed during sample preparation allow obtaining a complete MT precipitation and avoid oxidation of −SH groups, contamination by soluble low molecular weight thiols, and enzymatic protein degradation. Three measurements were performed for each treatment. The amount of MT was calculated as was described by Chiodi Boudet et al. (2013). The MT concentration was reported as μg of MT per gram of wet tissue.

2.5. Cd in metal rich granules (Cd-MRG)

The Cd in metal rich granules (Cd-MRG) in hepatopancreas tissue ($\approx$ approx. weight 150 μg) were obtained by differential centrifugation following the method of Wallace et al. (2003). The MRG fraction was digested with perchloric and nitric acid (1:3) according to the FAO/SIDA method (1983) to determine Cd bound to this subcellular fraction. The Cd concentration was measured with ICP-OES by triplicate. Analytical accuracy was determined using blanks and certified reference material of the National Research Council of Canada (LUTS-1, Lobster hepatopancreas). The values obtained in the reference material for Cd were between 24.37 and 26.7 mg Cd·kg^{-1} (24.9 ± 1.0), that they were within the confidence range of certified value (26.7 ± 0.6 mg Cd·kg^{-1}). The Cd-MRG concentration was reported as μg of Cd per gram of wet tissue.

2.6. SDS-PAGE and western blot analysis

The spectrometric determination is an analytical procedure based on several characteristics of MT, but it does not guarantee that the target molecule is a true MT. Therefore, SDS-PAGE and Western blot (WB) techniques were used to confirm the presence of MT in shrimp samples and estimate their molecular weight. The supernatants (containing MT) obtained from the MT assay were used. Protein concentration was determined by the bicinchoninic acid (Sigma Aldrich) method (Hill and Straka, 1988). The SDS-PAGE, which was performed according to Laemmli's procedure (Laemmli, 1970), consisted of 4.5% acrylamide stacking gel and a 12% resolving gel containing 0.1% SDS and 0.75 mm thick. Samples were heated at 100 °C for 4 min in SDS-mercaptoethanol loading buffer to minimize negative charge differences and disulfide linkages. Each sample (40 μl) was divided in half, and each 20 μl was applied (60 μg per lane) to gel. After electrophoresis, one gel was stained with Coomassie Brilliant Blue R-250. The molecular weights were

estimated by comparison of electrophoresis mobility with those of the standard marker proteins (ranging from 12000-225000 Da, Amersham Full-Range Rainbow Molecular Weight, GE Healthcare). A commercially pure standard of MT (apoMT 1A/2B rabbit mixture, Bestenbalt, Estonia) was used as positive control. In addition, the SDS-PAGE gel was electroblotted by semidry protein transfer to a nitrocellulose membrane (Novex®, Semi-Dry Blotter, Invitrogen). The nitrocellulose membrane was blocked for 12 h with 5% dried milk in TRIS-buffered saline solution pH 7.5 containing 0.1% (v/v) Tween-20 (TBST). Then, it was incubated for 1h with a 1:500 dilution of anti-MT polyclonal rabbit antibody, washed with TBST solution, and incubated for 2h with 1:10000 dilution of anti-rabbit antibody conjugated with alkaline phosphatase (Sigma-Aldrich, USA). The blotted protein was revealed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution.

2.7. Statistical analysis

The data were checked for variance homogeneity by Levene's test and for distribution normality by Shapiro-Wilk's test. Significant differences were assessed by parametric tests: t-test and one-way analysis of variance (ANOVA) followed by the post-hoc Tukey test if the conditions were met, or with non-parametric tests: U-Mann-Whitney and Kruskall-Wallis with post-hoc Dunn test (Zar, 2010). The significance level was p < 0.05. All statistical analyses were performed using STATISTICA version 8.0 (Statsoft, Inc.).

3. Results

The analytical concentrations of Cd in water samples were between 95% and 99% of the nominal value. The measured concentration (mean ± standard deviation) for 12.26 μg Cd·L^{-1} was 11.98 ± 0.22 μg Cd·L^{-1}; while the analytical determination for 3.06 μg Cd·L^{-1} was not possible due to the limit of detection of the technique (5 μg Cd·L^{-1}). The survival of NR (non-tolerant) and LP (tolerant) shrimp exposed to 3.06 μg Cd·L^{-1}, varied from 98 to 100% along exposure and depuration, as well as for controls. In the case of tolerant shrimp exposed to 12.26 μg Cd·L^{-1}, the survival began to decrease from 10d (92%), declining to 87% at 15d. During depuration assays, the survival values varied from 85 to 89%.
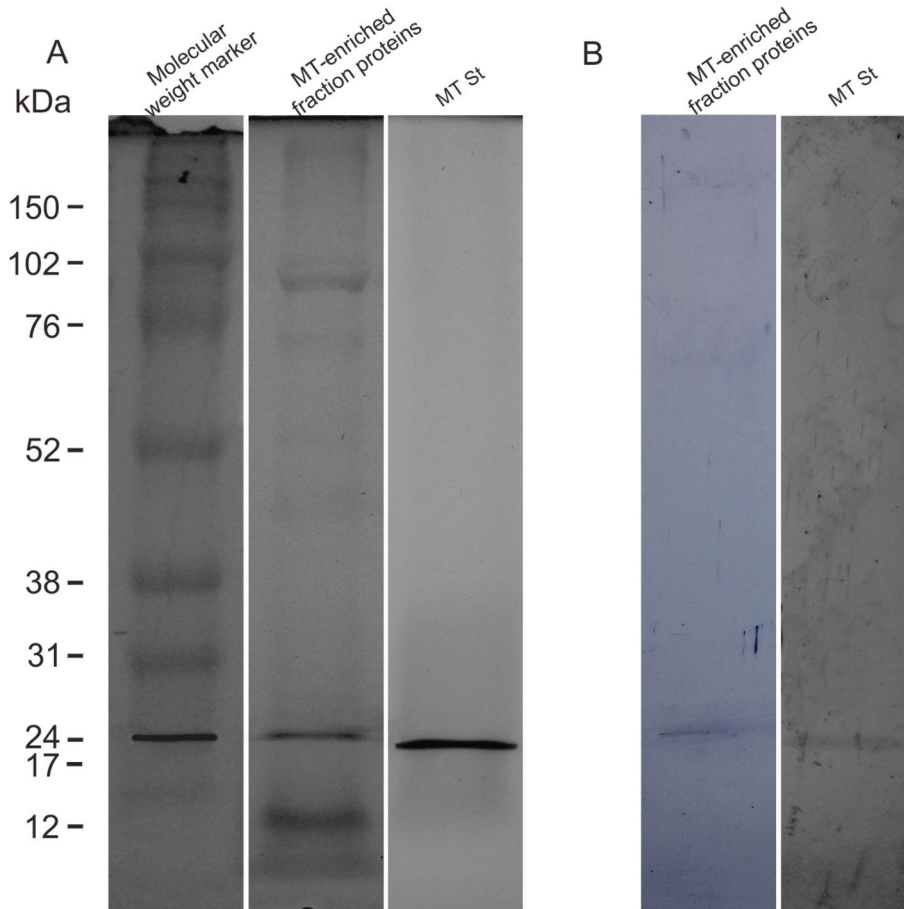
3.1. SDS-PAGE and WB analysis

The SDS-PAGE assay showed several bands, one of which had a relative molecular weight similar to the pure MT standard used as positive control (25 kDa vs 24 kDa, respectively) (Fig. 1A). To confirm this, a WB analysis was performed with anti-MT antibody. As expected, the antibody reacted with MT standard band showing that the band of 25 kDa was revealed by anti-MT antibody (Fig. 1B).

3.2. MT concentrations

The exposure of NR shrimp to 3.06 μg Cd·L^{-1} produced a significant increase in MT levels for all exposure times (t-test, p < 0.05) with respect to their controls (Fig. 2 A). In addition, it was observed a significant increase along exposure, reaching the maximum level at 10d (ANOVA with post-hoc Tukey test, p < 0.05). The concentrations in control shrimp did not show differences among them. In the first week of depuration, the MT levels decreased significantly (ANOVA with post-hoc Tukey test, p < 0.05) with respect to those observed in the exposure assay. During the following weeks, levels remained with no significant changes (ANOVA, p > 0.05). The concentrations in control shrimps did not show differences during depuration period except those for 7d, which were significantly lower than 14d and 21d (ANOVA, post-hoc Tukey test, p < 0.05).

Fig. 1. SDS-PAGE and Western Blot analysis. Proteins (60 μg) of MT-enriched fraction and MT commercial standard were analyzed by SDS-PAGE (A). Western blotting test of both samples were performed and bands were revealed with anti-MT polyclonal antibody (B).

In contrast, the exposure of LP shrimp to 3.06 μgCd·L^{-1} did not cause an increase in MT levels (Fig. 2 B), with no significant differences both respect to their control and among the different exposure times. In addition, controls did not show significant differences among them, except those for 10d, which were higher than the rest (ANOVA with post-hoc Tukey test, $p < 0.05$). During depuration no significant variations in MT levels were observed (ANOVA, $p > 0.05$), either with the control or among days.

In the experiment of 12.26 μgCd·L^{-1}, during the first week (3 and 7d), MT levels remained unchanged (Fig. 2 C). In contrast, for 10 and 15d, an increase was observed with respect to their controls (t-test, p < 0.05) as well as with 3d of exposure (ANOVA with post-hoc Tukey test, $p < 0.05$). During the first two weeks of depuration, the MT levels were unchanged with respect to 10 and 15d of exposure. In addition, concentrations at 7 and 14d were significantly higher than those for 21 and 28d (ANOVA, post-hoc Tukey test, $p < 0.05$). MT levels in controls were unchanged along depuration period, except those for 7d, which were significantly higher than 14d (ANOVA, post-hoc Tukey test, $p < 0.05$).

3.3. Cd in metal rich granules

The exposure of NR shrimp to 3.06 μg Cd·L^{-1} produced a significant increase of Cd-MRG concentrations with respect to their controls (t-test and U-Mann Whitney test, $p < 0.05$) for all times (Fig. 3 A). In addition, a gradual increase with the exposure time was observed, reaching the maximum concentrations at 15d (ANOVA with post-hoc Tukey test, $p < 0.05$). In the first week of

depuration (7d), it was observed a sharp decrease respect to the 15d of exposure (t-test, $p < 0.05$), remaining unchanged during the rest of the assay. However, the concentrations for all remained significantly higher than their controls (t-test and U-Mann Whitney test, $p < 0.05$). Unfortunately, it was not possible to analyze the depuration at 28d due to lack of samples. The Cd-MRG levels in controls did not show differences during the exposure and depuration period.

The exposure of LP shrimp to 3.06 μg Cd·L^{-1} produced a significant increase of Cd-MRG concentrations with respect to their controls (t-test and U-Mann Whitney test, $p < 0.05$) for all times (Fig. 3 B). The concentrations reached the maximum value at 15d (ANOVA with post-hoc Tukey test, $p < 0.05$). Similarly to NR shrimp, during the first week of depuration there was a significant decrease of Cd-MRG concentrations respect to 15d of exposure (t-test, $p < 0.05$), remaining unchanged during the rest of the assay. At all depuration times, the concentrations were significantly higher than their controls (t-test and U-Mann Whitney test, $p < 0.05$). It should be mentioned that the concentrations in the LP shrimp doubled, and even tripled in some cases, relative to those observed in the NR shrimp (both exposed to 3.06) for all exposure and purification times.

For LP shrimp exposed to 12.26 μg Cd·L^{-1}, there was a significant increase of Cd-MRG concentrations for all times with respect to their controls (U-Mann Whitney test, p < 0.05) (Fig. 3 C). The concentrations reached the maximum value at 15d, which were significantly higher than those of 3 and 7d (ANOVA with post-hoc Tukey test, $p < 0.05$). In similar way, during the first week of
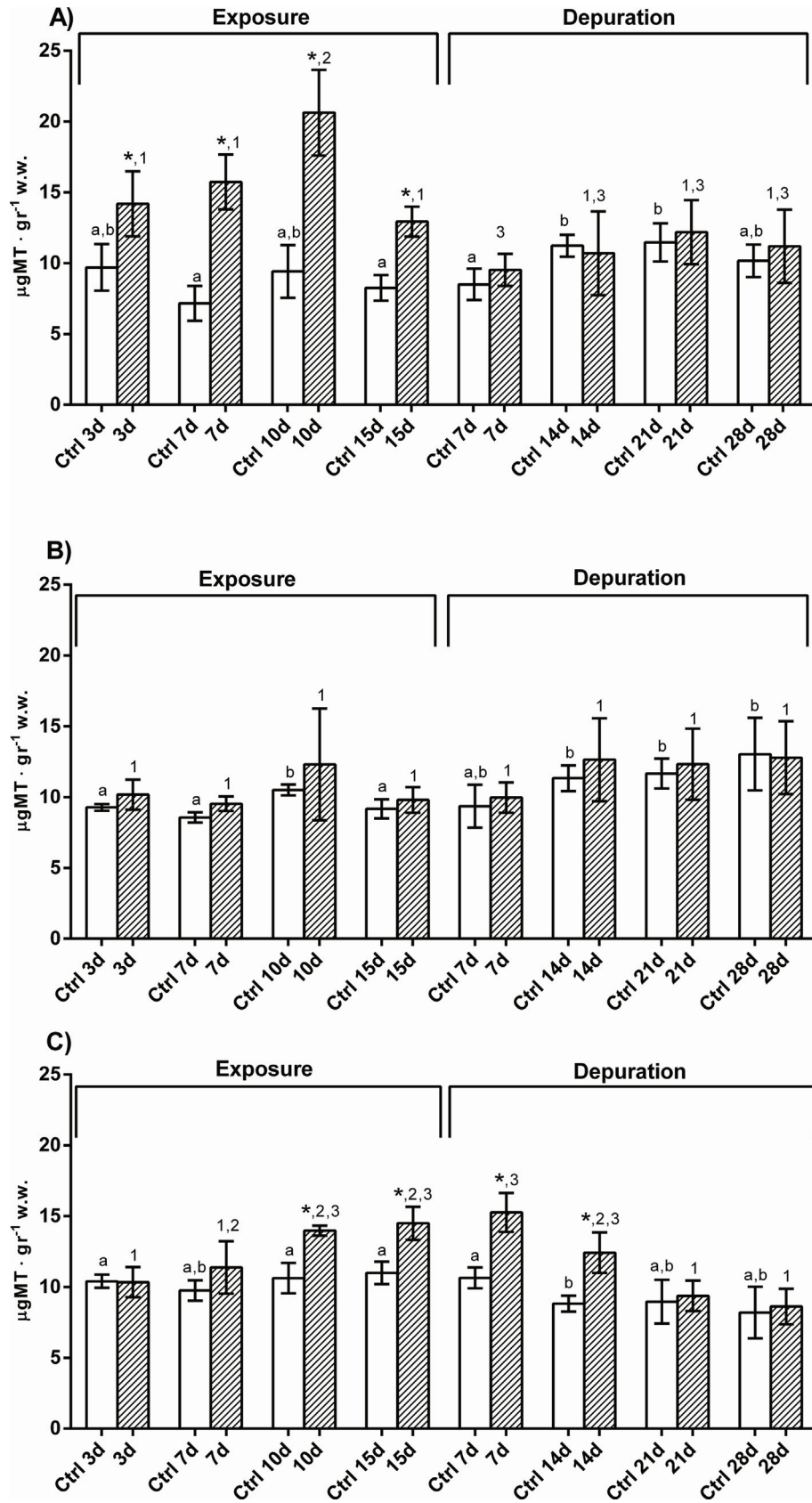
Fig. 2. Metallothionein levels (μgMT·gr^{-1} wet weight, mean $\pm$ SD) in hepatopancreas of *P. argentinus*: (A) NR (non-tolerant) shrimp exposed to 3.06 μgCd·L^{-1}; (B) LP (tolerant) shrimp exposed to 3.06 μgCd·L^{-1}; and (C) LP shrimp exposed to 12.26 μgCd·L^{-1}. The significance was tested between each treatment and their control (treatments with asterisk differ significantly at the 95% level), between different exposure times (same number indicates absence of significant differences at the 95% level), and between different controls (same letter indicates absence of significant differences at the 95% level).
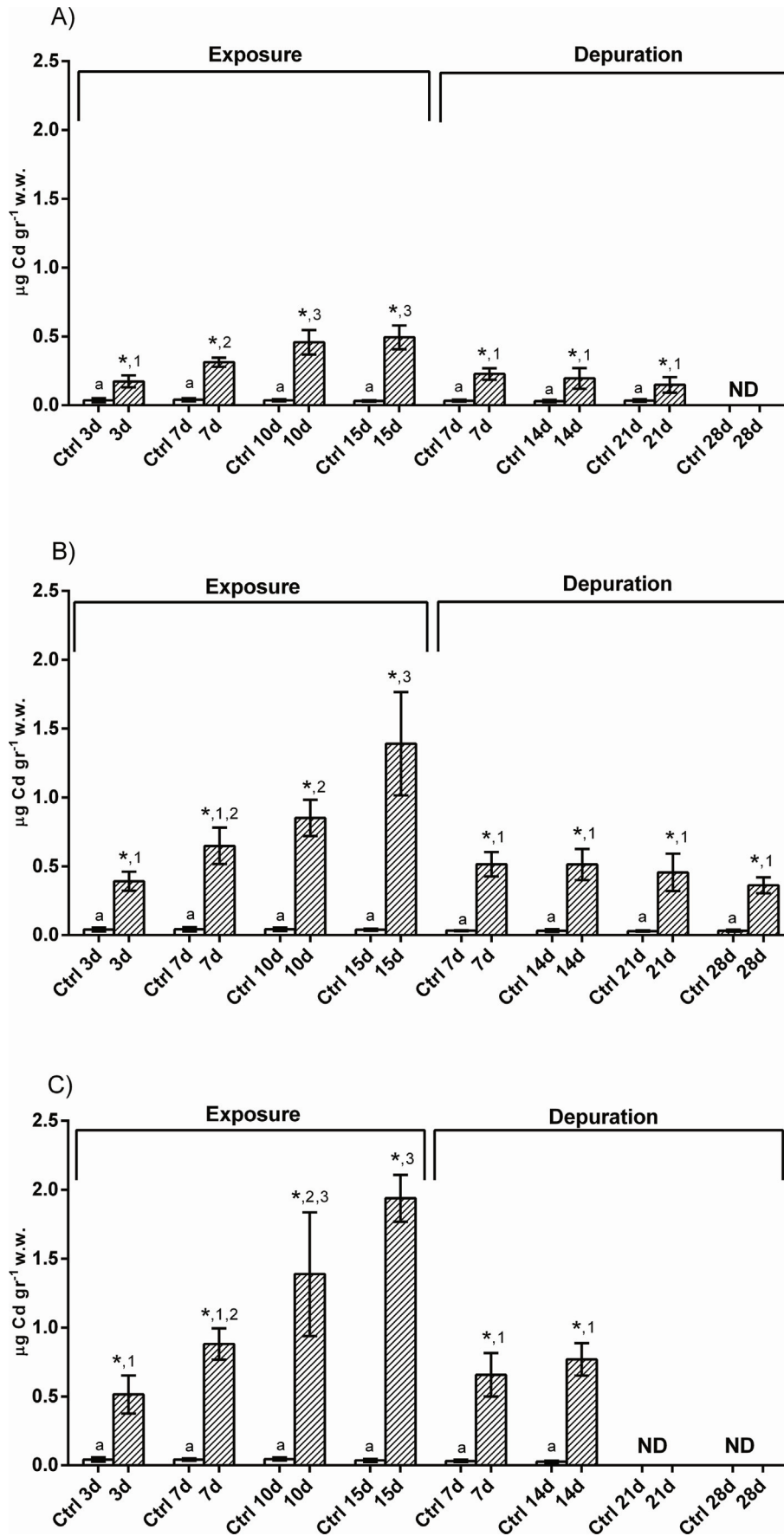
Fig. 3. Cadmium in metal rich granules (μgCd·L^{-1} w.w, mean ± SD) in hepatopancreas of *P. argentinus*: (A) NR shrimp exposed to 3.06 μgCd·L^{-1}; (B) LP shrimp exposed to 3.06 μgCd·L^{-1}; and (C) LP shrimp exposed to 12.26 μgCd·L^{-1}. The significance was tested between each treatment and their control (treatments with asterisk differ significantly at the 95% level), between different exposure times (same number indicates absence of significant differences at the 95% level) and between different controls (same letter indicates absence of significant differences at the 95% level). ND: not determined.

depuration there was a sharp decrease of Cd-MRG concentrations respect to 15d of exposure (*t*-test, $p < 0.05$), without differences between 7 and 14d concentrations. Unfortunately, it was not possible to determine Cd-MRG at 21 and 28d of depuration due to lack of samples. The Cd-MRG levels in controls did not show differences during the exposure and depuration period.

4. Discussion

4.1. Occurrence of MTs in shrimp hepatopancreas

Recent studies have reported the presence of MT in *P. argentinus* by spectrophotometric analysis (Chiodi Boudet et al., 2013) and differential pulse polarography (Bertrand et al., 2015, 2016). However, a biochemical analysis by SDS-PAGE and WB had not been performed until this study. These techniques identified the MT protein using a proper primary and secondary antibody; demonstrating the occurrence of 24 kDa MT in shrimp hepatopancreas. The molecular weight of this putative MT coincides with the average mass of MTs found in several aquatic invertebrate species. Mackay et al. (1993) reported two different groups of MTs in the mussel *Mytilus edulis* having apparent molecular weight of 10 and 20 kDa, respectively. MTs with molecular weight ranging from 7 to 25 kDa were found in heavy metal-exposed marine mussel (Viarengo et al., 1997). On the other hand, studies performed in the polychaete *Eurythoe complanata*, exposed to different metals, revealed MTs of molecular weights between 10 and 20 kDa, as well as, heavier MTs (>60 kDa) (Marcano et al., 1996). In the American oyster *Crassostrea virginica* exposed to Cd and Cu, two MT-like proteins with molecular weights of 10 kDa and 24 kDa were detected (Engel, 1999). Likewise, SDS-PAGE assays conducted with the amphipod *Gammanus locusta* revealed a putative MT of 23 kDa inducible by Cu exposure (Correia et al., 2004). These discrepancies related to the molecular weight (6–7 kD) can be explained by the unusual properties of MT; their multiple sulfhydryl groups lead to the formation of multimeric complexes, and therefore overestimate its molecular weight (Correia et al., 2004; Costa et al., 2008).

4.2. Strategies of cadmium detoxification

The development of strategies to avoid metal induced toxicity often occurs out of necessity through exposure to elevated concentrations (Khan et al., 2011). Consequently, prior natural exposure may confer a degree of tolerance that is absent in populations from uncontaminated areas. Tolerant crustaceans generally possess physiological or biochemical mechanisms that allow them to cope with elevated levels of bioavailable metals in the environment (Mason and Jenkins, 1995; Vigneron et al., 2015). These mechanisms include sequestration by MTs (Mason and Jenkins, 1995; Roesijadi, 1992) and formation of MRG (Klerks and Bartholomew, 1991; Mason and Jenkins, 1995). The current study indicated that both mechanisms were present in *P. argentinus*, although there were variations between tolerant and non-tolerant populations.

The MT induction was the main detoxification pathway of Cd for the non-tolerant population (NR). Its synthesis was induced from 3d of exposure, suggesting that the physiological response to Cd contamination was fast. Similar results have been reported in other studies (Barka et al., 2001; Bodar et al., 1988; Del Ramo et al., 1995; Martinez et al., 1996; Viarengo et al., 1985), which indicates that Cd is a potent inducer of MT synthesis (Klaassen, 2001). The maximum capacity of induction was occurred at 10d of exposure, which involved levels up to 23.91 μgMT$\cdot$g^{-1} w.w., two-fold higher than control levels. In the copepod *Tigriopus brevicornis*, the maximum capacity occurred with the 24h of exposure with levels close to 1.6 μgMT$\cdot$g^{-1} w.w., which were 5-fold higher than the controls (Barka

et al., 2001). Also, the brachiopod *Artemia parthenogenetica* and the amphipod *Echinogammarus echinosetosus* reached their maximum induction capacity at 24 h of exposure, with values up to 110 and 9 μgMT$\cdot$g^{-1} w.w. (Martinez et al., 1996), respectively. On the other hand, the decapod *Litopenaeus vannamei* reached its maximum capacity of induction at 56 days of exposure with levels close to 250 μgMT$\cdot$g^{-1} w.w. (Wu and Chen, 2005). In all these cases, organisms came from uncontaminated environments. Therefore, it appears that MT response to Cd exposure is very variable among crustaceans. This variability can be due to numerous factors specific to each species, such as size, sex, and stage of moult (Amiard et al., 2006), which does not allow a direct comparison. However, characterization of the MT response (time and capacity) provides insight into the interspecific differences of vulnerability to Cd contamination. In general, a high capacity of MT induction is related to a lower vulnerability to metal contamination (Del Ramo et al., 1995; Martinez et al., 1993; Nordberg and Nordberg, 2009). In the case of *P. argentinus*, the observed levels of MT induction, among the lowest reported for crustaceans, could explain the high sensitivity to Cd for the species (Chiodi Boudet et al., 2013). The decrease in MT levels at 15d of exposure could be a manifestation of the toxic effect of Cd. This idea is supported by the findings of Wu and Chen (2005), which showed that MT induction in the shrimp *L. vannamei* was limited after long-term exposure. In the amphipod, *Orchestia gammarellus*, the absence of MT induction or even a depletion of MT concentrations were observed at high Cd doses (Mouneyrac et al., 2002). It is generally agreed that Cd can cause a decrease in MT levels due to an impact on the normal structure and functioning of hepatopancreas, causing physiological and metabolic changes (Wu and Chen, 2005). A previous study of *P. argentinus* demonstrated an increase of lipid peroxidation (LPO) levels at 15d of exposure and histological alterations in the hepatopancreatic tissue (Chiodi Boudet et al., 2015), indicating the toxic effects of Cd. Lipid peroxidation (LPO), is one of the main biomarkers of oxidative damage, and it has been reported as a major contributor to the loss of cellular function (Hermes-Lima et al., 1995). The decrease in MT levels continued during depuration until eventually returning to the initial levels, probably due to the elimination of the stressor. Moksnes et al. (1995) observed a similar pattern in *L. vannamei*, attributing the loss of MT to the decrease in internal Cd concentrations, which would be related to the decrease of available Cd.

The formation of Cd granules was also present in the non-tolerant population, although their levels were very low in comparison to those observed in the tolerant population. Similar results were reported in the oligochaetes *Hediste diversicolor* and *Limnodrilus hoffmeisteri*, where non-resistant worms from unpolluted sites sequestered the majority of Cd into MT (Berthet et al., 2003 and Wallace et al., 1998, respectively). Some authors postulated that the formation of metal granules is a mechanism for long-term storage, while the MT is involved in time scales shorter (Brown, 1982; Roesijadi, 1992).

The tolerant population (LP), also presented the two mechanisms (MT and MRG), although the MRG formation was the main detoxification pathway of Cd. This is consistent with earlier studies in invertebrates from metal-polluted habitats, depositing large amounts of metals in the form of granules. For instance, the oligochaete *L. hoffmeisteiri* (Wallace et al., 1998) and polychaetes *H. diversicolor* and *Neanthes japonica* (Berthet et al., 2003; Fan et al., 2015) preferred to sequester Cd into MRG; the amphipods *Corophium volutator* and *O. gammarellus* from copper-contaminated sites formed Cu insoluble granules in cells of the hepatopancreas (Icely and Nott, 1980; Nassiri et al., 2000). Many authors (Bodar et al., 1990; Corrêa et al., 2002; Leland and Kuwabara, 1985)

consider the presence of MRG as a physiological adaptation to the accumulation of toxic metals. Apparently, the sequestration into MRG may be more efficient in detoxifying Cd than MTs (Goto and Wallace, 2009; Wallace et al., 1998), allowing organisms living in polluted habitats to tolerate higher levels of Cd.

As mentioned above, there was no MT induction at lower concentration (3.06 μgCd·L^{-1}) at any time, while at highest concentration (12.26 μgCd·L^{-1}) there was a slight MT induction at the end of the exposure. In this case, the lack of induction would not seem to be related to a toxic effect of Cd, taking into account that the non-tolerant population exposed to 3.06 μgCd·L^{-1} induces its synthesis, doubling the MT levels. The maintenance of a tolerance mechanism may be energetically expensive (Postma et al., 1995; Voets et al., 2009). Increased metal resistance is often associated with a trade-offs in energetic cost and fitness (Campbell et al., 2005; Kwok et al., 2009). For instance, Xie and Klerks (2004) demonstrated that the killifish *Heterandria formosa* with a Cd resistance through multigeneration exposure, exhibited a significantly lower fecundity and produced smaller sized offspring than the control populations. A similar situation could be occurring in the tolerant population of *P. argentinus*, in which it has been reported a low level of fitness indicated by small adult size, low percentage of ovigerous females during the breeding season, fewer eggs, high loss of eggs and low fertility (Ituarte, 2008). On the other hand, organisms in good condition, as reported by Chiodi Boudet et al. (2015) for NR shrimp (non-tolerant population), might be able to invest more energy in metal detoxification (Voets et al., 2009). Protein synthesis is energetically costly (Hawkins, 1991), while MRG formation would have little or no cost (Simkiss, 1991, 1996; Davies and Simkiss, 1996). This could explain the differences observed between tolerant and non-tolerant shrimp with respect to MT synthesis. Given the physical condition of the tolerant population, the Cd-MRG formation is more advantageous than MT synthesis.

The different strategies adopted by each population could have trophic consequences due to the Cd transfer, and potentially cause secondary toxic effects in their predators (Baines et al., 2002; Ni et al., 2000). As trace metals bound to MT are readily solubilized by the digestive processes of a predator, metals associated with these proteins are thought to be more available to a predator than those with MRG (Wallace et al., 2003; Zhang and Wang, 2006; Goto and Wallace, 2009), although these consequences must be tested in *P. argentinus* in future evaluations.

The depuration period allowed reaching initial MT values in those groups where its induction was observed. In the case of Cd-MRG, the purification period was not sufficient to reach the initial values, which was confirm only for the tolerant population exposed to 3.06 μgCd · L^{-1}. As already mentioned, some depuration times could not be analyzed for the other two assays due to the lack of sample. Both populations showed a decrease in MRG levels during the first week of depuration, which implies that the granules were eliminated. The highest amount of MRG is generally found in senescent R-cells in the proximal region of the hepatopancreas tubules (Corrêa et al., 2002; Vogt and Quinitio, 1994; Vogt, 2015). These cells do not exhibit apocrine secretion, therefore contaminants must remain inside the cells until cellular death (Vogt and Quinitio, 1994). Some authors have reported that granules are eliminated during the epithelial renovation at the end of each digestive cycle, and then are excreted through the faeces (Hopkin and Nott, 1979; Vogt and Quinitio, 1994). In this process, senescent cells and the mineral concretions are released while the new epithelium is constructed below (Brown, 1982). On the other hand, part of the epithelium of hepatopancreas is renewed in each molting cycle (Hopkin, 1989), and the granules could be also eliminated. In this context, it is noteworthy that during the first

days of depuration it was observed a large number of molts during assays for both populations, although it was only an observation. This event coincided with the decrease in Cd-MRG levels. The granules pattern observed in *P. argentinus* during depuration appeared to be related to the moult, with a sharp decrease during the first week which stabilized during the remainder of depuration.

5. Conclusions

Our results revealed that both MT and MRG play important roles in the storage and detoxification of Cd in *P. argentinus*. They were differences in Cd detoxification between non-tolerant and Cd-tolerant shrimp populations indicating different strategies of metal handling. The formation of Cd-MRG was the main mechanism for detoxification in the tolerant population, indicating its fundamental role in the tolerance of this population. In contrast, MT induction was the main detoxification pathway of Cd in the non-tolerant population. These differences could be related to their environmental history and the health status of the studied shrimp populations. Our results show the variable sensitivity of this species and provide insight for the interpretation of results from both in laboratory and in field studies.

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