Fish Physiol Biochem (2014) 40:645–657
DOI 10.1007/s10695-013-9873-8

Lead accumulation and its effects on the branchial physiology of *Prochilodus lineatus*

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Received: 21 June 2013 / Accepted: 30 September 2013 / Published online: 11 October 2013

Abstract The purpose of this work was to determine the tissue accumulation of lead (Pb) and its effects on osmoregulatory processes of the freshwater fish *Prochilodus lineatus*. Juvenile fish were exposed to Pb (from 1.7 to 0.7 mg of dissolved Pb L^{-1}) for 6, 24 and 96 h and Pb accumulation was analyzed in the gills, liver, kidneys, blood cells and muscle. The following parameters were also analyzed: hematologic (hemoglobin content, hematocrit and number of red blood cells), metabolic (blood glucose), endocrine (blood cortisol), osmo ionic (plasma osmolality and Na^+, K^+, Cl^- and Ca^{+2} concentrations), gill enzymes (Na^+/K^+-ATPase and carbonic anhydrase), chloride cell (CC) density and CC location in the gills. Pb accumulated in all the analyzed tissues, with the kidneys showing the highest concentration, followed by the gills and liver. The lowest Pb concentrations were found in blood cells and muscle. Pb promoted an increase in blood glucose after 6 and 24 h exposure. Gill Na^+/K^+-ATPase was inhibited after 24 h of exposure, but its activity was restored at 96 h, probably due to the increase in CC in gill lamellae. Plasma Na^+ was reduced after 6 and 96 h, while K^+ concentrations increased at all the experimental times. Fish exposed to Pb showed reduced plasma Ca^{+2} at all experimental periods. Hematologic parameters remained unchanged. Overall, this study demonstrated that Pb interferes in osmoregulatory processes of *P. lineatus* and the proliferation of CC in the gills is a response in order to reestablish adequate ion concentrations.

Keywords Bioaccumulation · Blood glucose · Chloride cells · Hematology · Na^+/K^+-ATPase · Plasma ions

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Introduction

Lead (Pb) is a non-biodegradable element that is not essential to the functions of organisms and there are no efficient pathways of metabolization and elimination, thus its bioaccumulation in different organs and tissues of fishes exposed to Pb is a common occurrence (Peakall and Burger 2003). The concentration of metal in each tissue varies mainly as a function of the species exposed (Roach et al. 2008), the metal species (Vinodhini and Narayanan 2008) and exposure time (Grosell et al. 2006). In many fish species, the liver and the kidney are usually targets for the retention of large amounts of metals due to their high metabolic activity

and the production of metallothioneins, which bind to metal ions (Cicik et al. 2004). High metal accumulation can also occur in the gills, due to their close contact with the environment (Ay et al. 1999; Rogers et al. 2003; Vinodhini and Narayanan 2008). In addition to the functions of respiration, acid-base regulation and excretion of nitrogenous wastes, the gills of freshwater fishes must actively take up ions from the water in order to maintain a favorable internal environment for homeostasis, but Pb accumulation in this organ may impair its function (Rogers et al. 2003).

Osmoregulation is particularly impaired in the presence of metals, as a consequence of the inhibition of the enzymes Na^+/K^+-ATPase (NKA) (Ahern and Morris 1998; Atli and Canli 2007; Ay et al. 1999; Rogers et al. 2003) and Ca^+-ATPase (Rogers and Wood 2004), which results in changes in internal ion concentrations. Some metals are also able to interfere in the function of carbonic anhydrase (CA) (Morgan et al. 1997; Rogers et al. 2005; Skaggs and Henry 2002) and may therefore indirectly hinder the uptake of Na^+ and Cl^- (Evans 1987). Calcium levels may also undergo changes in the presence of metals, such as lead (MacDonald et al. 2002; Rogers and Wood 2004), which is described as calcium antagonists and uses calcium uptake pathway to enter the animal.

Since the presence of metals, such as lead, interferes on the concentration of different plasma ions, the organism has to counteract these ionic disturbances. One way to reestablish the normal concentration of ions is to increase their uptake, and the production of a new population of cells that work toward this goal is a possible approach to increase the ion influx rate (Perry 1997). Circumstances that expose freshwater fishes to stressful situations stimulate the production of cortisol (Mancera and McCormick 2007). The main functions of this hormone are the regulation of ion uptake and of the energetic balance (Ramesh et al. 2009), which are situations commonly found in the presence of a stressor. Upon the release of this hormone, the organism responds by multiplying the chloride cells (CCs), also known as mitochondria-rich cells, in the gills in order to optimize its osmoregulatory processes and return to its normal levels of plasma ions (Mancera and McCormick 2007; Ramesh et al. 2009). The CCs are usually inserted in filaments, and their occurrence in the lamellae is infrequent. However, in certain environmental conditions, CCs are more commonly found in the lamellar epithelium (Camargo et al. 2009). In freshwater fishes, cell migration from the filament to the lamellae may result from the need to increase ion uptake (Hwang and Lee 2007).

In this context, studies aimed at identifying the mode of action of lead on the physiology of freshwater teleosts are highly relevant, with emphasis on the processes of ion uptake through the gills. Extensive research has focused on determining how lead affects the uptake of ions such as calcium, sodium and chloride in the rainbow trout (*Oncorhynchus mykiss*) (Rogers et al. 2003, 2005; Rogers and Wood 2004). Similarly, more studies of the same parameters are needed for other freshwater fishes. In a previous work, Martinez et al. (2004) showed that the Neotropical freshwater fish *Prochilodus lineatus* acutely exposed to lead presented histopathological gill lesions and temporary disturbances in sodium regulation. Thus, the goal of the present research was to better understand the effects of lead (Pb) on the branchial physiology of *P. lineatus* and also to determine Pb accumulation in different tissues of this fish. This species was chosen because it represents a fish commonly found in rivers of the south and southeast regions of Brazil and it is sensitive to a variety of pollutants (Camargo et al. 2009; Nascimento et al. 2012; Simonato et al. 2008).

Materials and methods

Juveniles of *P. lineatus* (Valenciennes 1847) ($n = 116$) weighing 7.6 ± 2 g and measuring 8.8 ± 0.8 cm in length (mean $\pm$ SD) were supplied by the fish hatchery station of the State University of Londrina, Paraná, Brazil. Fish were acclimated in laboratory for at least 5 days, in 300-L tank with dechlorinated water, constant aeration and a photoperiod of 10 h light:14 h dark. The chemical and physical characteristics of the water were monitored continuously (T: 23.6 ± 0.5 °C; pH: 7.4 ± 0.1; dissolved oxygen: 7.4 ± 0.3 mg O_2 L^{-1}; conductivity: 336.4 ± 6.8 μS cm^{-1}; hardness: 41.3 ± 5.5 mg $CaCO_3$ L^{-1}). During acclimation, animals were fed with commercial fish food with 36 % protein every 2 days, and the feeding was suspended 24 h before the beginning and during the toxicity tests.

After acclimation, groups of fish were transferred to glass aquaria (100 L) filled with 80 L of dechlorinated water (control group–CTR) or water containing lead in

a nominal concentration of 3 mg Pb L^{-1} (experimental group–Pb). Lead was added to the water as $Pb(NO_3)_2$ (Vetec, Brazil) and this concentration was chosen based on lead determinations in rivers of northern Paraná state (Yabe and Oliveira 1998). Eight to ten fish were placed in each aquarium, in order to keep the maximum density at 1 g of fish per liter of water. Acute static toxicity tests were performed for each experimental period (6, 24 and 96 h) in independent experiments and for each exposure time CTR and Pb groups were run simultaneously.

The water temperature, pH, dissolved oxygen and conductivity of each aquarium were monitored throughout the tests using a multiparameter water quality meter (Hanna HI9828, USA) and remained stable (mean ± SE) 23.65 ± 0.33 °C; 7.40 ± 0.10; 7.17 ± 0.23 mg O_2 L^{-1} and 63.5 ± 0.83 µS cm^{-1}, respectively. Water hardness was determined by the EDTA titrimetric method and the values remained stable at 41.3 ± 5.5 mg $CaCO_3$ L^{-1}. Water samples were collected from the CTR aquaria at the end of each experiment (6, 24 and 96 h) and from the Pb aquaria after 6, 24, 48, 72 and 96 h, for lead determination. The concentration of total Pb was determined in samples of non-filtered water and the concentration of dissolved Pb was determined in water samples filtered through a 0.45-µm syringe filter (Millipore Millex HV/PVDF); for both analyses, samples were acidified by addition of HNO_3 and stored at 4 °C until metal determination.

At the end of each experimental period, the fish were anesthetized with benzocaine diluted in water (0.1 g L^{-1}) and blood was withdrawn from the caudal vein using a syringe rinsed with heparin. The fish were then killed by medullar section to remove the gills, liver, kidney and muscle. The branchial arches were processed for lead accumulation, immunohistochemical assay against Na^+/K^+-ATPase and determination of Na^+/K^+-ATPase and CA activity. Liver, kidney and muscle samples were stored dry at −80 °C. After sampling, the blood was centrifuged (1,870*g*, 10 min), the blood cells were stored at −80 °C for lead accumulation analysis, and the plasma samples were frozen (−20 °C) for osmolality, glucose, cortisol and ions (Na^+, K^+, Cl^- and Ca^{2+}) analyses.

Determination of lead

The organs and the blood cells were digested at 60 °C for 48 h in 1 N HNO_3 (Suprapur, Merck), centrifuged (3,600*g*, 20 min) and the supernatant was used for Pb measurement. Water acidified samples and tissue digests were analyzed for Pb using graphite furnace atomic absorption spectrophotometry (AAnalyst 700, Perkin Elmer, USA) against a reference Pb standard solution (Specsol, Brazil). Tissue concentrations are represented as mg Pb per wet weight of tissue (mg Pb g wet $weight^{-1}$) and water concentrations as mg Pb per water volume (mg Pb L^{-1}).

Hematologic parameters

Blood samples were used to analyze hematocrit (Htc), by centrifugation in glass capillaries and hemoglobin content (Hb), by the cyanmethemoglobin method in a spectrophotometer (540 nm, Libra S32, Biochrom, UK) at 540 nm, using a commercial kit (Labtest Diagnóstica, Brazil). The number of red blood cells per cubic millimeter of blood (RBC) was counted on an improved Neubauer hemocytometer using blood samples fixed in formol citrate.

Blood glucose and cortisol

Plasma glucose was determined by the glucose oxidase method, using a commercial kit (Labtest Diagnóstica, Brazil), at 505 nm, in a multilabel plate reader (Victor 3, Perkin Elmer, USA). Plasma cortisol was determined by immunoassay using a commercial kit (Diagnostic Systems Laboratories Inc., USA), at 450 nm, in a microplate reader (450 nm, ELX 800, BioTek, USA).

Osmolality and ion concentrations (Na^+, K^+, Cl^- and Ca^{2+})

Osmolality was measured with a freezing point osmometer (Osmomat 030, Gonotec, Germany). Plasma sodium and potassium were analyzed in samples diluted in deionized water, using a flame photometer (900, Analyser, Brazil). Chloride concentration was determined by the mercury thiocyanate method at 470 nm in a multilabel plate reader (Victor 3, Perkin Elmer, USA) using a commercial kit (Labtest, Brazil). Calcium ion was analyzed in diluted plasma samples, using lanthanum oxide as modifier, by flame atomic absorption spectrophotometry (AAnalyst 700, Perkin Elmer, USA).

Enzyme analyses: Na^+/K^+-ATPase and carbonic anhydrase

After removing the gills, the arches were washed, stored in SEI buffer (sucrose 0.3 M, Na_2EDTA 0.1 mM, imidazole 0.03 M, β-mercaptoethanol 10 mM, pH 7.4) and frozen (−20 °C). The branchial filaments were separated, homogenized in SEI buffer and centrifuged (7,500*g*, 15 min, 4 °C). The supernatant was employed to measure the NKA activity according to Quabius et al. (1997). Enzyme activity was measured indirectly through the production of inorganic phosphate from the breakdown of ATP, in samples incubated with a buffer solution (NaCl 100 mM, $MgCl_2$ 8 mM, imidazole 30 mM, EDTA 0.1 mM, ATP 3 mM, pH 7.6) containing KCl (5 mM) or ouabain (2.5 mM). A 650 µM phosphorus solution (Sigma-Aldrich, USA) was used as standard. The assays were performed in a microplate reader (620 nm, ELX 800, BioTek, USA).

For the determination of the CA activity, the branchial filaments were stored in a freezer (−20 °C), thawed, homogenized with a buffer solution (mannitol 225 mM, sucrose 75 mM, TRIS 10 mM, NaH_2PO_4 10 mM, pH 7.4), and centrifuged (7,500*g*, 15 min, 4 °C). A sample of the supernatant was added to the same buffer and a solution of CO_2-saturated water. The decrease in pH was monitored during 20 s. The method was established by Vitale et al. (1999) and is based on the catalysis of a CO_2-saturated solution with a corresponding release of H^+, and decrease in pH as result. The protein concentration was quantified in the gill homogenates according to Lowry et al. (1951), using bovine serum albumin as standard.

Immunohistochemical analysis: chloride cells

A branchial arch was fixed with Bouin's solution for 6 h and stored in alcohol 70 % until the beginning of histological processing. Alcohol dehydration was performed with an increasing series of ethanol solutions, followed by diaphanization in xylol and impregnation with paraffin. The blocks containing a branchial arch were cut sagitally (8 µm thick) and 8–10 slices were placed on each slide. The immunohistochemical technique was then applied to mark the CCs, using anti-NKA antibodies, as described by Dang et al. (2000a). The slides were incubated with mouse monoclonal antibody for NKA (IgGα5, Development Studies Hybridoma Bank, University of Iowa, USA) and with goat anti-mouse IgG peroxidase-conjugated antibody (GAMPO, Sigma). Following dehydration and slide mounting, the CCs were stained with tris-buffered saline (0.5 M Tris-base, pH 7.4) containing diaminobenzidine (DAB-Ni) and hydrogen peroxide.

The CC count was performed in an optical microscope (DM2500, Leica, Germany), using an image analyzing software (Leica Qwin, Germany). For each fish, all the CCs in five random filaments were counted, differentiating them by their location (filament or lamella). The results were expressed as the number of CC per millimeter of filament or lamella.

Statistical analysis

Considering that the toxicity tests for each exposure period (6, 24 and 96 h) were run independently, the results of the biochemical and physiological parameters of the control and respective treated group, for each exposure period, were compared using Student's *t* test or nonparametric Mann–Whitney test, depending on data distribution (normality and homogeneity of variances). As lead concentrations in tissues of control fish, after each period of exposure, did not vary significantly, only one control value (mean value obtained from the control fish for the three periods of exposure) was calculated for each tissue. The lead concentrations in each tissue were compared among control and different periods of exposure by one-way analysis of variance (one-way ANOVA) or the Kruskal-Wallis test, according to the distribution of the data (normality and homogeneity of variance). Values of $P < 0.05$ were considered statistically different. Data are presented as mean ±1 standard error of the mean (SE).

Results

The mean Pb concentration found in experimental aquaria was 2.24 mg L^{-1} of total Pb and 1.18 mg L^{-1} of dissolved Pb, but this concentration varied throughout the experiment (Table 1). Total Pb, which started at 3.35 mg L^{-1}, declined to 42 % of the initial concentration in 96 h. Similarly, dissolved Pb, which started at 1.69 mg L^{-1}, dropped to 41 % of the initial concentration in 96 h. The mean dissolved Pb concentration was approximately 53 % of the total Pb concentration. In the control aquaria, the total Pb concentration did not exceed 0.13 mg L^{-1}.

Table 1 Concentrations of total and dissolved lead (mg L^{-1}) in the water from control (CTR) and experimental (Pb) aquaria, measured at each experimental period (6, 24 and 96 h) and at intermediate periods (48 and 72 h) only in the water from experimental aquaria (Pb)

Time (h)	CTR		Pb	
	Total	Dissolved	Total	Dissolved
6	0.13	nd	3.35	1.69
24	0.08	0.03	2.51	1.77
48	–	–	2.52	0.97
72	–	–	1.43	0.80
96	nd	nd	1.43	0.70

nd not detected

Tissue lead accumulation

The results clearly showed Pb accumulation in all the analyzed tissues of the experimental fish (Fig. 1) as Pb concentrations were significantly higher from their respective control at all exposure times ($P < 0.001$). However, this accumulation occurred distinctly in the different tissues. The pattern of Pb burden in *P. lineatus* tissues after acute exposure was independent of time and occurred in the following decreasing order: Kidney > Gills > Liver > Blood > Muscle (Fig. 1). Muscle was the tissue presenting the lowest Pb concentrations, which remain between 0.15 and 0.22 μg Pb g $tissue^{-1}$ (Fig. 1a). Blood cells and liver presented a gradual accumulation of metal over time. The mean values of Pb in blood cells started at 0.64 ± 0.1 μg Pb g $tissue^{-1}$ and reached about four times this value in 96 h (Fig. 1b). In the liver, metal accumulation increased significantly along the experimental periods, varying from 1.98 ± 0.5 μg Pb g $tissue^{-1}$after 6 h to almost five times this value after 96 h of exposure (Fig. 1c). In contrast to the increase found in the liver, Pb concentration in the gills increased only up to 24 h, when it reached 27.5 ± 3.9 μg Pb g $tissue^{-1}$, fivefold higher than that found after 6 h (Fig. 1d). The highest Pb accumulation (110 ± 18.2 μg Pb g $tissue^{-1}$) was found in the kidneys after 96 h of exposure to the metal, i.e., fourfold higher than that found after 24 h, and eightfold higher than that found after 6 h (Fig. 1e).

Hematologic parameters

The hemoglobin content in blood, the number of red blood cells (RBC) and the hematocrit of fish exposed to Pb did not show any significant changes during the entire experimental period. The results of the hematologic parameters are shown in Table 2.

Metabolic and endocrine parameters

Plasma glucose concentration was significantly higher in fish exposed to Pb for 6 h (78 % increase, $P < 0.001$) and 24 h (80 % increase, $P = 0.026$) than in those exposed only to water, but returned to the initial levels after 96 h (Fig. 2a). Plasma cortisol concentration did not show significant changes in fish exposed to Pb, at any of the experimental periods (Fig. 2b).

Osmo-ionic parameters

Pb exposure caused a significant decrease in plasma osmolality after 6 h of exposure ($P < 0.001$) (Fig. 3a). Significant decreases in the plasma sodium concentration were also observed after 6 h ($P < 0.001$) and 96 h ($P = 0.0021$) (Fig. 3b) and in chloride after 24 h ($P = 0.038$) (Fig. 3c). In contrast, the potassium concentrations were significantly augmented after all the exposure times (6 h: $P = 0.015$, 24 h: $P < 0.001$, 96 h: $P = 0.021$) (Fig. 3d). Plasma calcium concentration of fish exposed to Pb decreased at all the experimental periods when compared with that of the control fish (6 h: $P = 0.007$, 24 h: $P < 0.001$, 96 h: $P = 0.02$) (Fig. 3e).

Enzyme parameters

After 24 h of exposure to Pb, gill NKA activity was significantly lower ($P = 0.017$) than in respective control (Fig. 4a). After 96 h this enzyme showed a total recovery of its activity in fish exposed to Pb. In contrast, CA activity was not significantly affected by the presence of Pb in water at any of the exposure times analyzed, although it exhibited a tendency to decline at all the experimental periods (Fig. 4b).

Cellular parameters

In normal conditions, the CCs in the gills of *P. lineatus* are typically located in the interlamellar region of the filaments, rarely occurring in the lamellae (Fig. 5a). The fish exposed to Pb presented an unusual occurrence of CCs in the branchial lamellae (Fig. 5b and

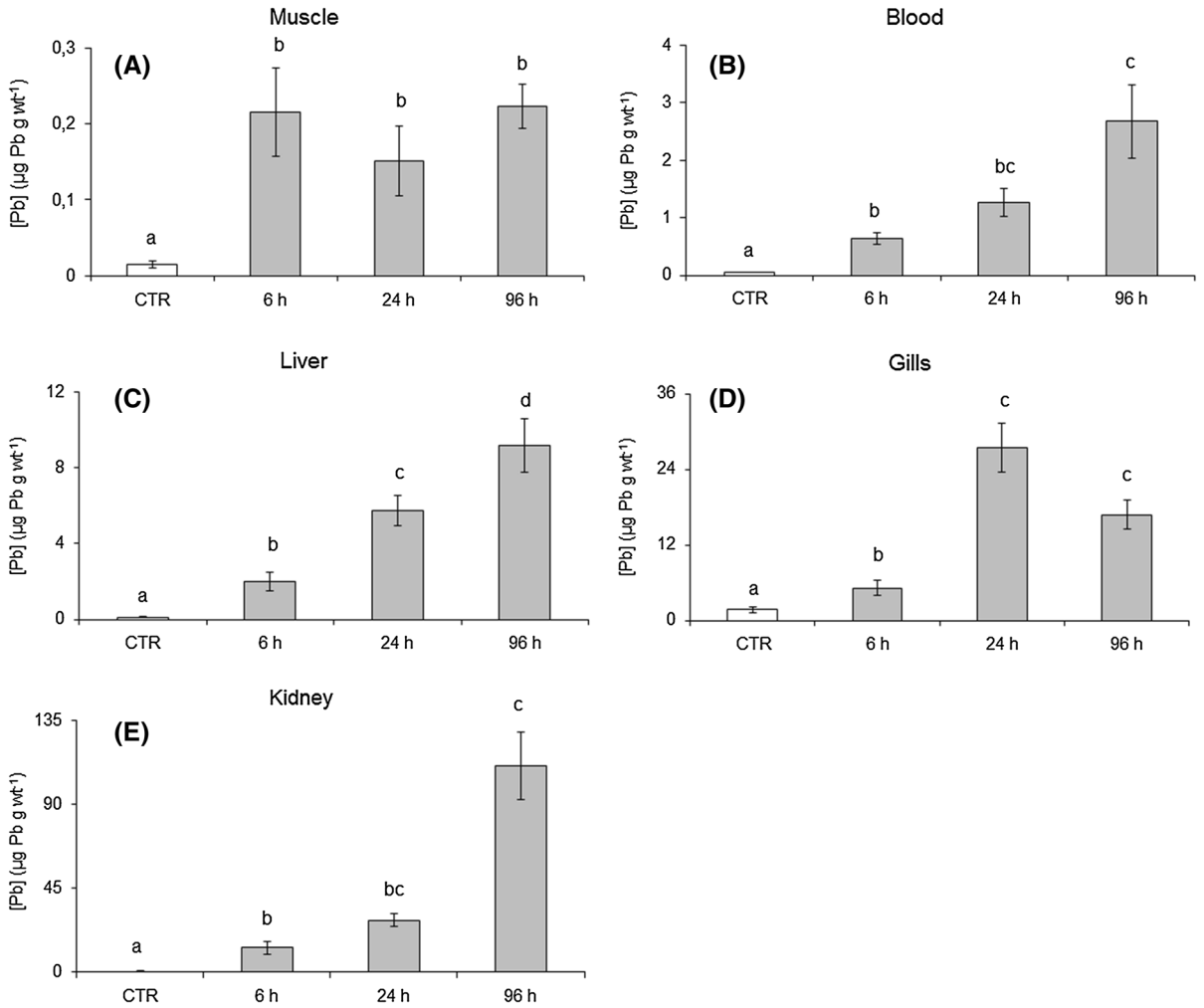

Fig. 1 Lead concentrations (mean ± SE) in muscle (**a**), blood cells (**b**), liver (**c**), gills (**d**) and kidney (**e**) of *P. lineatus* exposed to lead for 6, 24 and 96 h (n 6–8) or only to clean water (CTR) for up to 96 h (n 24–28). Different letters indicate significant difference between groups ($P < 0.05$)

Table 2 Hemoglobin content, hematocrit and the number of red blood cells (RBC) in blood of *P. lineatus* exposed to lead (Pb) or only water (CTR) for 6, 24 and 96 h

Time (h)	Hemoglobin (g dL^{-1})		Hematocrit (%)		RBC (x 10^6 mm^{-3})	
	CTR	Pb	CTR	Pb	CTR	Pb
6	6.68 ± 0.58 (8)	7.63 ± 2.00 (8)	29.8 ± 3.2 (8)	29.3 ± 5.6 (8)	2.43 ± 0.69 (8)	2.38 ± 0.64 (8)
24	6.94 ± 1.67 (10)	8.30 ± 2.59 (10)	32.1 ± 6.3 (10)	33.6 ± 6.2 (10)	2.27 ± 0.40 (10)	2.07 ± 0.29 (10)
96	6.03 ± 1.72 (7)	6.53 ± 2.51 (8)	24.0 ± 2.7 (8)	26.6 ± 5.3 (7)	1.58 ± 0.27 (7)	1.45 ± 0.22 (7)

Results are mean ± SE (n)

Fig. 6) at all the experimental periods when compared with their respective controls (6 h: $P = 0.019$, 24 h: $P = 0.014$, 96 h: $P = 0.02$). However, only after 6 h exposure, this increase was sufficiently high to determine a significantly higher total number of CCs ($P = 0.027$) (Fig. 6).

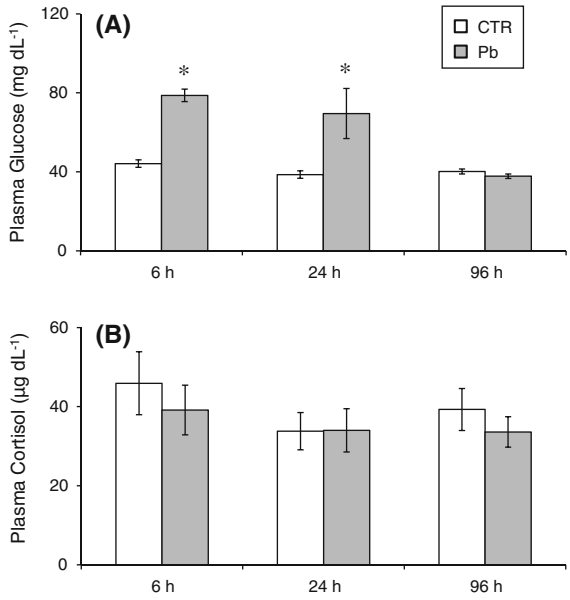

Fig. 2 Concentrations (mean ± SE, *n* 6–10) of plasma glucose (**a**) and cortisol (**b**) in *P. lineatus* exposed to lead (Pb) or only water (CTR) for 6, 24 and 96 h. *Asterisk* indicates significant difference compared to the respective CTR group ($P < 0.05$)

Discussion

The concentration of Pb in natural river waters has been estimated at 5 µg L^{-1}, but, in aquatic environments near to steel and iron industries and lead production and processing operations much higher concentrations can be found (ATSDR 2007). The Pb concentration used in this work was based on lead determinations in rivers of northern Paraná state, Brazil (Yabe and Oliveira 1998) and previous works have already shown that this same Pb concentration affects water and ion movements in the freshwater crab *Dilocarcinus pagei* (Amado et al. 2006) and promotes DNA damage in gill and liver cells and erythrocytes of *P. lineatus* (Monteiro et al. 2011). The decrease in Pb concentrations in experimental aquaria over time is due to precipitation, adsorption and absorption, which are phenomena common to metal species (Paquin et al. 2002).

The Pb distribution identified in *P. lineatus* after 6 h of exposure (kidney > gills > liver > blood > muscle) remained the same in all the other experimental periods. From the first hours of Pb exposure, the organ that accumulated the highest amount was the kidney, which plays an important role in the excretion of toxic substances (Streit 1998), and much of the Pb that enters the body is eliminated through the urine, after passing through glomerular filtration (Alves and Wood 2006). The kidney also plays an essential role in the water and electrolyte balance and in the maintenance of a stable internal environment (Palaniappan et al. 2009) and numerous channels, transport mechanisms and enzymes can be found in the renal cells (Hwang and Lee 2008), many of which have a high affinity for metals such as Pb (Patel et al. 2006). Analyses of accumulated Pb in distinct portions of the kidney have demonstrated that the posterior segment of the kidney retains more metal than the anterior portion, due to its greater involvement in ion reabsorption (Alves and Wood 2006). Due to its functions, the kidney is an organ of high metabolic activity that is able to produce metallothioneins (MT), which act as protectors against the action of metals (Cicik et al. 2004). Histopathological studies have shown that the presence of lead may also induce the formation of inclusion bodies in the cells of the renal tubules of rats (Moore and Goyer 1974) and birds (Locke et al. 1966). These bodies which are precipitates of lead-binding proteins, help to prevent Pb from continuing in circulation, and may be one of the reasons for the occurrence of a large quantity of Pb in the kidney of rainbow trout (*O. mykiss*) after acute exposure (Patel et al. 2006). As a result of these two processes, much of the circulating Pb may be retained in the kidney to prevent damage to other organs, which would explain the high renal levels of the metal found in this study, as well as its considerable increase over time.

Pb accumulation in the liver was marked by successive increases at all the experimental periods, possibly indicating that equilibrium had not yet been reached. The liver accumulates metal due to its role as a storage and detoxification organ (Klavins et al. 2009; Nussey et al. 2000; Palaniappan et al. 2009), and will invariably accumulate Pb if it is present in the animal's body. Here, too, the production of MT is described as one of the possible reasons for the accumulation of metals (Cicik et al. 2004). This fact was demonstrated by the significant increase in liver MT content observed after 6 and 24 h exposure of *P. lineatus* to Pb (Monteiro et al. 2011).

In a large part of the studies on metal accumulation in freshwater teleosts, the muscle was found to be the tissue that accumulates the lowest amount of metals (Alves and Wood 2006; Cicik et al. 2004; Klavins et al. 2009). In the present study, the Pb concentration

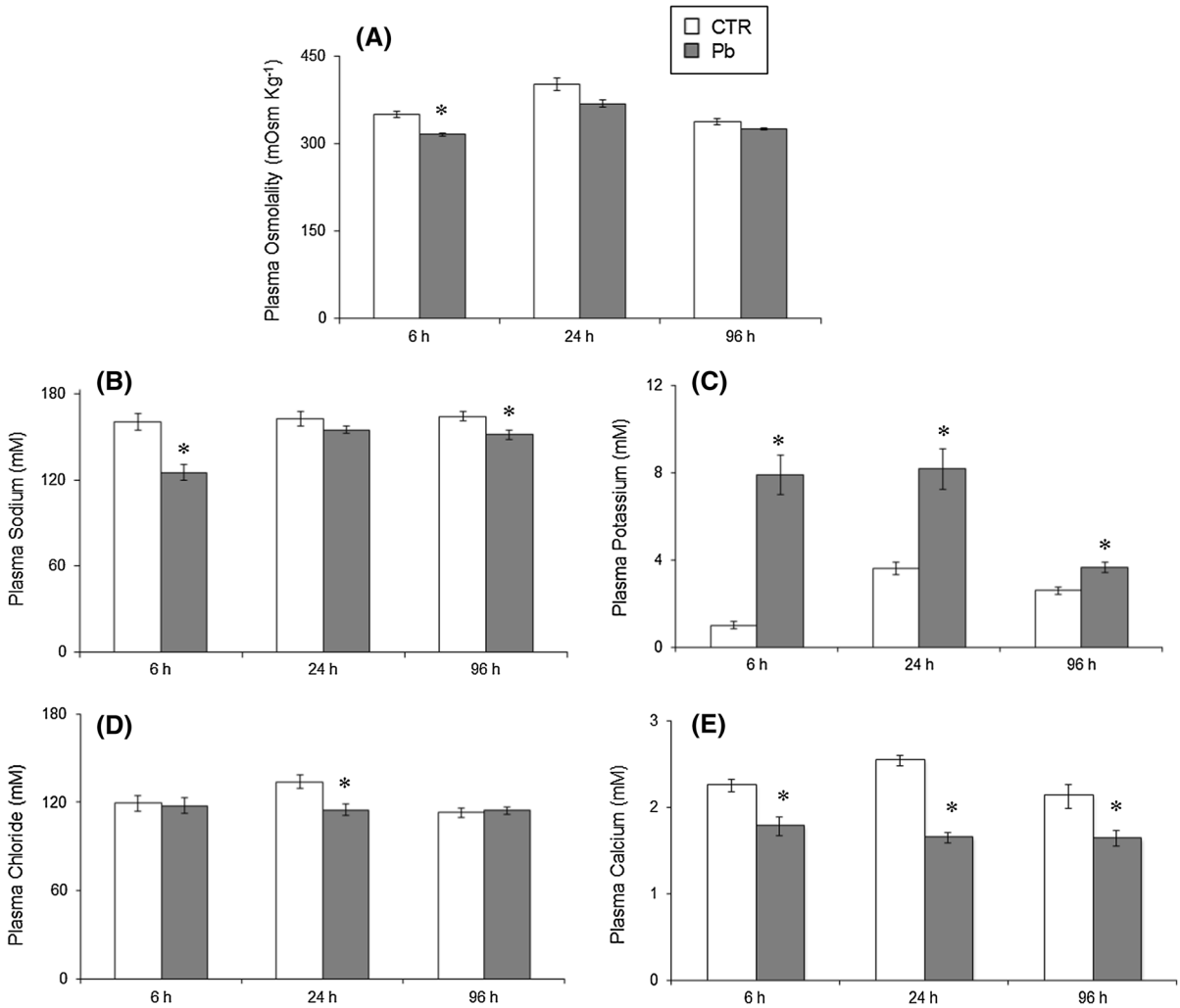

Fig. 3 Values (mean ± SE, *n* 5–10) of plasma osmolality (**a**) and concentrations of sodium (**b**), potassium (**c**), chloride (**d**) and calcium (**e**) in *P. lineatus* exposed to lead (Pb) or only water (CTR) for 6, 24 and 96 h. *Asterisk* indicates significant difference compared to the respective CTR group ($P < 0.05$)

found in muscle in the initial period remained unchanged throughout all the other experimental periods, which may indicate that the capacity of this tissue to retain metal had already been reached in the first hours of exposure, or that other regulatory mechanisms were activated. It should be noted, however, that the total mass of muscle tissue is much larger than that of the other organs analyzed, which would favor the metal's dissipation throughout the animal's body (Palaniappan et al. 2009). In studies to verify the total quantity of accumulated Pb in rainbow trout, the muscle tissue was found to account for 12 % of accumulated metal (Alves and Wood 2006). However, an isolated analysis of this tissue should not be seen as the only available biomonitoring tool, since Pb concentrations, even if present in water and in other tissues, may remain below the limits of detection in muscle, as has been found in the case of *P. lineatus* and *Pterodoras granulosus* by Villar et al. (2001).

The gills showed rapid accumulation of Pb during the initial periods (6 and 24 h), which becomes stable at 96 h exposure. This stabilization could be due to the decline of Pb levels in water over time, considering that Pb concentrations were reduced nearly by 60 % from 24 to 96 h exposure in the static system. Grosell et al. (2006) also observed a rapid increase in Pb in the gills of *Pimephales promelas* exposed to lead in a flow-through system, but, unlike the stabilization that

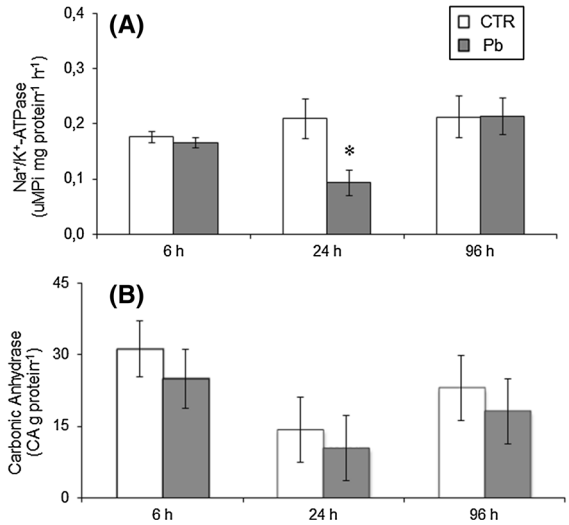

Fig. 4 Values (mean ± SE, *n* 7–10) of Na^+/K^+ATPase (**b**) and carbonic anhydrase (**b**) activities in the gills of *P. lineatus* exposed to lead (Pb) or only water (CTR) for 6, 24 and 96 h. *Asterisk* indicates significant difference compared to the respective CTR group ($P < 0.05$)

occurred in the present study, it continued to increase at a lower rate during 30 days exposure. On the other hand, it has been demonstrated that Pb accumulated in the gills of rainbow trout declines over time, possibly as a result of the depuration process (Alves and Wood 2006). It is also possible that the species *P. lineatus* used in this study presents adaptive strategies aimed at eliminating metal accumulated in certain types of cells, similarly to what has been demonstrated for aluminum (Camargo et al. 2009).

After passing through the branchial epithelium, Pb reaches the blood stream and enters the erythrocytes. Almost 99 % of the Pb present in total blood is found inside the erythrocytes (Alves and Wood 2006). In the blood cell there is a marked preference for Pb (between 35 and 80 %) to bind to δ-aminolevulinic acid dehydratase (ALAD) (Bergdahl et al. 1998), an enzyme that is present in the RBCs and responsible for hemoglobin synthesis. The quantity of Pb that can associate with ALAD is limited, and does not allow for an undefined increase in blood Pb levels (Skerfving and Bergdahl 2007). Nonetheless, in the present study, it is possible that the saturation of lead-binding sites in erythrocytes of fish exposed to Pb had not been attained, since the results obtained for accumulated Pb in the erythrocytes revealed that the levels of this metal increased fourfold between 6 and 96 h of exposure.

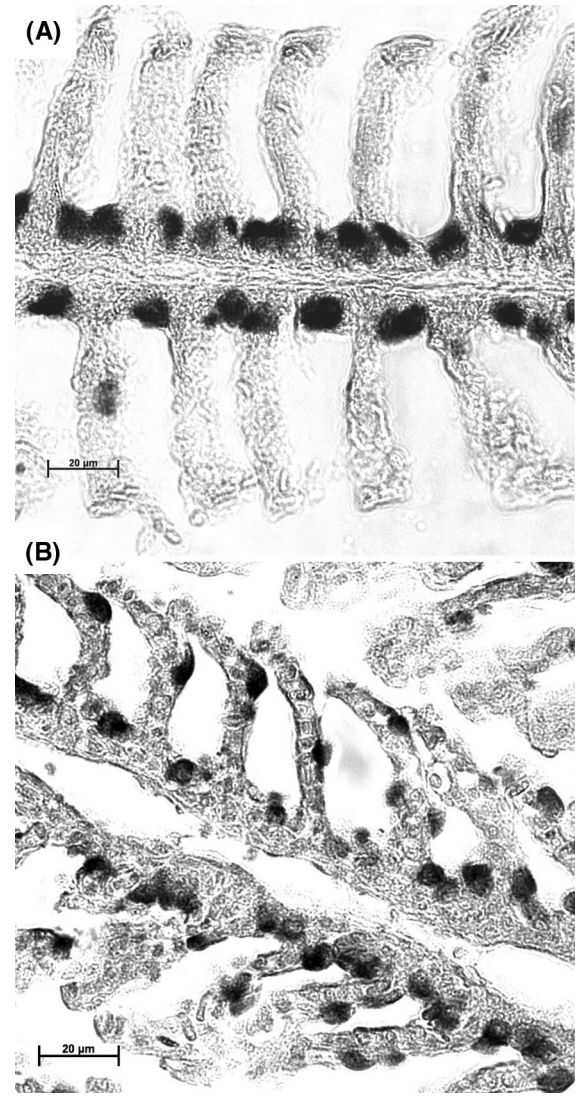

Fig. 5 Photomicrography showing the location of chloride cells by immunohistochemical technique for Na^+/K^+ATPase enzyme in *P. lineatus* exposed only to water (**a**) or to lead (**b**) for 96 h. Scale bars correspond to 20 µm

ALAD activity is highly sensitive to Pb (Costa et al. 2007; Hodson et al. 1980), and its inhibition may cause a diminished production of hemoglobin, followed by a decline in other hematologic indices (Ates et al. 2008). However, the results of hematologic analyses of the present study did not indicate the interference of Pb in blood cells. Similarly, the acute exposure of *P. lineatus* to higher concentrations of the same metal did not produce changes in the hematocrit (Martinez et al. 2004). Thus, short-term exposure of freshwater

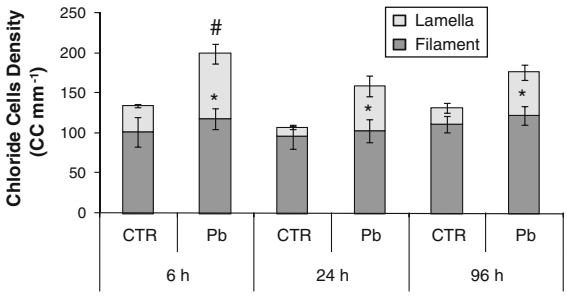

Fig. 6 Density (mean ± SE, $n = 4$) of chloride cells in the gills and their distribution in gill lamella and filament of *P. lineatus* exposed for 6, 24 and 96 h only to water (CTR) or to lead (Pb). *Asterisk* indicates density of CC in lamella different from respective CTR; *Hash* indicates density of CC in the whole gill (lamella + filament) different from respective CTR ($P < 0.05$)

teleosts to Pb does not seem sufficient to interfere in their hematologic parameters, since the exposure of rainbow trout to Pb for 96 h (Rogers et al. 2003) or the ingestion of dietary Pb (Alves et al. 2006) also caused no significant change in any of the hematologic parameters.

Among the osmo-ionic parameters, changes were found in plasma osmolality and concentrations of potassium, sodium, chloride and calcium ions. Dysfunctions in plasma ion concentrations were also found in rainbow trout (*O. mykiss*) after acute exposure to Pb, which were attributed to the interference of the metal on the ion uptake processes (Rogers et al. 2003, 2005). Similarly, other studies have demonstrated that the exposure of freshwater teleosts to Pb may result in the inhibition of NKA activity (Atli and Canli 2007; Ay et al. 1999; Rogers et al. 2003, 2005).

The decline of more than 50 % in NKA activity caused by Pb in the first 24 h of the experiment, and the subsequent reestablishment of normal levels after 96 h of exposure, suggests that the organism can overcome the metal effects on the osmoregulatory processes. Once the NKA activity has been impaired, the uptake of salts becomes deficient and the concentration of plasma ions is altered. The decline in plasma osmolality and sodium levels may serve as a signal for the release of cortisol, responsible for mobilizing energy reserves and stimulation of CC differentiation (Dang et al. 2000a; Mancera and McCormick 2007; McCormick 2001; Ramesh et al. 2009).

Although changes in plasma cortisol levels were not detected in fish exposed to Pb in any of the experimental periods, it is possible that it was released in the first hours of exposure, since the increase in glycemic levels after 6 and 24 h in fish exposed to Pb is an indication of this occurrence (Iwama et al. 2006). Other studies with *P. lineatus* exposed to other types of xenobiotics also detected no cortisol release after 6 h of exposure (Camargo et al. 2009; Nascimento et al. 2012; Simonato et al. 2008). This is probably due to the fact that, in the majority of teleosts species, this hormone is released between 30 min and 2 h after contact with the stressor (Barton 2002), returning to pre-treatment levels in at most a few hours after its release (Iwama et al. 2006). In fact Nascimento et al. (2012) showed a significant increase in plasma cortisol of *P. lineatus* after 1 and 3 h from the application of the air stress and a return to basal levels after 6 h.

In situations that promote the loss of ions the gills might exhibit stereotyped responses of CC proliferation (Perry 1997), particularly in the lamellae (Evans 1987; Wood 2001). Since a decline in plasma osmolality, sodium and calcium was identified, it is suspected that cortisol presumably acted upon stem cells in the gills, stimulating their differentiation into CCs. Like the case of *P. lineatus*, which, upon exposure to acid pH, presented a significant increase in the total number of CCs (Camargo et al. 2009), or the Nile tilapia (*Oreochromis mossambicus*), which, after exposure to copper, presented an atypical population of ionocytes scattered throughout the branchial lamellae (Dang et al. 2000b), the fish exposed to Pb showed a significant increase in the number of CCs in the lamellae, starting at the first hours of exposure. Nonetheless, only in the initial period of 6 h this increase in cells in the region of the lamellae was sufficiently high to alter the total number of branchial CCs, suggesting that part of the new cells was not maintained in the longer experimental periods. Branchial CCs are an easy target for metal accumulation due to their ion uptake function (Bury and Wood 1999), and the death of these cells through necrotic and/or apoptotic processes is a defense strategy to eliminate the accumulated contaminant or to replace damaged cells (Bury et al. 1998; Camargo et al. 2009; Dang et al. 2000b; Li et al. 1998). Thus, the less evident increase in CCs in fish exposed to Pb for 24 and 96 h compared to the increase that occurred in 6 h demonstrates that, despite the attempt to compensate for the loss of ions through cell differentiation, the exclusion of parts of these cells is necessary in order to

eliminate the accumulated metal and repair the damage caused by it. The results found for Pb accumulation in the gills supports this hypothesis.

This increase in the density of mitochondria-rich cells is aimed at augmenting the NKA enzyme population, thus improving the ion uptake activity (McCormick 2001). However, the analysis of this enzyme's activity indicated that it was inhibited by more than 50 % in the experimental period of 24 h, illustrating the interference of Pb in the ion transport mechanism. This occurrence solely in the intermediate sampling period may indicate that the metal takes some time to begin acting, and also that the animal later resorted to adjustments to correct this problem. These adjustments are related with the increase in the number of cells, which is already visible in the first hours of exposure. Nonetheless, the decrease in NKA activity could not be prevented even with this new population of CCs, since newly differentiated cells are immature and have a limited ion absorption capacity (Camargo et al. 2009; Dang et al. 2000b). The recovery of the enzyme's activity was only visible after 96 h of exposure to Pb, when its activity was reestablished 102 % in relation to the control, with the probable maturing of the cells (Dang et al. 2000a).

Calcium is considered an essential ion to the establishment of epithelial permeability (Flik and Verbost 1995). Metals such as cobalt, zinc, cadmium and also lead have proved to be calcium antagonists, using its absorption pathways to enter the animal and hindering the absorption of this ion (Bury and Wood 1999; MacDonald et al. 2002; Rogers and Wood 2004).When plasma calcium concentrations fall below normal, many functions related to its presence may be impaired. Metal ions that hinder the hyper-regulation of calcium may act directly or indirectly on the occlusion junctions, interfering on gills ionic permeability of freshwater fishes, enabling the efflux of other ions (Evans 1987). Calcium channels in branchial cells are described as the main pathways for the entry of Pb in freshwater teleosts, and there is evidence that Pb is able to block these channels. To exit the cell toward the animal's bloodstream, this metal uses Na^+/Ca^{2+}exchangers or ATP-dependent calcium pumps, and may, in the same way, inhibit the activity of these transport mechanisms (Atli and Canli 2007; MacDonald et al. 2002; Rogers et al. 2003). The disturbance of Ca^{2+} homeostasis induced by lead is not exclusively a branchial phenomenon, but is in part a result of inhibition of active tubular reabsorption by the kidney (Patel et al. 2006). Thus, the significant decline in plasma calcium levels found in *P. lineatus* exposed to Pb during the three experimental periods reinforces the idea that this metal inhibits the hyper-regulation of calcium ions, which may result of disrupted Ca^{2+} homeostasis due to Pb interactions at both the gill and kidney (Mager 2012). Moreover, a series of other studies have reported similar results for freshwater organisms such as crustaceans (Ahern and Morris 1998) and other teleost species (Rogers et al. 2003; Rogers and Wood 2004).

During the experiment, only a transitory significant change in the plasma chloride level was observed after 24 h of exposure. Changes in plasma chloride concentrations may be associated with disorders in CA activity, which is involved in hyper-ionic regulation, since the uptake of Na^+ and Cl^- is, to some extent, dependent on the products of its activity (Evans 1987; Souza-Bastos and Freire 2009). It has been reported that the activity of CA is markedly inhibited in the presence of several metal species (Morgan et al. 1997; Skaggs and Henry 2002), including Pb (Rogers et al. 2005). The cytosolic CA activity of *P. lineatus* exposed to Pb showed a tendency to decline (by about 25 %) at all experimental periods, but no significant changes were observed. Therefore, the variation found in chloride ions may be seen as a transitory one resulting from the attempts to adjust other parameters.

Final remarks

This study showed that Pb causes disturbance on plasma ion concentrations of *P. lineatus*, reducing the sodium and calcium concentrations and augmenting the plasma potassium levels. NKA was also changed after exposure to the metal, unlike CA, whose activity remained unaltered. The number of CCs in gill lamellae of fish exposed to Pb increased significantly, as did the plasma glucose in the initial experimental period. The hematologic parameters did not change significantly. These results suggest that after entering the fish, Pb can bind to exchangers inserted in the basolateral membrane, preventing their normal function and causing inefficient ion uptake, with the consequent disruption of normal plasma ion concentrations. In its attempt to overcome the imbalance and reestablish homeostatic equilibrium, the organism responds by promoting cellular differentiation in the gills, which are responsible for ion uptake. Concomitantly, the metal ions that enter the fish via

transepithelial absorption through the gills reach various other tissues through the bloodstream, accumulating in organs whose function is to remove the toxic substance from circulation, providing protection for the organism. Further studies are needed to substantiate the proposed mechanism of Pb uptake through the gills, the entry pathways of Pb, and its action on the proteins involved in osmoregulation and branchial permeability, as well as the hormones involved in the proliferation of CCs in the gills.

Acknowledgments The authors thank the Hatchery Station of State University of Londrina (EPUEL) for the supply of fish and the Brazilian Council for Scientific and Technological Development (CNPq) for the financial support to this work (grant no 476910/2011-0). M.N. Fernandes and C.B.R. Martinez are research fellows from CNPq and members of the Brazilian Institute of Aquatic Toxicology (INCT-TA, CNPq: 573949/2008-5).

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