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Osmoregulation and tissue water regulation in the freshwater red crab *Dilocarcinus pagei* (Crustacea, Decapoda), and the effect of waterborne inorganic lead

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Abstract

Inorganic lead has been measured in high concentrations in certain streams in Brazil. This study has evaluated the osmoregulatory effects of lead on the native freshwater red crab *Dilocarcinus pagei*. In order to probe its osmoregulatory and tissue volume regulatory capabilities and how it would be affected by lead, the crab has been submitted to individual and combined salt and chemical stresses (Pb^{2+}). Male crabs were exposed for 10 days to either: (1) control (freshwater, FW), (2) brackish water of salinity 15 (BW), (3) inorganic lead in freshwater (FWPb), and (4) inorganic lead in brackish water (BWPb), 2.7 mgPb/L. *In vivo*, whole crabs lost weight transiently when exposed to Pb^{2+}, both in FW and in BW. Haemolymph osmolality and ion concentrations increased and remained elevated upon exposure of crabs to BW, with or without Pb^{2+}, showing a trend to hyper-conformation. *In vitro*, muscle weight decreased in isosmotic conditions upon exposure to Pb^{2+}. Na^+, Cl^-, and ninhydrin positive substances (NPS) were increased in muscle exposed to hyperosmotic saline, well above what would be expected from simple efflux of water, suggesting a partial regulatory volume increase (RVI) capacity. This partial RVI involves the Na^+, K^+, $2Cl^-$-cotransporter and the Na^+/H^+ exchanger on Na^+ and Cl^- uptake, as judged from further decreases in muscle weight in the presence of the respective inhibitors. A breakdown of proteins into NPS seems to follow the uptake of inorganic ions. Pb^{2+} has affected water and ion movements in *D. pagei* both in the whole animal and in the isolated tissue. This study has highlighted the relevance of evaluating tissue volume regulation in aquatic animals confronted with metal polluted waters.

Keywords: Amiloride; Cell volume regulation; DIDS; Furosemide; Osmotic and ionic regulation

1. Introduction

Osmoregulatory mechanisms are employed to counteract osmotic stresses and to maintain the differences between the extracellular medium and the external environment, providing an adequate osmotic environment for the cells. Changes in extracellular concentrations determine the gain or loss of cell water, which is reflected in change in weight (Kévers et al., 1979; Pierce, 1982; Deaton, 1990; Scemes et al., 1991; Souza and Scemes, 2000). This movement of water activates cell volume regulatory mechanisms. The processes leading to recovery of normal cell volume after either swelling or shrinking are known as regulatory volume decrease (RVD) or regulatory volume increase (RVI), respectively. Cell volume regulation can be a necessity not only upon exposure to anisosmotic media, but also as a result of cellular metabolism (Macknight et al., 1994; Hoffmann and Dunham, 1995; Lang et al., 1998; Russell, 2000).

Early studies considered that volume regulatory mechanisms only involved fluxes of inorganic ions. The participation of organic solutes was only later demonstrated (Péqueux et al., 1979; Deaton, 1990, 1994; Bishop et al., 1994; Hoffmann and Dunham, 1995; Neufeld and Wright, 1996; Lang and Waldegger, 1997; Lang et al., 1998). Among the transport systems activated after hyperosmotic stress and the subsequent RVI, are the Na^+–K^+–$2Cl^-$ cotransporter and the Na^+/H^+ and Cl^-/HCO_3^- exchangers, generating Na^+, K^+ and Cl^- uptake. The uptake of organic solutes may or may not depend upon Na^+ fluxes (Hoffmann and Dunham, 1995; Lang et al., 1998; Wehner et al., 2003). The intracellular concentration of aminoacids nor-

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mally increase upon hyperosmotic stress, to aid in RVI, as a result of protein hydrolysis and/or uptake (Péqueux, 1995).

Besides eventual extracellular osmotic disturbances resulting from variations in the external media, aquatic animals have to cope with a great variety of chemical substances released into their habitat (Péqueux, 1995; Ahern and Morris, 1998, 1999). Metals such as lead (Pb^{2+}) have been measured in high concentrations in streams in northern Paraná, Brazil. Some areas near automotive battery factories exhibit Pb^{2+} levels as high as 4.5 mg/L (Yabe and Oliveira, 1998). Physiological responses elicited in crustaceans by sublethal effects of heavy metals present in freshwater bodies include reduction in ion uptake by the branchial epithelium (Péqueux, 1995; Hebel et al., 1997; Ahern and Morris, 1998). There is also evidence of metals penetrating into crustacean organs, thus exerting their effects on internal tissues as well (Hebel et al., 1997; Burgos and Rainbow, 1998; Engel et al., 2001). Since the internalized metal can reach all tissues of the animal, besides its specific effect in particular organs, it may also affect tissue volume regulation in general. Although the effect of heavy metals on several behavioral, biochemical, physiological parameters of crustaceans have been reported, including their integration into an "holistic approach" (Hebel et al., 1997), their effect on cell volume regulation has not been analyzed before. The combined effect of hyperosmotic stress with that of a heavy metal such as lead (Pb^{2+}) has not been reported in the literature for freshwater crustaceans. By presenting an osmotic challenge to the animal, one can better assay the volume regulatory capacity of its cells, which is actually a fundamental cellular capacity related to dealing with various stresses (Péqueux, 1995).

This study has investigated the osmoregulatory capability of the freshwater red crab *Dilocarcinus pagei*, which is endemic to the Amazon and Paraguai/Paraná river basins of South America (Magalhães, 1991). This species can be found in contaminated Paraná streams (Souza, M.M., unpublished observations); in addition to the contamination by metals, these waters also exhibit variable levels of conductivity (from 90.0 to 260.0 $\mu S\,cm^{-1}$) as a reflection of industrial and domestic effluents. This situation means that a possible synergistic interaction between chemical and "saline" stressors on animal physiology may occur, and warrants further investigation (Péqueux, 1995). Thus, the questions considered in this study were: (1) Does Pb^{2+} affect red crab osmoregulation, and its tissue volume regulatory capabilities? (2) Is the crab's muscle tissue able to perform RVI under hyperosmotic shock? (3) If so, what are the pathways responsible for that RVI?

2. Materials and methods

2.1. Animals

D. pagei Stimpson, 1861 (Brachyura, Trichodactylidae) is a freshwater crustacean species commonly known as the red crab. Intermoult male crabs (average 18 g) were collected, at night, in northern Paraná State, Brazil (23°16′S and 51°03′W) and were brought to the laboratory at the State University of Londrina. In the laboratory, animals were kept in tanks (140 L) of aerated artesian well water ~10 cm deep, around 6 L (conductivity 90–110 $\mu S\,cm^{-1}$). They were fed twice a week with tiny pieces of fish or ground beef. The laboratory was kept at 20–25 °C (room temperature) with a 14-h light:10-h dark photoperiod. The animals were kept in these conditions for at least 4 days, to acclimate prior to the experiments.

2.2. In vivo experiments

In order to assess the osmoregulatory capacity of the red crab under hyperosmotic stress, crabs were exposed for 10 days, in individual containers of 3 L capacity, each filled with 300 mL of aerated water. This volume was enough to cover one animal and guarantee that they would be under water. Four different conditions have been tested: (1) artesian well water (freshwater, FW); (2) artesian well water + $Pb(NO_3)_2$, FWPb; (3) artesian well water + marine salt (15 g/L, salinity 15), BW; (4) artesian well water + marine salt (15 g/L, salinity 15) + $Pb(NO_3)_2$, BWPb. Evaporated marine salt was obtained from a local aquarium shop. The concentration of lead used (5 mg $Pb(NO_3)_2$/L) yielded 2.7 mg Pb^{2+}/L or 13 μM in the water, measured by atomic absorption spectrometry. This concentration was based on lead determinations in rivers of northern Paraná state (Yabe and Oliveira, 1998). Seven crabs were used for each experimental group. The salinity of the brackish water used was based on experiments by Augusto and McNamara (personal communication), which demonstrated that *D. pagei* could endure this salt concentration. During the whole experimental time of 10 days, the water of the individual vials was changed once after 5 days, and the crabs were fed each 3 days.

Before exposure to the different experimental conditions, crabs were weighed and a haemolymph sample (50–100 μL) was withdrawn from the arthrodial membrane. Weighing and haemolymph sampling was repeated after 24 h, and again every 24 h, until 10 days of exposure were completed. Temperature and pH of the water were measured daily during the experiments. Haemolymph osmolality (mOsm/kg H_2O) was measured by a vapor pressure osmometer (VAPRO 5520 Wescor, Logan, USA). Na^+ and K^+ concentrations were determined by flame photometry (Analiser 900, São Paulo, Brazil), after samples had been diluted in distilled water, using NaCl or KCl as standards. Cl^- was measured using a colorimetric commercially available kit (Labtest, Lagoa Santa, Brazil). Cl^- ions react with mercury thiocyanate in the presence of ferric nitrate, and absorbance is read at 470 nm (1203 UV Schimadzu Spectrophotometer, Kyoto, Japan).

2.3. In vitro experiment

The specific conditions of the *in vitro* experiment were chosen based on the *in vivo* results for the ions in the haemolymph (mean increase of 40% of the measured ions). The control saline for the *in vitro* analysis was isosmotic to FW-acclimated crab haemolymph, as described by Onken and Mcnamara (2002). It contained (in mM) NaCl 200; KCl 5; $NaHCO_3$ 2; $CaCl_2$ 10; Hepes acid and Na^+ salt 5; dextrose 5. Measured osmolality was 362 mOsm/kg H_2O. The experimental hyperosmotic saline

contained 140% of the control levels of NaCl, KCl and $NaHCO_3$ (531 mOsm/kg H_2O of measured osmolality). For the *in vitro* assays the claw extensor muscle was removed from the claw and allowed to rest for 2 h at 4 °C.

2.4. Volume estimate

In order to estimate muscle volume change under hyperosmotic shock, the wet weight of the muscle was recorded every 15 min during 2 h of exposure. Muscle slices (~80 mg) were at first incubated for 15 min in control saline and then carefully blotted on filter paper, weighed to 1 mg precision, (Gehaka BG-200, Curitiba, Brazil), and immersed in the hyperosmotic saline. The accuracy of the weighing method was evaluated with muscle kept in isosmotic saline, following the same protocol as that for the slices in experimental salines. To assess a direct effect of Pb^{2+} on cell volume regulation, as an indicator of membrane permeability change, muscle slices were also submitted to isosmotic saline in the presence of lead [$Pb(NO_3)_2$, 5 mg/L, 2.7 mg Pb^{2+}/L or 13 μM].

2.5. Inorganic and organic osmolyte contents

Two experimental times were chosen for the determination of muscle tissue osmolyte content, 60 and 90 min of exposure to hyperosmotic saline. In the experiment using saline with Pb^{2+}, sampling for tissue content determination occurred at 60 min. These times were defined after the results from the volume change experiment (Figs. 3A and 4A, respectively). At these chosen times, muscle slices were weighed and then processed for either ionic content or determination of ninhydrin positive substances (NPS). For the determination of ionic content, muscle slices were dried for 24 h at 60 °C, and exposed to HNO_3 0.75N for another 24 h for ionic extraction. After centrifugation (5000 × *g*, 5 min), the supernatant was restored at −20 °C to the ions analysis, and the pellet received NaOH 1.0N for protein solubilization, so that ions could be expressed as a ratio to mg of total protein. Concentrations of Na^+, K^+ and Cl^- were measured as described for the haemolymph, and protein concentrations were measured by the colorimetric method of Lowry et al. (1951). The method consists of the copper-treated protein reaction with Folin reagent in pH 10. Ninhydrin-positive substances (NPS) were measured according to Clark (1968), adapted here for smaller samples. The method measures the total ninhydrin-positive nitrogen, in citrate buffer. No labeling of extracellular space was performed here, thus some of the measured ions or NPS may not be intracellular. This leads to a certain error, but this error was systematic. Therefore, only percentual variations were provided in the Section 3. Although absolute magnitudes might be unreal, the direction of change was not.

2.6. Analysis of inorganic osmolyte pathways

Blockers of ionic transporters were employed in the volume evaluation assay, in order to investigate the role of each transporter in RVI, upon hyperosmotic stress *in vitro*. The choice of the blockers was according to the results of variation in Na^+, K^+, and Cl^-, in muscle ionic content from the *in vitro* experiments with the isolated muscle (Fig. 4B). Furosemide (100 μM, inhibits Na^+–K^+–$2Cl^-$ cotransporter) and amiloride (100 μM, inhibits the Na^+/H^+ cation exchanger) were obtained from Neoquímica (Goiás, Brazil); DIDS (2,2′-disulphidric acid 4,4′-diisothiocyanostilbene, 50 and 100 μM, inhibits the Cl^-/HCO_3^- anion exchanger) was obtained from Sigma (St. Louis, USA). Tissue slices were preincubated for 15 min with the inhibitor in control isosmotic FW saline, before being exposed to the hyperosmotic saline with the inhibitor, in order to ensure that they would bind to the transporters; and also to evaluate if this transporter could be involved in volume maintenance in isosmotic environments.

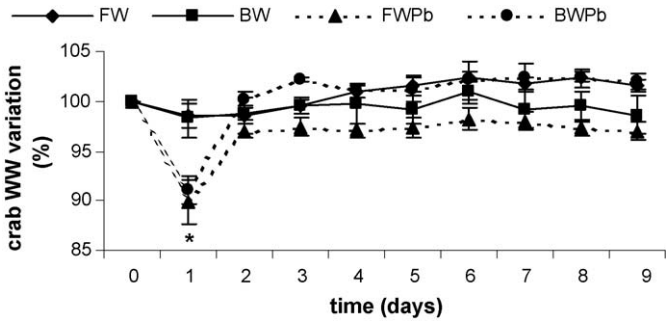

Fig. 1. Wet weight variation of animals, with days of exposure to control or experimental conditions: freshwater (FW), brackish water (BW), both with or without the addition of lead (Pb). $^*p < 0.05$, $n = 3$–7.

2.7. Statistical analysis

Data are presented as mean ± standard error of the mean. Groups were compared using one way analysis of variance within treatment groups (*in vivo* results), with the post hoc test of Student–Newman–Keuls, SNK), or through Student's *t*-tests when comparing with a control group, whenever appropriate, always with *p* of 0.05. *In vivo* results (Figs. 1 and 2) would have been more appropriately analyzed using repeated measures two-way ANOVAs. However, this was attempted, but data failed for normality and no transformation of data was successful in generating a normal distribution, preventing us from using this statistical approach.

3. Results

3.1. In vivo experiment

Exposure of the crabs to both solutions with lead resulted in a significant decrease of 10% of total body weight after 1 day ($p < 0.05$). No change in weight was observed upon exposure to brackish water (BW) alone (Fig. 1). Exposure of the crabs to BW in the presence of lead (BWPb) resulted in mortality: four animals died (three at 48 h and the fourth after 120 h), reducing the sample size from seven to three.

There was a significant increase in osmolality after 1 day of exposure to BW and BWPb, $p < 0.05$ (Fig. 2A). Similarly,

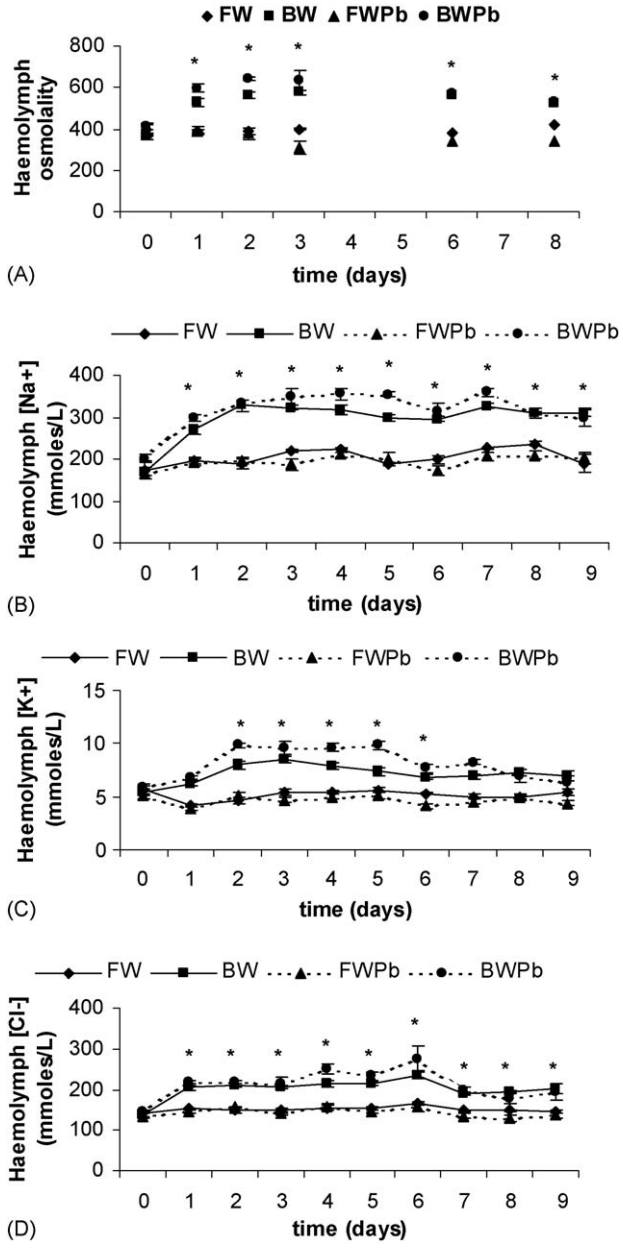

Fig. 2. Osmolality (mOsm/kg H_2O) (A) and ionic concentrations (mM) Na^+ (B), Cl^- (C), K^+ (D) in the haemolymph of crabs exposed for 10 days to control or experimental conditions: freshwater (FW); brackish water (BW), both with or without the addition of lead (Pb). $^*p < 0.05$, $n = 3$–7. Measured osmolality and ion concentrations in the water were: FW: osmolality 41 mOsm/kg H_2O, Na^+ 0.06 mM, Cl^- 0.28 mM, and K^+ 0.04 mM. BW: osmolality 391 mOsm/kg H_2O, Na^+ 260 mM, Cl^- 216 mM, and K^+ 4.8 mM.

haemolymph Na^+, Cl^- and K^+ were elevated during exposure to BW and BWPb exposures. No change was detected when Pb^{2+} was added to FW (Fig. 2B–D). Na^+ and Cl^- were significantly increased after 1 day of exposure to BW and BWPb ($p < 0.05$), but K^+ only after the second day: Na^+ by ~60%, and Cl^- and K^+ by ~50% (Fig. 2B–D). K^+ concentration in haemolymph returned to control levels after 6 days, $p > 0.05$ (Fig. 2C).

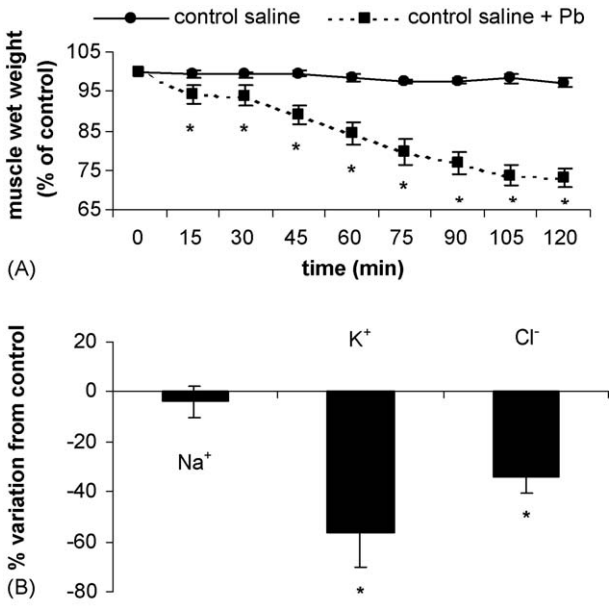

Fig. 3. Time course of muscle tissue wet weight (A, % of initial weight) during exposure to control saline or control saline with lead (+Pb), when compared to the time zero. $^*p < 0.05$, $n = 7$. Percent variation in the ionic content (B) of muscle tissue submitted to control saline with lead (for 60 min), when compared to the ionic content in the absence of lead, in the control saline. $^*p < 0.05$, $n = 4$–10.

3.2. In vitro experiment

In order to test for the effect of the observed haemolymph changes on cell osmoionic regulation, slices of claw opener muscle were directly exposed to osmotic and chemical stresses. No

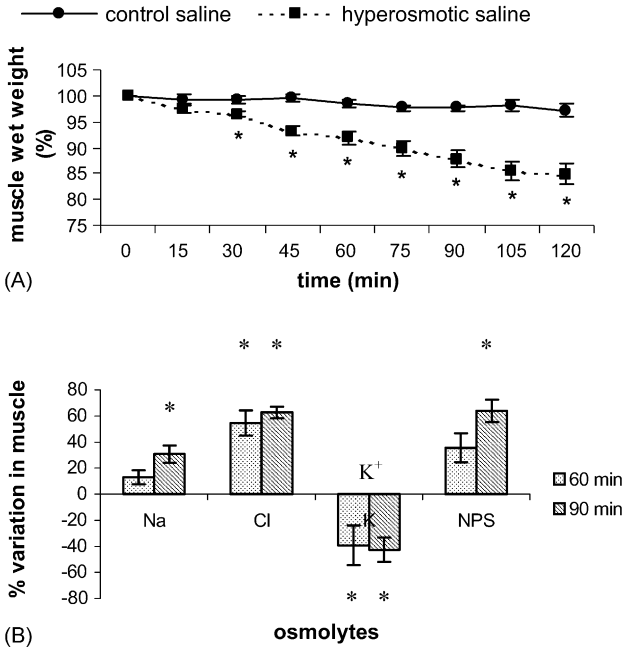

Fig. 4. Time course of muscle tissue wet weight (A, % of initial weight) during exposure to control saline or hyperosmotic saline, when compared to the time zero. $^*p < 0.05$, $n = 7$. Percent variation in the osmolyte content (B) of muscle tissue submitted to hyperosmotic saline (for 60 and 90 min), when compared to the osmolyte content in the control saline. $^*p < 0.05$, $n = 5$–10.

change in muscle volume was detected during 120 min of exposure to control saline, $p > 0.05$ (Fig. 3A).

3.3. Analysis of the effect of lead on cell permeability

The *in vivo* results of crab body weight have indicated that the addition of Pb^{2+} to fresh water (FWPb) led to significant crab weight reduction without change in haemolymph ionic concentrations, $p < 0.05$ (Figs. 1 and 2). Consistently, muscle slices displayed weight reduction in the presence of Pb^{2+}, with a significant fall of $27.0 \pm 2.3\%$ after 120 min of exposure to control saline + Pb, $p < 0.05$ (Fig. 3A). Muscle K^+ and Cl^- content also showed a significant decrease respectively of ~50% and 30% after 1 h of exposure, $p < 0.05$ (Fig. 3B).

3.4. Exposure to hyperosmotic saline

During the exposure to hyperosmotic saline, muscle slices significantly lost around 15% in weight ($p < 0.05$; Fig. 4A). After 90 min in hyperosmotic saline, Cl^- significantly increased by 62% (from 1140 ± 100 nmoles/mg protein to 1761 ± 171 nmoles/mg protein, $n = 8$–9, $p < 0.05$) and Na^+ by 23% (from 1383 ± 107 nmoles/mg protein to 1808 ± 171 nmoles/mg protein, $n = 7$–8; $p < 0.05$; Fig. 4B). On the contrary, K^+ significantly decreased 42% (from 137.3 ± 21.9 nmoles/mg protein to 78.7 ± 7.4 nmoles/mg protein, $p < 0.05$). At the same time, ninhydrin positive substances (NPS) showed an significantly increase of 49% of exposure to hyperosmotic saline (from 362.2 ± 33.2 μg/g wet weight to 539.9 ± 50.9 μg/g wet weight (Fig. 4B), when compared to control values, $p < 0.05$. Values after 60 min showed the same trends. However, only the Cl^- and K^+ concentrations were significantly different from control values, $p < 0.05$ (Fig. 4B).

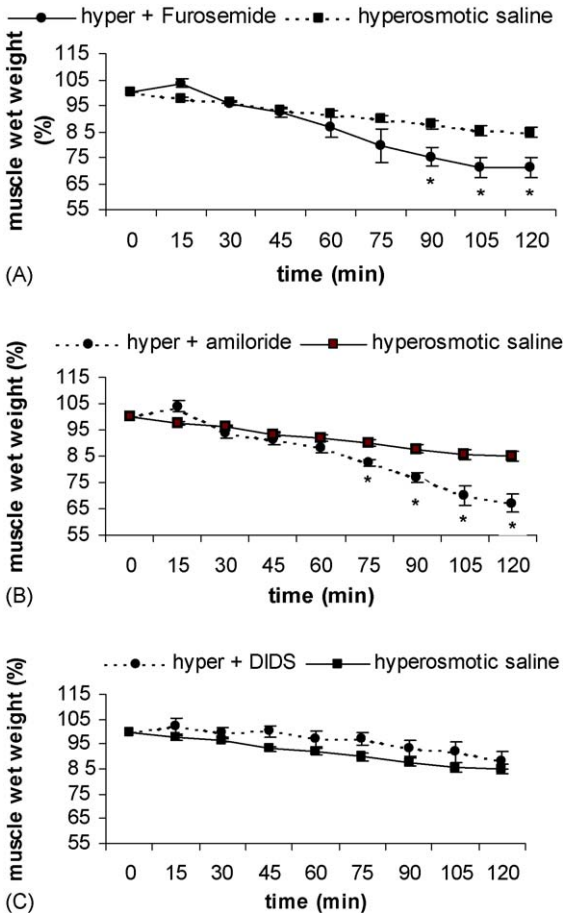

Fig. 5. Time course of muscle tissue wet weight (in percentage of initial weight) upon exposure to hyperosmotic saline with or without ionic transporters blockers: (A) furosemide 100 μM; (B) amiloride 100 μM and (C) DIDS 50 μM. $^*p < 0.05$, $n = 4$–8.

3.5. Analysis of the pathway for inorganic osmolytes

When crab muscle slices were exposed to hyperosmotic saline with the addition of furosemide (100 μM) there was a significantly larger decrease in weight ($p < 0.05$), which is 15–20% more than that observed during the exposure to the hyperosmotic saline alone, reaching a 30–35% decrease, Fig. 5A). The same pattern was observed when amiloride (100 μM) was added to the hyperosmotic saline with a 30–35% weight reduction, by 120 min, which was significantly different from the control, $p < 0.05$ (Fig. 5B). On the other hand, when DIDS (50 and 100 μM, 100 μM is not shown) was added to the hyperosmotic saline, no difference was observed from the weight reduction already observed with the hyperosmotic saline alone (Fig. 5C).

4. Discussion

Haemolymph concentrations of Na^+, K^+, Cl^-, and accordingly, osmolality, have increased upon exposure of the freshwater crab *D. pagei* to raised salinity, either with or without Pb^{2+}, compatible with hyper-conforming behavior in brackish water (BW) of salinity 15 (Fig. 2). The presence of Pb^{2+} in control fresh water (FW) medium did not affect the crab ionic and osmotic homeostasis, as judged from the haemolymph concentrations measured under this experimental condition. The lack of effect of Pb^{2+} on haemolymph ionic concentrations (also in BW) is not the most common response (reviews in Péqueux, 1995; Hebel et al., 1997). However, this has been reported before, for the FW crayfish *Cherax destructor* (Ahern and Morris, 1998). Even though these authors reported no change in haemolymph osmolality and sodium, they have paradoxically detected a decrease in Na^+ influx and a reduction of branchial Na^+/K^+-ATPase activity upon Pb^{2+} contamination (21 days, 0.5 mg Pb^{2+}/L) (Ahern and Morris, 1998). This apparent paradox could also apply to *D. pagei*, exposed to 2.7 mg Pb^{2+}/L. Unfortunately, this issue will not be possibly solved with the present results, and will have to await future studies, with measurements of ion fluxes and Na, K-ATPase activities. In *D. pagei*, haemolymph concentrations may not be changed by Pb^{2+}, simply because ion and water fluxes are hampered to the same magnitude, as possibly happened to *C. destructor* (Ahern and Morris, 1998). Additionally, Pb^{2+} did not affect the expected raised salt entry into the haemolymph of *D. pagei* in brackish water, BW (Fig. 2). The addition of Pb^{2+} to either FW or BW led to a transient ~10% whole animal weight reduction after 1 day (Fig. 1). There are at least two possible explanations for this finding. And both explanations are

not mutually exclusive. The first explanation would be with Pb^{2+} acting as a potent diuretic, as reported for mammalian kidneys (review in Loghman-Adham, 1997). The second explanation would be a transient reduced rate of osmotic water movement through the permeable surfaces in FW (that is, reduced apparent water permeability to AWP). If this was accompanied by an associated reduced salt uptake, the haemolymph concentrations might not change in FW. Indeed, Pb^{2+} was the most potent heavy metal in promoting reduction in apparent water permeability in the green crab *Carcinus maenas*, even after only 1 hour of exposure (Rasmussen et al., 1995). Furthermore, the effect of reducing AWP produced by zinc was also transient in the shrimp *Crangon crangon*, occurring after 1–3 days of exposure, but with values returning to normal after 6 days (Rasmussen et al., 1995). This transient effect could be a direct effect on water fluxes, but could also be an indirect effect, involving cardiorespiratory mechanisms. Nevertheless, an opposite effect of lead on water permeability has also been reported. Astrocytes exposed to Pb^{2+} show a significant (40%) increase in water permeability through AQP4 (Gunnarson et al., 2005). The indirect effect through cardiac and respiratory functions could be part of a physiological compensatory response to the metal (Rasmussen et al., 1995), with reduction in gill perfusion rates, or cardiac arrest (review in Hebel et al., 1997). In BW of salinity 15 (∼450 mOsm/kg H_2O), the crab is still hyperosmotic to the medium, though only slightly (haemolymph of ∼600 mOsm/kg H_2O). Thus, in BW, even with a putative transient reduction in gill perfusion rates caused by the presence of lead, there would still be an increased salt entry because of the increased salt content of the medium (haemolymph concentrations increase, Fig. 2). The putative diuretic action of Pb^{2+} in crustacean antennal glands proposed above would actually also be consistent with this last hypothesis of reduced water entry into the crab. Both actions of lead might be occurring at the same time: reduction in apparent water permeability, and increased diuresis. From the widely reported major role of antennal glands in extracellular volume regulation (e.g., Schoffeniels and Dandrifosse, 1994; Péqueux, 1995; Lin et al., 2000), it is not unreasonable to predict that a reduction in haemolymph pressure (reflected in 10% of weight reduction) will lead to a reduction in urine formation and output after a few more days of exposure to Pb^{2+} in both salinities. In addition, when simply exposed to BW without the disturbing effects of Pb^{2+}, the reduction in water entry from the reduced gradient seems to immediately generate decreased urine output, as there was no reduction in the crab weight. It is important to add, *D. pagei* does not produce dilute urine in FW. Its urine is isosmotic (∼380 mOsm/kg H_2O) to the haemolymph (Augusto, 2005). Decapods have a more complex circulation compared to other crustaceans, and develop sufficiently high haemolymph pressure as to drive pressure filtration through the filtration slits of the coelomosac podocytes (Icely and Nott, 1979; McGaw, 2005). This fact lends support to the contention of the action of Pb^{2+} as diuretic, as in the mammalian kidney (Loghman-Adham, 1997).

Inorganic lead could be affecting apparent gill water permeability, either directly or through its effect on gill hemodynamics, and could also be affecting ion uptake mechanisms, as discussed above. *In vitro*, Pb^{2+} added to the control isosmotic saline resulted in water loss, concomitant with KCl loss (Fig. 3). The latter could be due to inhibition of the Na^+/K^+ pump. Heavy metals are known to inhibit the activity of the Na^+/K^+ pump (Péqueux, 1995—mercury; Hebel et al., 1997—copper; Postel et al., 1998—cadmium; Ahern and Morris, 1998—lead). Also, red blood cells, from fish to humans, display K^+ efflux activated by Pb^{2+} (Corchs et al., 2001; Silkin et al., 2001). Pb^{2+} would thus have both whole-organism effects, as well as cellular effects, affecting ion and water fluxes through the cellular membranes. Such an effect on cellular volume regulation would naturally ensue upon Pb^{2+} internalization in impacted water bodies (Hebel et al., 1997; Engel et al., 2001; Lorenzon et al., 2001).

The haemolymph changes detected in the *in vivo* experiments demonstrated that *D. pagei* deals with an increase in ambient salinity from FW to salinity 15 through osmotic and ionic conformation, thus transferring the osmotic challenge to its cells. The *in vitro* experiments were thus performed in order to investigate the cellular volume capabilities of the crab muscle cells. The hyperosmotic saline prepared for the exposure of the muscle slices was 40% more concentrated than the freshwater control saline (isosmotic to the FW crab), in accordance with the results from the *in vivo* experiments. Upon exposure to hyperosmotic saline in the *in vitro* experiments, muscle weight progressively decreased, reaching a 15% reduction after 120 min (Fig. 4). The magnitude of the reduction was less than would be expected from an osmometric behavior of the cells, in the absence of any volume regulatory capacity. The osmometric behavior expected from Van't Hoff's law would mean $\pi_1/\pi_2 = 362/531 = 0.68$, from π_1, control saline (362 mOsm/kg H_2O) and π_2, hyperosmotic saline (531 mOsm/kg H_2O), or 32% in volume reduction. The smaller than expected volume reduction (from weight reduction) indicates some activation of mechanisms to act in RVI. However, the tissue loses weight/water and does not recover entirely. Other cell types in other organisms have been shown to display partial or complete RVI, such as cells of the Malpighian tubule of *Rhodnius neglectus* (Arenstein et al., 1995). Some mammalian cells do not display complete volume regulation after shrinking, such as mammalian cardiomyocytes (Deaton, 1997) and most of mammalian renal epithelial cells (Sun and Hebert, 1994). In crustaceans, axons of the green crab *Carcinus maenas* were unable to perform a partial RVI (Kévers et al., 1979).

This partial capacity to perform RVI avoiding steeper reduction in tissue volume in crab myocytes is also evidenced by the increased muscle tissue levels of Na^+, Cl^-, and NPS when in hyperosmotic saline (Fig. 4). The increase in these inorganic and organic osmolytes was higher (30–60% after 90 min) than would be expected from simple concentration derived from water loss (∼12%) (Fig. 4). Increase in muscle NPS when the animal is in medium of higher salinity has been widely reported in crustaceans (Péqueux, 1995), as for example, in the freshwater Malaysian giant prawn *Macrobrachium rosenbergii* (Tan and Choong, 1981), and in the postlarvae of the marine shrimp *Penaeus aztecus* (Bishop and Burton, 1993). The reduction in tissue levels of K^+, however, was surprising. This ion would be expected to have its concentration increased from water loss in hyperosmotic saline. This reduction in tissue K^+ could well

be the result of reduced activity of the Na, K-ATPase to prevent further loss of solutes (Whalley et al., 1993; Caruso-Neves and Lopes, 2000). A putative inhibition of the activity of the Na^+/K^+-ATPase could be at least the partial explanation in both situations: in the presence of lead, and when the muscle was submitted to hyperosmotic shock. The increase in tissue NPS somewhat later (90 min × 60 min) than the increase in tissue Cl^- would be expected, since the increase in the organic osmolytes signifies a breakdown of cellular proteins, as the saline bath did not contain amino acids. It is more likely that there is breakdown of proteins than *de novo* synthesis of amino acids; the relevant literature shows that cell shrinkage stimulates proteolysis and inhibits protein synthesis (Waldegger et al., 1997; Lang et al., 1998).

The pathways for Na^+ and Cl^- entry during RVI into muscle cells of *D. pagei* have been investigated using inhibitors of ion transporters. With the addition of furosemide and amiloride, respectively inhibiting the Na^+–K^+–$2Cl^-$ cotransporter and the Na^+/H^+ exchanger, the tissue has lost more water than the control tissue in hyperosmotic saline (Fig. 5). The result indicates participation of these transporters in solute uptake for partial RVI. Additionally, amiloride may be inhibiting Na^+ channels (Na^+ channels of the A6 kidney cell culture blocked by amiloride 50 μM, Cucu et al., 2003). In general, the Na^+/H^+ exchanger operates coupled with the anion exchanger Cl^-/HCO_3^- for intracellular pH homeostasis (Hoffmann and Dunham, 1995; Lang et al., 1998; Wehner et al., 2003). However, the anion exchanger inhibitor DIDS did not affect muscle wet weight when compared to values in hyperosmotic saline alone. Na^+–K^+–$2Cl^-$ is actually known for participating in general volume regulation in animal cells, generating net flux of solutes in either direction with respect to the cell membrane, depending on the electrochemical gradients for its three transported ions (Russell, 2000). The cation exchanger actually does not promote net solute flux, so its participation is somewhat more indirect, and probably coupled to another transporter, not investigated here, such as for example, the K^+/H^+ exchanger, leading to K^+ efflux according to its expected electrochemical gradient, preserving intracellular pH from significant changes, thus maybe also contributing to the observed tissue K^+ decrease. This transporter is discussed as activated in coelenterate cells during RVD (La Spada et al., 1999) but its involvement during RVI is not known. The response of *D. pagei* muscle tissue to increase in extracellular medium osmolality seems to be of partial RVI involving NaCl uptake, followed by NPS formation. The mobilization of organic osmolytes following an inorganic phase seems to be a metabolically parsimonious strategy, already described in other cells types under anisosmotic challenge (Neufeld and Wright, 1996; Souza et al., 2000).

In conclusion, the questions posed in the Introduction have been answered: inorganic lead has been shown to affect the osmo-ionic homeostasis of the FW crab, at least transiently in the first 24 h of exposure, through a modification of water/ion fluxes and/or diuretic action. Lead also caused K^+ and water efflux in muscle cells placed in isosmotic saline, affecting cell volume in isosmotic conditions. It could be demonstrated that muscle cells of this FW crab display a certain degree of RVI upon volume loss in hyperosmotic medium, through NaCl uptake at least partially via the Na^+, K^+, $2Cl^-$ and Na^+/H^+ transporters, and further through the generation of NPS. This study has shown how important it is not only to demonstrate the whole animal effects of waterborne metals, but also their direct effects on cells and tissues, when internalized by the animals. Additionally, the demonstration of the pathways for solute movement in cell volume regulation is an important step in the understanding of the specific targets of action of pollutants such as heavy metals on aquatic animals. It is also relevant to remark that, although *D. pagei* would never naturally face an osmotic shock of salinity 15 such as that provided here, it retains a significant capacity to deal with salt in the water, inherited from its seawater ancestry.

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