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How aluminium exposure promotes osmoregulatory disturbances in the neotropical freshwater fish *Prochilus lineatus*

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ABSTRACT

The aim of this study was to understand the effects of the interaction between aluminium and low pH in a native fish species *Prochilodus lineatus*. Thus, juveniles of this neotropical fish species were exposed to 196 $\mu g L^{-1}$ of dissolved aluminium in acid water (Al group), only to acid water (pH group) or to water with neutral pH (CTR group) for 6, 24 and 96 h. Al effects were evaluated with regard to hematological parameters (hemoglobin, hematocrit and red blood cell number), plasma ions and osmolarity, density and distribution of chloride cells (CC), Na^+/K^+–ATPase activity in the gills, metabolic (protein and glucose) and endocrine (cortisol) parameters. The fish exposed to Al had increased hematological and metabolic parameters in relation to the CTR group after all periods of exposure. In fish exposed to Al for 24 and 96 h plasma ions and osmolarity were significantly lower and the identification of the enzyme Na^+/K^+–ATPase by immunohistochemistry indicated a reduction in the number of CC in the gills. Enzyme activity was 50% lower in fish exposed to Al in all experimental times. Taken together these results showed that acute exposure to Al causes an ionic unbalance, probably related to the effects of Al on Na^+/K^+–ATPase activity, on the distribution and number of chloride cells in the gills as well as the effects associated with the stress response caused by the presence of the metal.

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1. Introduction

Aluminium is the most abundant metal on earth and mostly occurs as oxide and silicate of aluminium (Ščančar et al., 2004). Aluminium is also found in the atmospheric air of the big cities and industrialized areas (Casarini et al., 2001), and is used as a flocculation agent in water treatment (Silva et al., 2007). Some areas of England, United States and Czech Republic have high concentrations of Al in their river waters, reaching up to 1350 $\mu g L^{-1}$ of total Al due to air pollution and acid rain (Guibaud and Gualthier, 2003). In Brazil, this metal is naturally found in the Amazon region soil where the water of the rivers and streams has naturally low pH (Hara and Oliveira, 2004; Artaxo et al., 2005; Horbe et al., 2005). In the state of São Paulo (Southeast Brazil), 35% of the examined surface waters destined for public consumption contain high levels of dissolved Al (100–5700 $\mu g L^{-1}$) (CETESB, 2008). Even though, according to the Brazilian law, the limits for dissolved Al in freshwater is between 100 and 200 $\mu g L^{-1}$ (CONAMA 357, 2005).

Aluminium is toxic to fishes and most studies on Al toxicity are restricted to fish species from the Northern hemisphere (McCartney et al., 2003; Monette and McCormick, 2008). In the tropical and neotropical areas such studies are still rare (Barcarolli and Martinez, 2004), leaving a gap in the knowledge of the physiological effects of Al in neotropical fishes where the high temperature of natural water may increase toxicity (Lydersen et al., 1990).

Most of the studies on Al toxicity in fish are related to the pH of water, as the solubility of Al increases linearly with the reduction in pH increasing the presence of inorganic Al, the form of Al most toxic to fish (Gensemer and Playle, 1999). In contrast, acidity, by itself, causes several effects in fishes such as hematological (Wood and McDonald, 1982; Carvalho and Fernandes, 2006), endocrine and metabolic (Cole et al., 2001) and reproductive (Vuorinen et al., 2003) disturbances. However, when acidity is associated with Al in water the effects are concentrated mainly in the gills and all physiological processes related to this organ (Waring and Brown, 1995; Cole et al., 2001; Teien et al., 2006). The gills are a multi-functional organ playing an important role in osmoregulation of fish (Hwang and Lee, 2008). This organ represents the main target-organ of pollutants due to its extensive surface area in contact with the external environment and the very thin barrier between the environmental water and internal milieu of fish (Dang et al., 2000; Cerqueira and Fernandes, 2002). Gills are the most affected organ by Al contaminated water (Dietrich and Schlatter, 1989; Playle and Wood, 1990; Peuranen et al., 1993).

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In the gills the main cells related to ionic regulation are the chloride cells (Hirose et al., 2003), which are located mostly in the gill filaments, close to the base of the lamellae (Perry, 1997; Hirose et al., 2003). Chloride cells (CC) are large and round cells characterized by numerous mitochondria and an extensive tubular membrane system containing a high density of Na^+/K^+–ATPase activity (Dang et al., 2000).

In order to maintain their body fluid and mineral homeostasis, freshwater teleosts compensate for diffusive ion loss and osmotic gain of water by actively absorbing Na^+, Cl^- and Ca^{2+} through the gills and producing large volumes of diluted urine, respectively (Hirose et al., 2003). Thus, ion analysis and plasma osmolarity, associated with the determination of density and localization of CC, besides Na^+/K^+–ATPase activity, which actively transport ions through the gills, should be informative for the understanding of the mechanism of Al toxicity in freshwater teleosts (Peuranen et al., 1993; Vuorinen et al., 2003). Therefore, in the present study an integrated approach examining plasma ions and osmolarity, CC distribution and density, gill Na^+/K^+–ATPase activity, and hematological parameters besides others associated with stress response, was employed in order to evaluate the effect of acute exposure to aluminium in acid pH on the osmoregulation of the *Prochilodus lineatus* fish. This species was chosen because it represents a neotropical fish commonly found in rivers of the south and southeast regions of Brazil, and it is also considered a potential bioindicator species (Martinez et al., 2004; Takasusuki et al., 2004; Simonato et al., 2006).

2. Materials and methods

Juveniles *P. lineatus* (Valenciennes, 1847) (n = 115) weighing 20.07 ± 6.08 g and total length equals 12.23 ± 1.23 cm (mean ± SD) were obtained from the hatchery station of State University of Londrina. Prior to experiments, the fish were acclimated, for 7 days, in 300 L tanks with non-chlorinated water, constant aeration and a photoperiod of 12 h:12 h. During acclimation, the animals were fed with commercial pellet food with 36% protein (Guabi®, BR) every 2 days, and the feeding was suspended 24 h before the beginning of the toxicity tests. The physical and chemical parameters of the water were continuously monitored (T = 21.8 ± 0.9 °C; pH 7.5 ± 0.1; DO = 7.5 ± 0.7 mg O_2 L^{-1}; conductivity = 133.4 ± 9.7 μS cm^{-1}; hardness = 42.5 ± 6.0 mg $CaCO_3$ L^{-1}).

After acclimation, groups of fish (6 or 7 per aquarium) were transferred to glass aquaria (100 L each) containing water as follows: fish of the control group (CTR) to water with neutral pH; fish of pH group (pH) to water with acid pH (5.0); fish of Al group (Al) to water in acid pH (5.0) + aluminium. Acid pH in water was obtained by the addition of 50% HCl and the aluminium was added to water as $Al_2(SO_4)_3$. The toxicity tests in each experimental time (6, 24 and 96 h) were performed in separated aquaria. All toxicity tests were carried out in duplicate.

During the tests, water was monitored for temperature, pH, dissolved oxygen and conductivity. Samples of water collected immediately after each experimental period were analysed for Al concentration, using atomic absorption spectrophotometry. The concentration of total Al was determined in samples of non-filtered water and the concentration of dissolved Al was determined in filtered water samples (0.45 μm); for both analyses, samples were acidified with HNO_3.

At the end of each experimental period, fish were anaesthetised with benzocaine (0.1 g L^{-1}) and a blood sample was withdrawn, via the caudal vein, using heparinised plastic syringes. The animals were then killed by medullar section and the gills were removed and processed for immunohistochemical against Na^+/K^+–ATPase and enzyme assay. Immediately after sampling, the blood was centrifuged (10 min, 10,000 × g) and samples of plasma were frozen (−20 °C) for osmolarity, ion concentrations, cortisol, glucose and protein analyses.

2.1. Hematological analysis

Blood samples were used to determine the hematocrit (Hct), hemoglobin content (Hb) and red blood cells count (RBC). Hct was determined by micro-hematocrit technique, using heparinised capillary tubes and centrifuged for 5 min in an appropriate centrifuge. Hemoglobin was determined by the cyanometahemoglobin method, in a spectrophotometer (Libra S32, Biochrom, UK) using a commercial kit (Analysa, Brazil). Red blood cells were counted in blood samples fixed in formol-citrate buffer, using an improved Neubauer chamber under a light microscope (magnification of 400X).

2.2. Identification of chloride cells in the gills

The gills were washed with saline solution and samples from the gill were fixed in Bouin's fluid (6 h), dehydrated in ethanol crescent series and embedded in paraffin. Sagittal sections (8 μm in thickness) were made and processed according to the avidin–biotin–peroxidase complex (ABC) technique to visualize chloride cells, through the identification of Na^+/K^+–ATPase, according to the method described by Dang et al. (2000). Slides were incubated with a mouse monoclonal antibody to Na^+/K^+–ATPase (IgGα5) and goat-anti-mouse IgG was used as the second antiserum. Subsequently, 3-3-diaminobenzidine (DAB 0.05 M) in Tris-buffered saline (pH 7.4), containing H_2O_2 (0.03%) was applied. Finally, sections were dehydrated and mounted.

The chloride cells were quantified in relation to the filament length (mm) according to their localization: in the gill filament (CCF) or in the gill lamellae (CCL), using a photomicroscope (DM 2500, Leica, Germany) and an image analyser (Leica *Qwin*, Germany). For each section from the same fish, five filaments were randomly selected and measured for CC quantification. The results were expressed as the number of CC per mm of filament (mean ± SD).

2.3. Na^+/K^+–ATPase activity in the gills

After washing the gill arches, the gill filaments were removed and transferred to plastic tubes containing SEI buffer (sucrose 0.3 M, Na_2EDTA 0.1 mM, imidazole 0.03 M, β-mercaptoethanol 10 mM, pH 7.4) and then kept frozen (−20 °C) until the moment of enzyme assay. For assay, the gill filaments were homogenised with SEI buffer (10× volume) and centrifuged (10,000 × g, 15 min, 4 °C). The supernatant was used to determine Na^+/K^+–ATPase activity, according to the method described by Quabius et al. (1997) and adapted for a microplate reader by Nolan (2000). The assay consists of determining the phosphate released by the samples incubated in buffer (NaCl 100 mM, $MgCl_2$ 8 mM, imidazole 30 mM,

Table 1
Physical and chemical parameters of the water samples collected in the different groups during the tests (6, 24 and 96 h).

Parameter	CTR group	pH group	Al group
Temperature (°C)	22.4 ± 0.5	22.4 ± 0.5	22.5 ± 0.5
pH	7.6 ± 0.5	5.1 ± 0.3	5.2 ± 0.1
Dissolved oxygen (mg O_2 L^{-1})	7.6 ± 1.0	7.4 ± 0.9	7.5 ± 0.8
Conductivity (μS cm^{-1})	83.7 ± 23.6	104.1 ± 18.3	104.6 ± 15.0
Hardness (mg $CaCO_3$ L^{-1})	41.3 ± 7.9	41.6 ± 5.9	44.1 ± 7.3
Total Al (μg L^{-1})	ND	ND	438.0 ± 36.3
Dissolved Al (μg L^{-1})	ND	ND	196.0 ± 28.7

The values represent means ± SD (n = 5). ND: not detected.

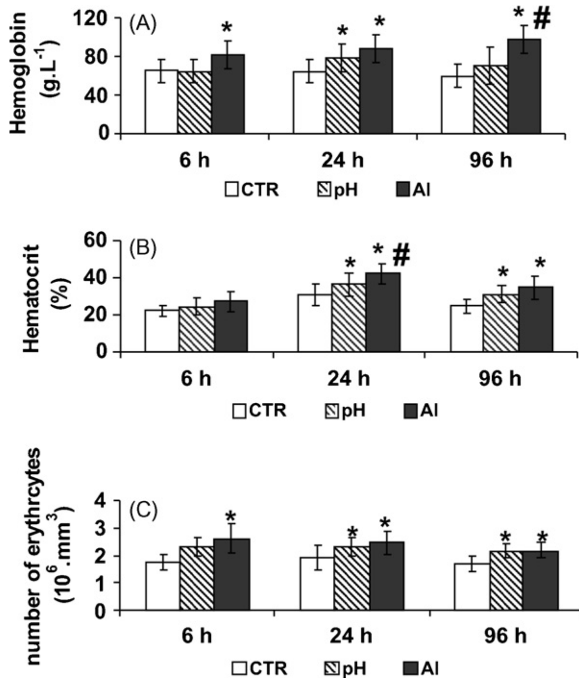

Fig. 1. Hemoglobin (A), hematocrit (B) and number of erythrocytes (C) of *P. lineatus* exposed to 6, 24 and 96 h to CTR, pH or Al groups. The bars indicate mean and the vertical lines, the SD (number of animals: 10–13). *Different from the CTR group and #different from the pH group for each experimental period ($P < 0.05$).

EDTA 0.1 mM, ATP 3 mM, pH 7.6) containing KCl (5 mM) or ouabain (2.5 mM). A solution of 0.65 mM phosphate (Sigma) was used as standard and the samples were analysed in triplicate at 620 nm in a microplate reader (ELX 800, BioTek, USA). Na^+/K^+–ATPase activity was expressed as μmol Pi/mg protein h^{-1}. Protein concentration was determined according to the method described by Lowry et al. (1951).

2.4. Plasma ions and osmolarity

The concentrations of Na^+ and K^+ were measured in plasma using a flame photometer (Analyser, Brazil). The concentration of Cl^- was determined with the thiocyanate method in spectrophotometer at 470 nm (commercial kit, Analisa, Brazil). Osmolarity was determined using a freezing point osmometer (Osmomat 030, Gonotec, Germany).

2.5. Plasma concentrations of cortisol, glucose and proteins

Cortisol was determined in plasma with a commercial immunoenzymatic assay kit (Diagnostic Systems, Laboratories, USA), and the absorbance was read in a microplate reader at 450 nm. The concentration of glucose was determined using a commercial colorimetric kit (Labstest, Brazil) at 505 nm in a spectrophotometer. Plasma protein concentration was determined according to the method described by Lowry et al. (1951), using bovine serum albumin (BSA) as standard.

2.6. Statistical analysis

The results are presented as means ± SD. The results obtained in each treatment (CTR, pH or Al), at each experimental time (6, 24 or 96 h), were compared using one-way analysis of variance (ANOVA) or Kruskall–Wallis test, depending on data normal distribution and homogeneity of variance. Differences were analysed by a post hoc Tukey test for all pairwise comparisons between treatments. Statistical significance was designated as $P < 0.05$.

3. Results

Only one fish of the Al group died after 24 h of exposure. No fish died in the other groups. Over the course of the study, the values of temperature, dissolved oxygen, conductivity and hardness did not varied significantly among control, pH and Al groups (Table 1). The mean pH values were 7.6 ± 0.5 in control groups and 5.1 ± 0.3 and 5.2 ± 0.1 in pH and Al groups, respectively. In Al groups, the mean concentration of total Al was 438 ± 36.35 μg L^{-1} and of dissolved Al was 196 ± 28.7 μg L^{-1} (Table 1).

3.1. Hematological parameters

Hemoglobin content was significantly greater in *P. lineatus* exposed to Al for 6 h (increased 24.3%), 24 h (increased 38.1%) and 96 h (increased 63.3%) in comparison to respective control fish. Fish exposed only to pH 5 showed a significant increase in Hb content (21.8%) only after 24 h exposure (Fig. 1A). The hematocrit of fish exposed to Al for 24 h (41.7 ± 5.5%) and 96 h

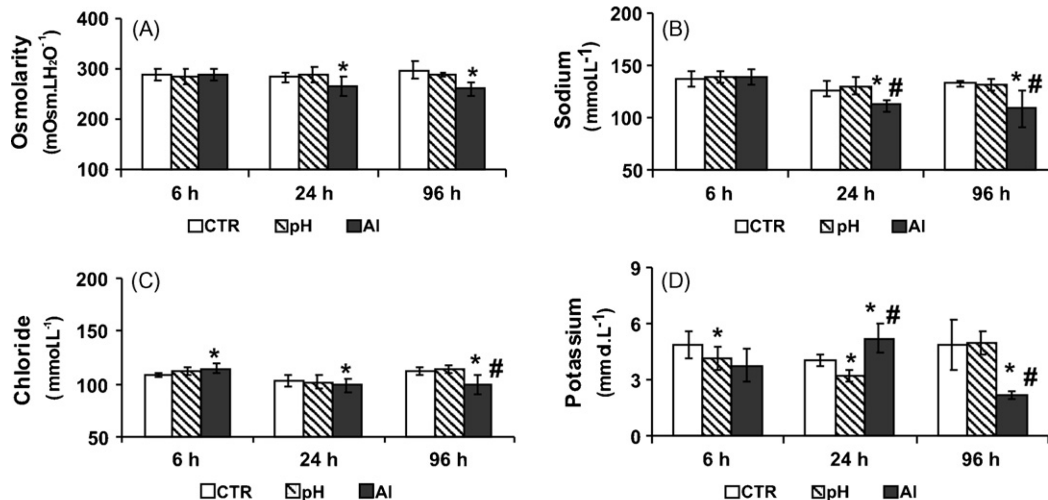

Fig. 2. Plasmatic osmolarity (A) and concentrations of sodium (B), chloride (C) and potassium (D) of *P. lineatus* exposed to 6, 24 and 96 h to CTR, pH or Al groups. The bars indicate mean and the vertical lines, the SD (number of animals: 10–13). *Different from the CTR group and #different from the pH group for each experimental period ($P < 0.05$).

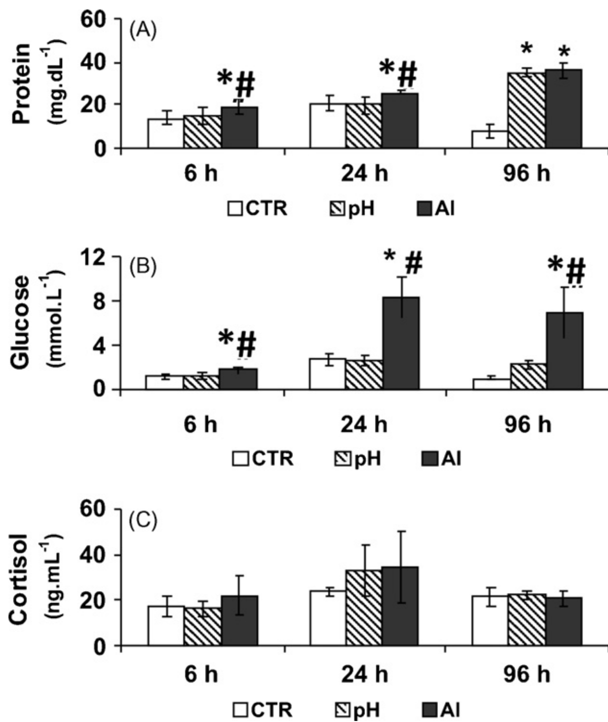

Fig. 3. Plasmatic concentrations of proteins (A), glucose (B) and cortisol (C) of *P. lineatus* exposed to 6, 24 and 96 h to CTR, pH or Al groups. The bars indicate mean and the vertical lines, the SD (number of animals: 10–13). *Different from the CTR group and #different from the pH group for each experimental period ($P < 0.05$).

(34.6 ± 6.2%) or only to pH 5 for 24 h (36.2 ± 5.9%) and 96 h (30.7 ± 4.8%) was significantly greater than those of the respective control groups (30.7 ± 5.6 and 24.5 ± 3.9). The exposure to Al or only to acid pH for 6 h did not affect fish hematocrit, which did not differ from control value (21.8 ± 3.2%) (Fig. 1B). The number of red blood cells also increased significantly after 24 and 96 h of exposure to Al (29.1% and 27.8%, respectively) and to pH 5 (21.2% and 27.2%, respectively), in relation to respective controls (Fig. 1C). After 6 h, only fish exposed to Al showed RBC counts significantly greater (48.3%) than respective control fish (Fig. 1C).

3.2. Plasma ions and osmolarity

Plasma osmolarity decreased significantly in fish exposed to Al for 24 h (6.8%) and 96 h (12.6%) in relation to respective controls (Fig. 2A). Sodium plasmatic concentrations were lower than respective control values after both time points, decreasing from 127.5 to 112.4 mM after 24 h and from 132.7 to 109.3 mM after 96 h of Al exposure (Fig. 2B). Plasma Cl^- levels of fish exposed to Al for 24 and 96 h were also significantly lower than control fish (decreased 3.9% and 11.7%, respectively) (Fig. 2C). Plasma osmolarity as well as the plasmatic concentrations of sodium and chloride of fish exposed to pH 5 did not differ from control values throughout the study (Fig. 2A–C). Plasma K^+ concentrations showed large variability among different exposure times and experimental groups (Fig. 2D).

3.3. Plasma protein, glucose and cortisol

Plasma protein levels of Al exposed fish were significantly greater than in control fish after 6 h (34.7%), 24 h (24%) and 96 h (364%). In fish exposed only to pH 5, for 6 and 24 h, plasma protein

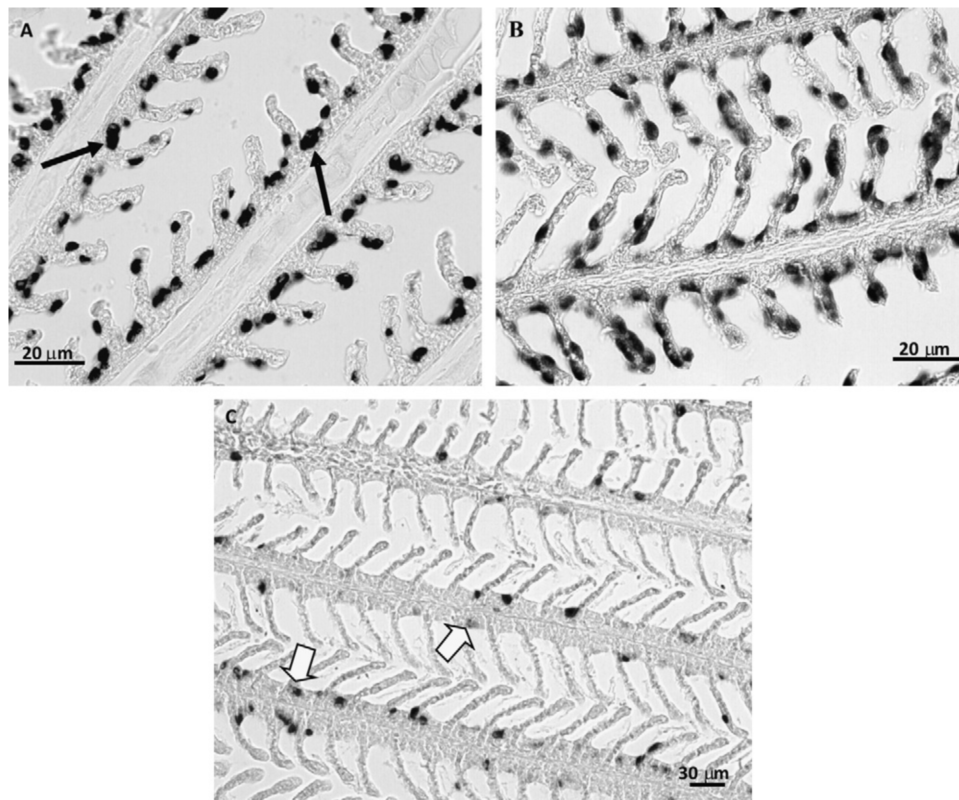

Fig. 4. Immunohistochemistry location of the Na^+/K^+–ATPase enzyme in the chloride cells (CC) of *P. lineatus* used in the experiment of 96 h. The arrows indicate strong immunoreactivity of Na^+/K^+–ATPase in the fish of CTR group (A). In the gills of fish exposed to Al group (C), white arrows indicate CC weakly stained and also a small number of CC, and in the fish exposed to pH group (B) is noted an increased number of lamellar and filamental CC. Scale bar: 30 μm.

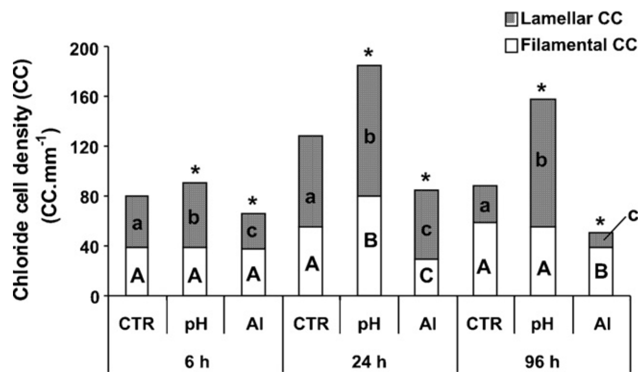

Fig. 5. Density of chloride cells in the lamellar and filamental regions of the gills of *P. lineatus* exposed to 6, 24 and 96 h to CTR, pH or Al groups. *Different from the total number of CC in the respective CTR group; small letters are related to the lamellar CC and capital letters are related to filamental CC. Different letters indicates statistical difference for each parameter in each experimental period ($P < 0.05$).

levels were not affected, but increased significantly (352%) in relation to control group after 96 h exposure (Fig. 3A). Fish exposed to Al also showed significantly higher levels of glucose after 6 h (45.3%), 24 h (213%) and 96 h (492%) than respective control groups (Fig. 3B). Plasma glucose levels were not significantly different between pH and respective control groups, in any experimental period (Fig. 3B). Plasma cortisol concentration did not change significantly among different treatments, in any experimental period, ranging from 16 to 34 ng mL^{-1} (Fig. 3C).

3.4. CC distribution and density

P. lineatus from the control group has large number of CC distributed throughout the filament and lamellar epithelium (Fig. 4A). The exposure to acid water did not change such CC distribution (Fig. 4B), however, after Al exposure CC in the lamella disappeared and those in the filament were extremely reduced (Fig. 4C). Fig. 5 shows the changes in CC density and localization in the gills of fish from control, pH and Al groups. In general, exposure to acid water induced an increase in CC density in both, filament and lamella (also shown in Fig. 4B). Conversely, aluminium exposure, although in acid water, resulted in significant reduction of CC density in the lamella after 6 h and in both filament and lamella after 24 and 96 h.

3.5. Gill Na^+/K^+–ATPase activity

Na^+/K^+–ATPase activity in the gills of fish exposed to Al, at all exposure times, showed significant inhibition (on average, 50% reduction) when compared to the animals from control groups (Fig. 6), which Na^+/K^+–ATPase activity was 1.22 μM Pi/mg protein h^{-1}. Acid exposure did not produce any significant difference in the Na^+/K^+–ATPase activity in relation to CTR groups (Fig. 6).

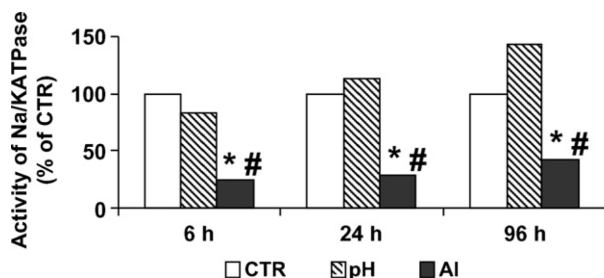

Fig. 6. Percentage of activity of Na^+/K^+–ATPase enzyme in the gills of *P. lineatus* exposed to 6, 24 and 96 h to CTR, pH or Al groups. *Different from the CTR group and #different from the pH group for each experimental period ($P < 0.05$).

4. Discussion

The neotropical freshwater fish *P. lineatus* exposed to aluminium, at low water pH (pH 5.0), exhibited osmoregulatory disruption indicated by plasma Na^+ and Cl^- concentration decrease, probably due to the reduction of chloride cells density in the gills and consequent reduced Na^+/K^+–ATPase activity. Stress condition, supported by high glucose content in plasma, may have corroborated to osmoregulatory disturbance.

The concentration of dissolved Al and the pH value used in this study have already been reported in surface waters in Brazil (Lara et al., 2001; Flues et al., 2002) due to natural causes or because of anthropogenic emissions. The concentration of 200 μg L^{-1} of dissolved Al corresponds to the maximal concentration allowed by the Brazilian guidelines for freshwater. However, the results of the present study clearly showed that this concentration promotes some serious effects on fish osmoregulation.

Water pH of 5 is not lethal to *P. lineatus* (Takasusuki et al., 2004) although it has been found to be the maximal tolerated for most freshwater fishes (Playle and Wood, 1990; Poléo, 1995; Waring and Brown, 1995). Values of pH between 6 and 9 are recommended for freshwater used for the protection of fish communities in Brazil (CONAMA 357, 2005). However, episodes of rapid acidification in continental water bodies may occur during ecological accidents. In such situations Al from the soil is mobilised providing high elevation of aluminium in its dissolved and more toxic form affecting fish (Monette and McCormick, 2008).

In the present study, the increased hematocrit and RBC counts after 24 and 96 h exposure to acid water and to Al in acid water cannot be considered as a good indicator of Al exposure, as suggested by Witters et al. (1996), at least, for *P. lineatus*. Changes in blood parameters of same species exposed only to low water pH (pH 4.5 at 20 and 30 °C) have been described by Carvalho and Fernandes (2006). The increased number of RBC and hemoglobin content may represent the secondary stress response, which leads to an increased RBC in the circulation, because of spleen contraction, to improve O_2 uptake for metabolism (Brown, 1993; Wendelaar Bonga, 1997; Hontela, 1998). Elevated blood parameters were also described in Salmoniformes fish, such as *Oncorhynchus mykiss* and *Salmo trutta*, and in the neotropical fish *Leporinus macrocephalus* after acute exposure to Al in acid water (Witters et al., 1990; Witters et al., 1996; Barcarolli and Martinez, 2004). In these works, Al concentrations varied from 15 to 200 μg L^{-1}. Poléo and Hytterød (2003) also registered elevated blood parameters in salmons exposed to Al concentrations from 28 to 359 μg L^{-1} in alkaline waters.

Stress response in *P. lineatus* was also indicated by the increased plasma glucose after 6 h of Al exposure. The elevation in plasma glucose is a typical response for any animal facing a stressing situation (Brown, 1993; Lohner et al., 2001) and it is mediated by the catecholamines and cortisol release. The increase in plasma glucose was the result of gluconeogenic processes or hepatic glucogenolysis to supply the increase in the energy demand caused by stress (Witters et al., 1996). Hyperglycaemia has been reported, by several authors, in fishes exposed to copper (Tavares-Dias et al., 2002), to aluminium (Witters et al., 1996; Barcarolli and Martinez, 2004) and other different stressing situations (Mommsen et al., 1999). As the catecholamines are rapidly eliminated from the circulation, the maintenance of the high plasma glucose levels, as observed in the present study, could have resulted from cortisol release, which might have occurred just after exposure to pollutant (Iwama et al., 2004). Cortisol is the main corticosteroid hormone in fish, and toxic agents can thereby interfere on its dynamics (Mommsen et al.,

1999). The absence of significant changes in plasma cortisol, in the present study, corroborates with the data previously reported by Langiano and Martinez (2008) and Pereira-Maduenho and Martinez (2008), for the same fish submitted to different stressful agent. In general, the increase in cortisol occurs between 0.5 and 1 h after exposure to a contaminant (Barton, 2002) returning to the basal levels after some minutes or few hours (Mommsen et al., 1999; Sloman et al., 2001; Iwama et al., 2004). The mobilisation of energy reserves as part of the stress response also includes protein metabolism (Heath, 1987; Adams et al., 1990; Mommsen et al., 1999). In the case of the present study, the high values of total protein observed in the animals exposed to Al may be the result of two events: protein mobilisation to meet the higher energy demand imposed by stress, or cell damage and proteins release due to direct action of the metal on the cells (Exley et al., 1991; Wilson, 1996).

Metals in water may act directly or indirectly via stress hormones in the gills causing changes in osmo-ionic homeostasis. Freshwater fish undergo passive influx of water and ions efflux and equilibrates the osmotic flux excreting large volume of diluted urine and taking actively ions by the gills (Evans et al., 2005; Lingwood et al., 2006; Hwang and Lee, 2008). Aluminium, at concentrations between 100 and 200 $\mu g\,L^{-1}$ and at a pH of near to 5.0, interacts with the gills and favour electrolytes loss (Dietrich and Schlatter, 1989; Exley et al., 1991). However, the present data suggest that Al interfered on sodium uptake by the gills of *P. lineatus*. Reductions on plasma ions concentrations in fish exposed to Al were already reported (Dietrich and Schlatter, 1989; Exley et al., 1991; Witters et al., 1996). Reductions in plasma sodium and chloride concentrations were found in *L. macrocephalus* exposed to Al in acid pH (Barcarolli and Martinez, 2004) and salmon in similar conditions (Monette and McCormick, 2008).

Decreased plasma ionic concentrations in stressed fishes cannot be related only to the reduced active uptake of ions, the increase in paracellular permeability of the branchial epithelium, which increases the passive efflux of ions, might represent another cause (Monette and McCormick, 2008). In this study, the analysis of chloride cells (CC) and of Na^+/K^+–ATPase was used as an approach to understand which stage of the osmoregulation process would be damaged in fish exposed to Al.

Na^+/K^+–ATPase is a protein that is linked to the cell membrane and it uses energy from the hydrolysis of ATP in order to transport $2K^+$ into the cell and $3Na^+$ out from the cell to the blood, being of great importance in the gills of teleosts (Lingwood et al., 2006; Silva et al., 2007; Hwang and Lee, 2008). There is a positive correlation between the staining of the CC and the activity of this enzyme in the branchial epithelium of fish (Dang et al., 2000). In the present study, the lower activity of Na^+/K^+–ATPase determined in the gills of the fish exposed to Al could be related to the lower activity of this enzyme in the poorly stained CC and to the smaller number of CC found in the filaments (due to the death of these cells through apoptosis and/or necrosis). Monette and McCormick (2008) also observed similar results in young salmon after acute exposure to Al in acid pH. These authors claim that the CCs are the main site for the accumulation of aluminium in the gills, and consequently the death of these cells would facilitate the elimination of Al from this organ. The few CC noted in the fish exposed to Al seemed to be displaced mostly to the lamellar region rather than in the filament. Dang et al. (2000) obtained similar results in *Oreochromis mossambicus* exposed to copper, i.e., both a decreased number of CC in the filaments and the migration of CC to the branchial lamellae. CC in gill lamellae would be closer to the bloodstream, facilitating ion uptake, which can also mean that these cells are more resistant to the metal than the CC that remain in the filament (Dang et al., 2000). Some of the CC that were found in the lamellae could represent immature cells as well, with a smaller quantity and/or activity of Na^+/K^+–ATPase, and therefore, they were less stained. The presence of these immature cells might represent the action of cortisol, which interferes with CC differentiation. In the present study, this idea would be supported by the occurrence of secondary stress responses (such as the increase in blood glucose and hematological parameters). Besides, it is important to point out that the immature CC could show greater concentrations of metallothioneins, proteins that could bind metals and protect the tissue from the direct action of metal ions (Dang et al., 2000).

The increase in the number of CC in fish exposed only to acid water, at all experimental periods may be related to the maintenance of acid–base balance (Clairborne et al., 2002; Sakuragui et al., 2003; Hwang and Lee, 2008), rather than directly related to ion uptake in these fish. Takasusuki et al. (2004) established a high tolerance for changes in water pH for *P. lineatus*, however, a pH of 4.5 is more stressful to this fish species than one of 8.0.

Until now, the exact role of CCs in the transport of Cl^- and Na^+ across the gills and in the acid–base regulation is not well established. The exchange of HCO_3^- for Cl^- together with the H^+/ATPase to eliminate H^+ creates an electrical potential that favours the influx of Na^+ (Hwang and Lee, 2008). This may explain, at least in part, the increasing of these cells in *P. lineatus* exposed to low water pH, allowing fish to maintain an efficient active excretion of H^+ and ion regulation. H^+/ATPase has been found in both the CC and pavement cells of the gill (Goss et al., 1998; Clairborne et al., 2002; Hwang and Lee, 2008).

In summary, the present study points out relevant results of the toxicity of Al in acid water to a neotropical fish species, showing that *P. lineatus* experienced osmoregulatory disturbances. The causes of ionic unbalance is probably related to the effects of Al on Na^+/K^+–ATPase activity, on the distribution and number of chloride cells in the gills as well as the effects associated with the stress response caused by the presence of the metal.

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