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Lethal and sublethal effects of cadmium in the white shrimp *Palaemonetes argentinus*: A comparison between populations from contaminated and reference sites

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ABSTRACT

In the present study, the acute toxicity of cadmium (Cd) in white shrimp (*Palaemonetes argentinus*) from a metal polluted lagoon (Los Padres, LP) and from unpolluted lagoon (Nahuel Ruca, NR) was evaluated. Both population, were exposed to 3.06, 12.26, 30.66, 61.32, 306 and 613.2 μg Cd·L^{-1} for 96-h. The sublethal effects of Cd were examined by two cellular biomarkers: metallothionein (MT) and lipid peroxidation (LPO). The seasonal variations of biomarkers in both lagoons were also evaluated.

P. argentinus demonstrated a high sensitivity to Cd, with values of 96-h LC50 lower and close to those of highly sensitive species; therefore, can be proposed as a good indicator species. The LC_{50} values of shrimp from LP (24-h: 269.8, 48-h: 67.45, 72-h: 30.66, 96-h: 24.50 μg Cd·L^{-1}) were higher than those from NR (24-h: 153.3, 48-h: 32.65, 72-h: 18.40, 96-h: 12.26 μg Cd·L^{-1}), indicating a higher tolerance to Cd, and it was related to their origin. Differential responses in terms of MT induction and LPO between populations were also detected. In NR shrimps, the MT synthesis was induced very fast (24-h) and even at the minimum concentration tested (3.06 μg Cd·L^{-1}), while no increases were observed in LPO levels. In contrast, the MT and LPO levels in LP shrimps were not increased relative to control, although they were more tolerant to Cd than those of NR; suggesting the presence of another mechanism involved in the detoxification of Cd. The differences in both sensitivity and biochemical responses to Cd may be related with their environmental histories.

1. Introduction

The genus *Palaemonetes* (Crustacea: Decapoda: Caridea) includes a geographically diverse group of fresh and brackish-water shrimp. The species of this genus are ecologically important as detritivores, predators of small invertebrates and prey for fish and birds (Buikema et al., 1980). Due to their feeding mode, these species are considered to play a key role in the nutrient cycle and consequently in the energy balance in their habitat (Reinsel et al., 2001). They are candidates for research due to its easy collection, handling and laboratory tests. All these features, along with sensitivity to toxicants, are reasons for their frequent use in acute and chronic toxicity tests (Buikema et al., 1980). Moreover, some species of the genus have been recently proposed as bioindicators of anthropogenic impact (Key et al., 2006).

The white shrimp *Palaemonetes argentinus* (Nobili, 1901) is an abundant, nonmigratory freshwater shrimp distributed in the littoral fluvial region of Argentina, Paraguay, Uruguay, and southern Brazil (Morrone and Lopreto, 1995). Its ecological significance for the area has been reported by several authors (Collins, 1999; Rodrigues Capítulo and Freyre, 1995). Many aspects of *P. argentinus* have been studied: population structure, reproduction, development, molt cycle, natural and artificial feeding, osmoregulation physiology, histology (Rodrigues Capítulo and Freyre, 1995; Nazary et al., 2000; Felix and Petriella, 2003; Collins, 1999; Charmantier and Anger, 1999; Sousa and Petriella, 2007), but there is little information regarding toxicology. Rodrigues Capítulo (1984) studied the effect of anionic detergents on survival and energy metabolism. Collins and Capello (2006) studied the acute toxicity of cypermethrin to juveniles of *P. argentinus*, while Montagna and Collins (2007) the effect of chlorpyrifos and endosulfan for adults.

Cadmium (Cd) is considered a very toxic metal, and industrial and agricultural activity has markedly increased it distribution in aquatic environments. Sources of Cd to aquatic habitats include mine drainage, metal smelting wastewater, runoff of agricultural fertilizers, and atmospheric fallout from fossil fuel combustion and refuse incineration (WHO, 1992). Cadmium is known to inhibit deoxyribonucleic acid (DNA) repair and cause lipid peroxidation (Stohs and Bagchi, 1995). Such effects have been demonstrated in

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crustaceans exposed to Cd through the evaluation of biochemical biomarkers (Amiard et al., 2006; Barata et al., 2005; Khan et al., 2011; Wu and Chen, 2005). Biomarkers are early-warning signals whose detection can avoid adverse effects (Van der Oost et al., 2003). Metallothioneins (MTs) and lipid peroxidation (LPO) are biomarkers widely used in crustacean research (Amiard et al., 2006; Barata et al., 2005; Dutra et al., 2008; Khan et al., 2011; Wu and Chen, 2005).

Metallothioneins (MTs) are low-molecular weight proteins rich in cysteine that bind metals and are found in all animal phyla. MTs play a primary role in the homeostasis of essential metals, such as copper (Cu) and zinc (Zn). However, non-essentials metals, particularly Cd, are also able to induce MT synthesis (Amiard et al., 2006; Roesijadi, 1996). Therefore, MTs have been regarded as an indicator of metal exposure and widely used as a tool for biomonitoring programs.

Lipid peroxidation (LPO) is considered an important biomarker of cell damage resulting from the interaction of free radicals with membrane lipids (Barata et al., 2005). It has been used extensively to assess the detrimental effects of various pollutants such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls (De Lafontaine et al., 2000), endosulfan, paraquat (Barata et al., 2005), and Cd, Cu, Zn (Khan et al., 2011).

Neither the sensitivity nor biomarker response of *P. argentinus* to Cd has been studied. Since organisms from polluted areas may react to metal exposure differently from animals living in pristine areas (Barata et al., 2002; Damiens et al., 2006; Khan et al., 2011; Legras et al., 2000; Lopes et al., 2005; Ross et al., 2002), the origin of shrimp was taken into consideration. This origin considered as "environmental history" by De Kock and Kramer (1994) may cause a variable response in MT induction (Damiens et al., 2006). The goal of this study was to evaluate the acute toxicity and sublethal effects (assessed through MT and LPO assay) of Cd in two populations of *P. argentinus* with different environmental histories, one from an unpolluted area (Nahuel Ruca lagoon) and the other from an area impacted with heavy metals (Los Padres lagoon).

2. Materials and methods

2.1. Sample sites

Shrimp were obtained from two, shallow lagoons with similar physicochemical characteristics, Nahuel Ruca (37°37′S-57°25′W) and Los Padres (37°57′S-57°44′W) lagoons, situated in the southeastern area of Buenos Aires Province, Argentina. They are typical lagoons of the Pampas's wetlands characterized by Quirós et al. (2002) as shallow lakes without stratification and naturally eutrophic. Their waters are characterized by low salinity, an alkaline pH and hard waters (Chiodi Boudet et al., 2008, 2010; Ituarte, 2008).

The lagoons are differentiated by the level of contamination. Los Padres lagoon (LP) is surrounded by horticultural fields of economic importance to the area (Osterrieth, 2002). These practices have been conducted for nearly 30 years (Osterrieth, 2002) and, coupled with the intensive use of fertilizers, are the primary cause of heavy metals in sediments and biota (Chiodi Boudet et al., 2008). Sediments of LP have high metal concentrations (0.7 μg $Cd \cdot g^{-1}$; 1 μg $Hg \cdot g^{-1}$; 72 μg $Cr \cdot g^{-1}$; 15.3 μg $As \cdot g^{-1}$, 119 μg $Zn \cdot g^{-1}$; Chiodi Boudet et al., 2008) that exceed levels considered as safe for the biota (Cd: 0.6 $\mu g \cdot g^{-1}$, Hg: 0.17 $\mu g \cdot g^{-1}$, Cr: 37.3 $\mu g \cdot g^{-1}$, As: 5.9 $\mu g \cdot g^{-1}$, Zn: 123 $\mu g \cdot g^{-1}$; CEQGS, 2002). In contrast, the Nahuel Ruca lagoon (NR), located within a biosphere reserve within the Program for Man and Biosfere (MAB, UNESCO) (Martínez, 2001), is considered an unpolluted area. Sediments of NR have low metal concentrations (0.15 μg $Cd \cdot g^{-1}$; 0.02 μg $Hg \cdot g^{-1}$; 28 μg $Zn \cdot g^{-1}$; Chiodi Boudet et al., 2010) which make this area a good reference site.

2.2. Collection and maintenance of organisms

The animals were cared for in accordance with guidelines of the Institutional Committee for Care and Use of Laboratory Animals of Mar del Plata University (CICUAL) based on the "Guide for the Care and Use of laboratory Animals" (2010, 8th Edition, National Research Council, The National Academies Press, Washington DC) and Directive 2010/63/UE of the European Parliament and of the Council on the protection of animals used for scientific purposes.

Shrimp (adults of both sexes at sexual rest) were collected with a hand net and transported to the laboratory. Acclimation was performed in 140 L aquaria with gently aerated freshwater and 12:12 h light/dark photoperiod for 3 days as recommended for genus *Palaemonetes* by Buikema et al. (1980). Water temperature was maintained at 17 ± 0.9 °C, pH at 8.3 ± 0.05 and water hardness was 235 mg $CaCO_3 \cdot L^{-1}$. Shrimp were fed Tetramin® flake food daily. The Cd content of the food was < 0.05 μg $Cd \cdot g^{-1}$. During acclimation, those groups that had more than two percent mortality were not used for the experiments following the criteria establish by Khan et al. (1988).

2.3. Reagents

The stock solution of Cd (613 mg $Cd \cdot L^{-1}$) was prepared from cadmium chloride ($\geq$ 99.99 percent, Sigma-Aldrich Chemical Corporation USA) and double distilled water (ddH_2O). The different cadmium concentrations assayed were prepared using a dilution series of the stock solution. The analytical Cd concentrations of each treatment at the beginning of the experiment were measured by anodic stripping voltammetry (ASV) (Andrade et al., 2006) with a detection limit <50 ppb. Cadmium standard (1000 mg Cd, $CdCl_2$ in H_2O, Titrisol® Merck) was used as a standard.

2.4. Acute toxicity test

Experimental conditions for the acute test (96-h) were based on Buikema et al. (1980) recommendations. We used acute static tests with water renewal every 48-h. Shrimp were exposed to the following nominal concentrations of Cd: 3.06, 12.26, 30.66, 61.32, 306 and 613.2 μg $Cd \cdot L^{-1}$. Before this stage, we performed a preliminary test to evaluate potential exposure concentrations. Each concentration was tested in triplicate with 100 individuals from each location in a 20 L, aerated glass aquarium along with controls. The shrimp were not fed during the experiments. The shrimp were checked daily and dead individuals were counted and removed. The absence of response to gentle mechanical stimulus was the criterion for death.

Median lethal concentrations (LC_{50}) at 24, 48, 72 and 96-h were determined using the trimmed Spearman–Karber method (Hamilton et al., 1977). The safe concentrations (SC) of Cd were determined by the method of Miller and Miller (1986) using the application factor 1/100th of the 96-h LC_{50} value. After the 96-h assays, all live shrimp were collected for subsequent analysis of biomarkers.

2.5. Seasonal variation in MT and LPO

To establish the profile of seasonal variation of MT and LPO levels, the collections were initiated in September 2010 and extended until August 2011. The seasons were defined as spring (September, October and November), summer (December, January and February), fall (March, April and May) and winter (June, July and August). Adult shrimp were captured in each of the four seasons and the hepatopancreas ($n=200$) were dissected *in situ*, frozen in liquid nitrogen and stored at -80 °C. Temperature, pH and hardness of water were measured *in situ*.

2.6. 24-h Bioassays

Assays were conducted to evaluate the biomarkers response on shrimps exposed to sublethal concentrations of Cd (based on acute toxicity test results). Shrimps ($n=200$) were exposed to 3.06, 12.26, 30.66, 61.32 μg $Cd \cdot L^{-1}$ for 24 h. The bioassays were conducted under similar conditions to that of acute toxicity test. Shrimp hepatopancreas were immediately, frozen in liquid nitrogen and stored at -80 °C.

2.7. Metallothionein assay and lipid peroxidation

The MT assay was performed according to the spectrometric method described by Viarengo et al. (1997). The absorbance was read at 412 nm, and MT concentration was quantified using reduced glutathione (GSH) as a reference standard. The amount of MT was calculated based on cysteine content in *Palaemonetes pugio* (17 cysteines, GenBank accession no. AY935987), assuming a similar SH group content in *P. argentinus* MT. The MT concentration was reported as μg of MT per gram of wet tissue.

Total lipid peroxidation (LPO) was measured according to Oakes and Van Der Kraak (2003) using the formation of thiobarbituric acid reactive substances (TBARS). Fluorescence was measured by excitation at 515 nm with an emission peak at 553 nm. The concentration was expressed as nmols TBARS per gram of tissue (wet weight), which was calculated from the fluorescence at 553 nm using tetramethoxypropane (TMP) as external standard.

2.8. Statistical analysis

All statistical analyses were performed using STATISTICA version 8.0 (Statsoft, Inc.). Significant differences were assessed by parametric tests: *t*-test and analysis of variance (ANOVA) followed by the post-hoc Tukey test, and non-parametric tests: *U*-Mann–Whitney and Kruskall–Wallis with post-hoc Dunn test; being previously checked the variance homogeneity by Levene's test (Zar, 1984). The significance level was $p<0.05$.

3. Results

For all the assays the analytical values of Cd in solution were between 94 and 99 percent of the nominal values for the concentration range between 12.26 and 613.2 μg Cd·L^{-1}. The effective concentrations (mean ± standard deviation, $n=3$) for the five solutions assayed (12.26, 30.66, 61.32, 306 and 613.2) were 11.64 ± 0.8, 29.81 ± 0.9, 58.84 ± 1.2, 304.03 ± 1.9, 604.87 ± 3.2 μg Cd·L^{-1}, respectively. The lowest concentration (3.06 μg Cd·L^{-1}) was not included in the analysis because it was below the detection limit of the method.

3.1. Acute toxicity test

The survival for control and 3.06 μg Cd·L^{-1} treatments at 96-h was 100 percent (Fig. 1). For 12.26 μg Cd·L^{-1}, the survival of NR shrimp began to decrease from 48-h of exposure, declining to 50 percent at 96-h, while for LP it was 94 percent. For 30.66 and 61.32 μg Cd·L^{-1}, the survival of NR shrimp was <50 percent after 48-h, while survival of LP shrimp was >50 percent. At the end of the experiment, survival rates for NR shrimp were 7 and 0 percent for 30.66 and 61.32 μg Cd·L^{-1} treatments, respectively, while for LP shrimp they were 22 and 6, respectively. Finally, for 306.0 and 613.2 μg Cd·L^{-1} was <40 percent at 24-h, reaching values close to zero percent at 48-h for both NR and LP shrimp.

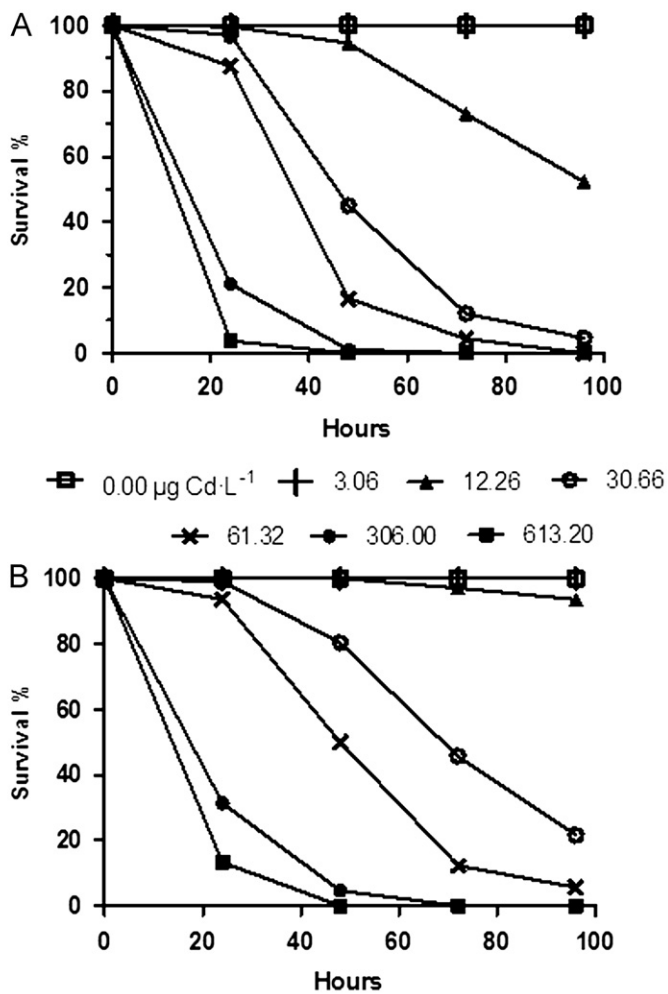

Fig. 1. Survival curves for *P. argentinus* from Nahuel Ruca (A) and Los Padres (B) lagoons exposed to Cd during 96 h.

Table 1
LC$_{50}$ values (μg Cd·L^{-1}) for 24, 48, 72 and 96 h with 95 percent confidence intervals (CI) of *Palaemonetes argentines* from Nahuel Ruca (NR) and Los Padres (LP) lagoons.

Hours	NR		LP	
	LC$_{50}$	CI	LC$_{50}$	CI
24	153.3	122.6–208.5	269.80	141.0–380.2
48	32.65	24.50–36.80	67.45	55.15–85.80
72	18.40	12.26–18.40	30.66	24.50–36.80
96	12.26	12.26–12.26	24.50	18.40–24.50

For concentrations >306.0 μg Cd·L^{-1} for 24-h exposure, lethargy (i.e., reduced swimming activity) and melanised gills were observed in shrimp from both locations, although these anomalies were a merely observation. No molting was observed during the 96-h study period.

Median lethal concentrations for LP shrimp were significantly higher (*t* test, $p<0.05$) than those for NR shrimp for all exposure times except for 24-h (Table 1). The safe concentration (SC) values of NR and LP shrimp were 0.123 and 0.245 μg·L^{-1}, respectively.

3.2. Seasonal variations of MT and LPO

Values of pH showed a very low range of variation in both lagoons and through the year, ranging from 8.9 to 9.2 in NR and 8.4–9.8 in LP. The temperature showed a seasonal variation from 8.1 °C (winter) to 26.6 °C (summer) in NR and from 8.0 °C (winter) to 23.2° (summer) in LP. The range values for water hardness were from 150.0 to 244.5 mg $CaCO_3$·L^{-1} in NR and from 172.3 to 242.3 mg $CaCO_3$·L^{-1} in LP.

The MT levels in hepatopancreas of shrimp collected from NR and LP lagoons showed seasonal variation (Fig. 2). In NR, the minimum MT values were observed in fall and winter, increasing in spring and reaching significant (ANOVA with post-hoc Tukey test, $p<0.05$) maximum values in summer. In LP, MT levels were maximum in fall and decreased significantly (ANOVA with post-hoc Tukey test, $p<0.05$) during winter–spring period, then began to increase in summer. When comparing MT levels during the year for both lagoons, spring–summer levels in shrimp from NR were significantly (*t* tests, $p<0.05$) higher than those of LP. In contrast, LP levels in fall were significantly (*t* test, $p<0.05$) higher than those of NR. In winter, no differences were found.

The LPO seasonal pattern was also different between lagoons. A gradual decrease from summer to spring was observed in NR. In contrast, LPO levels were higher during summer-fall period and lower in winter–spring for LP. No differences were observed in LPO values between lagoons in winter and spring, although in summer and fall the levels were significantly higher in NR shrimps (*t* tests, $p<0.05$).

3.3. 24-h Bioassays

Shrimp from both lagoons showed a differential MT response during 24-h exposure (Fig. 3). In NR, 3.06, 12.26 and 30.66 μg Cd·L^{-1} treatments presented similar MT levels that were significantly (Kruskall–Wallis with post-hoc Dunn test, $p<0.05$) higher than those of controls, but the exposure to 61.32 μg Cd·L^{-1} presented similar levels as the control. In LP, MT levels were similar among treatments, even with those of control. The comparison between lagoons showed no

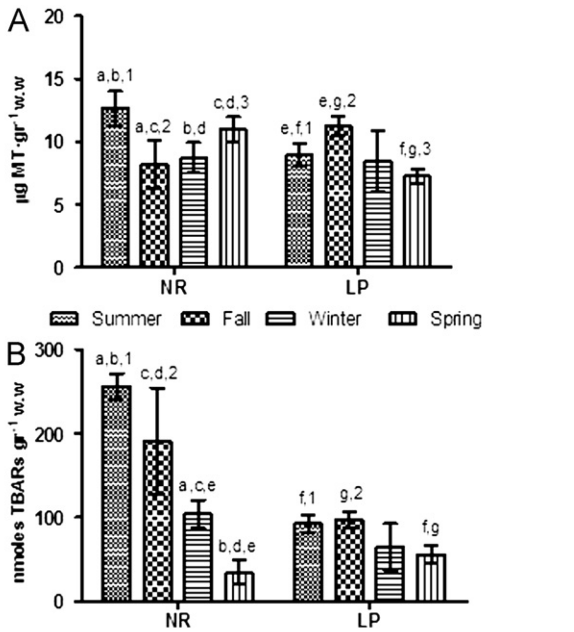

Fig. 2. Seasonal variations in metallothionein (MT) (A) and lipid peroxidation (LPO) (B) levels in hepatopancreas of *P. argentinus* from Nahuel Ruca (NR) and Los Padres (LP) lagoons. Values are mean $\pm$ SD. The significance was tested between seasons within a lagoon (bars with the same letter differ significantly at the 95 percent level) and between lagoons for a given season (bars with the same number differ significantly at the 95 percent level).

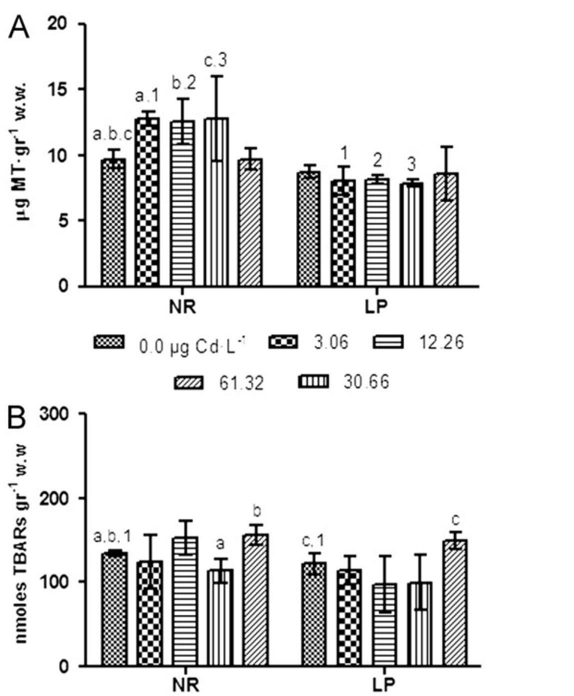

Fig. 3. Metallothionein (MT) (A) and lipid peroxidation (LPO) (B) levels in hepatopancreas of *P. argentinus* from Nahuel Ruca (NR) and Los Padres (LP) lagoons exposed to cadmium (Cd) during 24-h. Values are mean $\pm$ SD. The significance was tested between treatments within a lagoon (bars with the same letter differ significantly at the 95 percent level) and between lagoons for a given treatment (bars with the same number differ significantly at the 95 percent level).

differences for controls; but not for 3.06, 12.26 and 30.66 $\mu g\ Cd \cdot L^{-1}$ treatments that were significantly (*t* tests, $p < 0.05$) higher in NR.

LPO levels in NR showed a significant (*t* tests, $p < 0.05$) increase with respect to controls for 30.66 and 61.32 $\mu g\ Cd \cdot L^{-1}$, while in LP it was significantly elevated only for 61.32 $\mu g\ Cd \cdot L^{-1}$. No differences between lagoons were observed in LPO levels for each treatment, except in controls that were significantly (*U*-Mann–Whitney test, $p < 0.05$) higher for NR.

3.4. 96-h Bioassays

No shrimp survived exposure to 61.32, 306 and 613.2 $\mu g\ Cd \cdot L^{-1}$, so it was not possible be perform the analysis of MT and LPO. In addition, the low survival rate (< 10 percent) for 30.66 $\mu g\ Cd \cdot L^{-1}$ made statistical analysis difficult. The MT levels in NR for 3.06, 12.26 and 30.66 $\mu g\ Cd \cdot L^{-1}$ were significantly (*t* test, $p < 0.05$) higher than in controls and similar for the two locations (Fig. 4). In LP, MT levels found in all treatments did not differ from those of controls for both locations (*t* test, $p > 0.05$). The comparison between lagoons showed that control values of LP shrimp were significantly (*t* test, $p < 0.05$) higher than those of NR, but levels for treatments were higher (*t* test, $p < 0.05$) in NR shrimp.

For NR, the maximum value of LPO was found in 3.06 $\mu g\ Cd \cdot L^{-1}$, but no differences were found for other treatments and controls. In the case of LP, this biomarker was significantly (ANOVA with post-hoc Tukey test, $p < 0.05$) higher for 30.66 $\mu g\ Cd \cdot L^{-1}$ compared with 3.06, 12.26 $\mu g\ Cd \cdot L^{-1}$ and controls. The only treatment that showed significant (*t* test, $p < 0.05$) differences between lagoons was that of 30.66 $\mu g\ Cd \cdot L^{-1}$, being higher in LP shrimp. However, as mentioned, due to the low survival percentage of NR shrimps for 30.66 $\mu g\ Cd \cdot L^{-1}$, LPO values should not be considered representative.

4. Discussion

4.1. Acute toxicity

The toxicity of Cd to freshwater crustaceans is well documented, but not for *P. argentinus*. For example, the 96-h LC_{50} value of

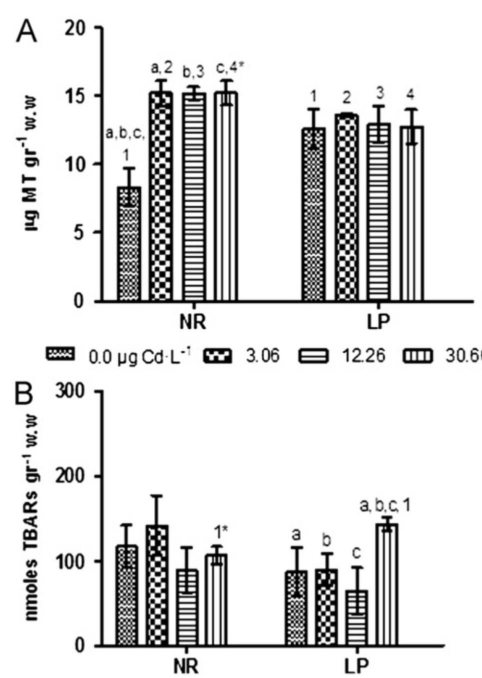

Fig. 4. Metallothionein (MT) (A) and lipid peroxidation (LPO) (B) levels in hepatopancreas of *P. argentinus* from Nahuel Ruca (NR) and Los Padres (LP) lagoons exposed to cadmium (Cd) during 96-h. Values are mean $\pm$ SD. * Survival below to 10 percent. The significance was tested between treatments within a lagoon (bars with the same letter differ significantly at the 95 percent level) and between lagoons for a given treatment (bars with the same number differ significantly at the 95 percent level).

Cd for adult *Macrobrachium nipponense* and *Macrobrachium rosenbergii* (freshwater decapods) have been reported as 53.9 and 74 μg $Cd \cdot L^{-1}$ (Kaoud and Eldahshan, 2010; Yang et al., 2008). Likewise, the 96-h LC_{50} of Cd for adult *Hyalella azteca* and *Hyalella curvispina* (freshwater amphipods) are 8.0 and 29.99 μg $Cd \cdot L^{-1}$ (García et al., 2010; Nebeker et al., 1986) and are considered the most sensitive freshwater crustaceans to Cd. Despite the great variability in LC_{50} for freshwater crustaceans, all of them are sensitive to Cd. The LC_{50} for *P. argentinus* from NR and LP populations are similar to the lowest values mentioned above, indicating high sensitivity to Cd.

As expected, the LC_{50} decreased with increased exposure time. Our results expand the knowledge about the sensitivity of *P. argentinus* to contaminants, highlighting the response of the species to a very toxic and ubiquitous pollutant as Cd. Thus, information on the toxicity of Cd could be used in the protection of aquatic life through the determination of safe concentration (Miller and Miller, 1986). In Argentina (Law No. 24051), the permissible level of Cd for the protection of freshwater aquatic life is 2 μg $Cd \cdot L^{-1}$ for water with a hardness of 60–120 $mg \cdot L^{-1}$ $CaCO_3$ (moderately hard water) and 0.2 μg $Cd \cdot L^{-1}$ for water with a hardness of 0–60 $mg \cdot L^{-1}$ $CaCO_3$ (soft water). Based on this regulation, the permissible level for both lagoons is 2 μg $Cd \cdot L^{-1}$. The SC values obtained for *P. argentinus* (0.123 and 0.245 μg $Cd \cdot L^{-1}$ for NR and LP, respectively), are below the maximum permissible level for aquatic life protection, suggesting that the level established up by Argentinian government is unsatisfactory. This finding highlights the importance of considering toxicological studies when setting environmental legislation.

The LP shrimp are more tolerant to Cd than those from NR. This higher tolerance (as measured by LC_{50}) may have resulted from pre-exposure to pollutants present in their natural environment. Sediments of LP have high metal concentrations (0.7 μg $Cd \cdot g^{-1}$; 1 μg $Hg \cdot g^{-1}$; 72 μg $Cr \cdot g^{-1}$; 15.3 μg $As \cdot g^{-1}$, 119 μg $Zn \cdot g^{-1}$; Chiodi Boudet et al., 2008) exceeding levels established as safe for the biota (Cd: 0.6 $\mu g \cdot g^{-1}$, Hg: 0.17 $\mu g \cdot g^{-1}$, Cr: 37.3 $\mu g \cdot g^{-1}$, As: 5.9 $\mu g \cdot g^{-1}$, Zn: 123 $\mu g \cdot g^{-1}$; CEQGS, 2002). In contrast, sediments from NR have low metal concentrations (0.15 μg $Cd \cdot g^{-1}$; 0.02 μg $Hg \cdot g^{-1}$; 28 μg $Zn \cdot g^{-1}$; Chiodi Boudet et al., 2010). Field and laboratory research has shown that, in some shrimp species (e.g., *Palaemon elegans* (Moraitou-Apostolopoulou et al., 1982) and *P. pugio* (Khan et al., 1988)), previous exposure to low concentrations of Cd can produce higher tolerance. Similarly, the larvae of *Crassostrea gigas* (bivalve mollusc species), naturally pre-exposed to Cd, Zn and Cu, presented high tolerance to Cu (Damiens et al., 2006). For LP population, the pre-exposure to a multimetal mixture may be the cause of the increased tolerance to Cd.

4.2. Seasonal variations of MT and LPO

Natural variation in biomarker levels must be understood before they can be used in the field (Mouneyrac et al., 2000). Different biotic and abiotic factors, including reproductive state, age, sex, temperature, salinity, and season may change levels of biochemical biomarkers whatever the contamination of the environment (Amiard et al., 2006). The influence of these factors on MT and LPO levels in crustaceans were reported by some authors (Dutra et al., 2008; Geffard et al., 2007; Legras et al., 2000). These natural variations may interfere with the estimation of levels induced by pollutants; therefore a seasonal sampling was conducted.

Seasonal variations were apparent for both biomarkers and the two populations of *P. argentinus*. MT levels in NR showed higher values in spring–summer and lower values in autumn–winter. The same pattern was reported for the marine crab *Pachygrapsus marmoratus* (Mouneyrac et al., 2001) and the freshwater amphipod *Gammarus pulex* (Geffard et al., 2007), which was related to the physiological condition, particularly with reproductive activity. These authors observed an increase in MT levels during the reproductive period. The breeding season of *P. argentinus* begins in September–October (spring) continues through February (end of summer) (Donatti, 1986), coincident with the highest values of MT. Therefore, the seasonal pattern of MT in NR appears related to the reproductive period, which in turn is linked to increased water temperatures (Donatti, 1986). In LP shrimps, the seasonal variation pattern was opposite to that observed in NR, and it was not consistent with other species. The life-history traits of *P. argentinus* populations from the Buenos Aires province were studied by Ituarte (2008), showing that shrimp (larvae and adults) from LP were: (1) smaller in size and weight, (2) the percentage of ovigerous females during the breeding season was low (<50 percent), (3) fertility was low, (4) there were fewer eggs and (5) egg loss was high (47 percent). Ituarte (2008) reported that the reproductive traits observed in this population suggest a particularly low level of fitness, probably reflecting unfavorable environmental conditions for the population dynamics. Kwok et al. (2009) reported that Cu resistance in a marine copepod species was associated with a fitness costs, exhibited by a lower fecundity and population growth rate. Therefore, contaminants present in LP lagoon could be the responsible for the poor body condition and reduced fecundity of the shrimp, which could also affect the natural variations of MT.

LPO levels showed a marked seasonality in the two populations, with higher values in summer-fall and lower in winter–spring, although this was more evident for NR. The same pattern was observed in both marine (*Aristeus antennatus* Antó et al., 2009), and freshwater (*Macrobrachium borellii* Lavarías et al., 2011) shrimp and the amphipod *H. curvispina* (Dutra et al., 2008). Dutra et al. (2008) attributed the increase levels of lipid peroxidation to the increase of light during summer months. Indeed, it was shown that UV radiation causes a rise of lipid peroxidation in *Daphnia longispina* (freshwater amphipod) under laboratory conditions (Vega and Pizarro, 2000). Moreover, Sroda and Cossu-Leguille (2011) observed a positive correlation between temperature and LPO levels in *Gammarus roeseli* (freshwater gammarid), which explained the high concentrations during warmer months (summer–autumn). As a result, the different water temperature between NR (26.6 °C) and LP (23.2 °C) during summer and the extended photoperiod may explain the difference between LPO levels of both lagoons.

In summary, due to the low impact of pollutants in the NR lagoon, other factors may change the levels of biomarkers. This reinforces the need to know the seasonality of MT and LPO to the properly interpret their presence.

4.3. 24-h and 96-h Assay

Once a toxic chemical enters an organism, several biochemical and physiological mechanisms attempt to counteract the toxic stress caused by the pollutant. Crustaceans do not have the ability to regulate the internal concentration of nonessential metals such as Cd (Rainbow, 1998). In caridean shrimp, all Cd taken up from solution is accumulated without excretion over at least a 28-day period (Rainbow and White, 1989), and the accumulated Cd is detoxified, generally as MT. The mechanism of cadmium tolerance is explained by induction of metallothionein, a cystein- rich cytoplasmic metal-binding protein. In general, the mechanism by which MTs is thought to play a role in Cd toxicity is through its ability to bind heavy metals and thereby render them biologically inactive (Klaassen, 2001). The MTs bind to Cd through its thiol groups (–SH) of cysteine, resulting in a high stable complex. This binding decreases the presence of free Cd and the chance to bind to macromolecules, reducing its toxicity.

In *P. argentinus* from NR, MT synthesis was induced with 24-h of exposure at the lowest dose of Cd, suggesting that the physiological response to Cd contamination is very fast. This was also observed in the amphipod *Echinogammarus echinosetosus*, in which an increase of MT content was observed after 24-h to 100 μg Cd·L^{-1} exposure (Martinez et al., 1996) or in *Artemia* spp under the same experimental conditions (Del Ramo et al., 1995). The increase of MT was time-dependent, although several studies reported that in crustaceans it was also concentration-dependent (Del Ramo et al., 1995; Wu and Chen, 2005; Pan and Zhang, 2006). The observed *plateau* at 24 and 96-h of exposure could indicate the maximum detoxification capacity of MTs. Moreover, the LPO levels did not increase, showing no oxidative damage caused by Cd. This could be evidence for the protective role of MT against oxidative damage. Similar results were reported for the shrimp *Chorocaris chacei* (Gonzalez-Rey et al., 2008) as well as the bivalves *Ruditapes decussatus* (Geret et al., 2003) and *C. gigas* (Damiens et al., 2006). Furthermore, the increase of LPO levels observed at 61.32 μg Cd·L^{-1} (24-h) coupled with the lack of MT induction for this treatment may support the protective role of those proteins. This lack of induction was related to the toxicity of Cd. Martinez et al. (1996) underlined the fact that MT induction in the amphipod *E. echinosetosus* was limited at the highest Cd dose tested (2000 μg/L). In another 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). The lack of induction in NR shrimps at maximum concentration could be the manifestation of this toxic effect.

In the case of shrimp from LP lagoon, a higher tolerance and low or absent oxidative stress suggest the presence of another mechanism in the detoxification of Cd. In invertebrates, two major mechanisms are involved in metal detoxification: metal binding proteins like MTs and insoluble metaliferous granules by biomineralisation (Amiard et al., 2006). The binding of metals by biomineralisation reduces the metallic free cations to induce MT (George and Olsson, 1994). As a result, is possible to observe high metal levels in crustaceans without an increase in MT levels (Barka et al., 2001; Mouneyrac et al., 2002; Pedersen et al., 1997). A correlation between metal rich granules production and increased tolerance has been observed in other species. In the oligochaete, *Limnodrilus hoffmeisteri*, no significant differences were found between non-resistant and Cd-resistant worms in the proportion of Cd bound to MT after 6 days of exposure to a high concentration of Cd (8.9 μM) (Klerks and Bartholomew, 1991). This resistance to Cd was ascribed to the formation of metal rich granules. The isopod *Asellus meridans* and the polychaete *Nereis diversicolor* from Cu-polluted environments had increased Cu-tolerance and produced Cu-rich granules (Brown, 1977; Bryan and Hummerstone, 1971). Biomineralisation explains the absence of MT induction in LP population, as well as their greater tolerance. However, their role in *P. argentinus* from LP will require further research.

5. Conclusions

The measurement of LC_{50} demonstrated that *P. argentinus* has a high sensitivity to Cd, being one of the most sensitive species of crustaceans. As a result, this species can be proposed as a good autochthonous bioindicator. In addition, the differential sensitivity observed between populations indicated that *P. argentinus* has the capacity to develop tolerance. Both MT and LPO are good early-warning tools of Cd exposure in the species. The environmental history of shrimp populations was reflected in their responses to Cd exposure and must be considered in risk assessment studies using natural populations of *P. argentinus*.

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