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RESEARCH ARTICLE

High waterborne Mg does not attenuate the toxic effects of Fe, Mn, and Ba on Na^+ regulation of Amazonian armored catfish tamoatá (*Hoplosternum litoralle*)

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Abstract

Formation water (FoW) is a by-product from oil and gas production and usually has high concentrations of soluble salts and metals. Calcium (Ca) and magnesium (Mg) have been shown to reduce the toxicity of metals to aquatic animals, and previous study showed that high waterborne Ca exerts mild effect against disturbances on Na^+ regulation in Amazonian armored catfish tamoatá (*Hoplosternum littorale*) acutely exposed to high Fe, Mn, and Ba levels. Here, we hypothesized that high Mg levels might also reduce the toxic effects of these metals on Na^+ regulation of tamoatá. The exposure to 5% FoW promoted an increase in Na^+ uptake and a rapid accumulation of Na^+ in all tissues analyzed (kidney<plasma<gills<carcass<liver), besides increasing the branchial activity of both NKA and v-type H^+-ATPase in fish. High waterborne Mg lowered Na^+ efflux rates and markedly inhibited Na^+ uptake, and also reduced both NKA activity and newly Na^+ accumulation in gills of fish. High Fe levels increased Na^+ net losses and inhibited Na^+ uptake in tamoatá. The diffusive Na^+ losses and the newly accumulated Na^+ in gills were reduced in fish exposed to high Mn and Ba. High waterborne Ba also inhibited NKA in gills, while both high Mn and Ba inhibited v-type H^+-ATPase in kidney of tamoatá. High Mg did not lessen the toxic effect of Fe on Na^+ net fluxes, and reduced even more Na^+ uptake and the newly Na^+ accumulation in gills and plasma, and did not prevent the inhibition of both NKA and v-type H^+-ATPases in kidney. Furthermore, Mg did not attenuate the effect of Mn on inhibition Na^+ uptake, keeping the activity of v-type H^+-ATPase in kidney significantly lowered. High Mg levels mildly attenuated the effects of Ba in Na^+ balance by increasing the new accumulation of Na^+ in liver, and restore the activity of both NKA and v-type H^+-ATPase in gills of tamoatá. Overall, high waterborne Mg does not have a strong contribution to, or have only minor effects, in protecting tamoatá against disruptions in Na^+ regulation mediated by high Fe, Mn, and Ba levels.

Keywords Na^+ efflux · Na^+ uptake · Newly accumulated Na^+ · Formation water · Freshwater fish · Na^+/K^+-ATPase · V-type H^+-ATPase · Metal toxicity

Introduction

Formation water is a by-product from oil and gas production. It is separated from oil and gas on the drilling platform and re-injected into the well or discharged (Caliani et al. 2009). In general, formation water has high concentrations of soluble salts and metals (Woodall et al. 2003; Jackson and Reddy 2007; Manfra et al. 2007; Baldisserotto et al. 2012). The composition of formation

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water is distinguished among the production fields but it is commonly constituted by high levels of iron (Fe) and manganese (Mn) (Woodall et al. 2003; Pantaleão et al. 2006; Manfra et al. 2007; Baldisserotto et al. 2012), and in some areas also by high barium (Ba) levels (Jackson and Reddy 2007; Baldisserotto et al. 2012). In this context, the formation water extracted from the Urucu platform, located near the Urucu River (Amazon, Brazil), contains very high levels of ions, Fe, Mn, and Ba (Baldisserotto et al. 2012). In spite of the precautions observed in the crude oil mining area of Urucu River, the local environment remains vulnerable to crude oil spills or formation water leakage.

The Urucu river and its tributaries are "black water" environments, which are recognized as acidic ion-poor waters (see Sioli 1984; Cunha and Pascoaloto 2009 for more details on the ionic composition and physico-chemical properties of "black water" rivers). A very rich local ichthyofauna is commonly found in these black water rivers (Costa and Freitas 2013) that evolved at this unusual conditions, displaying singular adjustments in ion regulation mechanism, particularly those related to Na^+ and Cl^- homeostasis, to cope with the low waterborne ionic levels (Gonzalez et al. 2005). Thus, in a scenario of formation water leakage, the raise in levels of major cations and metals might disrupt the osmoregulatory balance of fish, having deleterious consequences for the organisms and affecting their fitness and survival, which might negatively impact the whole local biodiversity.

It is widely recognized that toxic action of metals to aquatic organisms involves morphophysiological disruptions in gill epithelium, resulting in impairments in either gas exchange and/or imbalances on ionic regulation of animals, particularly on the control of paracellular diffusive movements, increasing the net ionic losses of animals to the environment (Paquin et al. 2002; Niyogi and Wood 2004). Studies had reported that formation water might adversely affects the physiology of fish. Exposure to 0.1 or 1.0% formation water for 6 weeks induced gill lamellae fusion and increased whole body cortisol levels in the marine turbot (*Scophthalmus maximus*) (Stephens et al. 2000). Female Atlantic cod (*Gadus morhua*) fed feed-paste containing 500 mg formation water/kg for 20 weeks presented endocrine disruption (Lie et al. 2009). In addition, the tamoatá, *Hoplosternum littorale*, an armored catfish found in ion poor waters of the Amazon (Val and Almeida-Val 1995), exposed to 5% formation water showed a 51-fold increase on whole body Na^+ influx, and a significant increase in accumulation of Na^+ in gills, liver, kidney, and plasma in relation to the control fish kept at well water (Baldisserotto et al. 2012).

Hardness cations as calcium (Ca) and magnesium (Mg) have been shown to reduce the toxicity of metals (e.g., Cu^{2+}, Ag^+, Zn^+, Cd^{2+}, and Pb^{2+}) to aquatic animals, through the competition between cations and metals for the binding ligand sites (BL) on the organisms (e.g., ionic transporters proteins in gills, skin, and gut), which results in decreased free metal ion activity (Pagenkopf 1983; Paquin et al. 2002; Niyogi and Wood 2004). In fact, the competition between hardness cations and metals for the binding to the BL decrease the amount of metal burden in tissue, leading to lower acute toxicity to aquatic organisms (i.e., reduced LC_{50}–96 h) (Pagenkopf 1983; De Schamphelaere and Janssen 2002; Paquin et al. 2002; Niyogi and Wood 2004). In addition, hardness cations at high waterborne levels can also reduce the toxicity of metals by controlling the branchial permeability to ions, decreasing the metal action on its toxic sites at the paracellular tight junctions, and lowering the ionic diffusive losses (Paquin et al. 2002; Niyogi and Wood 2004).

Previous study has shown that high waterborne Ca levels, similar to those found in 5% of formation water extracted from the Urucu Reserve, attenuated the negative effect of high waterborne Fe and Mn levels from the formation water on Na^+ fluxes of tamoatás (Baldisserotto et al. 2012). Tamoatás exposed to high waterborne Fe and Mn in combination with high Ca exhibited no stimulation in diffusive paracellular Na^+ losses, which was seen in fish exposed to Fe and Mn alone, and also avoided an compensative increase in Na^+ uptake in fish exposed to Mn + Ca. In addition, high Ca levels also help fish to avoid an excessive accumulation of Na^+ in liver following exposure to both Fe and Mn, which was also seen in kidney, plasma, and carcass of fish exposed to Mn + Ca (Baldisserotto et al. 2012). This formation water also present high Mg levels (Baldisserotto et al. 2012), but there is sparse information about the role of high Mg levels on the toxicity of metals in formation water to freshwater fish. Although the influence of Mg in reducing metals toxicity is thought be lower than Ca, mainly by the weaker binding affinity of Mg than Ca to the toxic sites in gills (Niyogi and Wood 2004), there are some evidences that Mg is an important factor modifying the acute toxicity of metals to freshwater organisms, as seen to *Daphnia magna* (De Schamphelaere and Janssen 2002), *Pimephales promelas* (Santore et al. 2001), *Oncorhynchus mykiss*, and *Oncorhynchus tshawytscha* (Welsh et al. 2000). However, there is a lack of information about the role of high Mg levels on acute toxicity of the most abundant metals in formation water to aquatic organisms, specially on its combined effects on the mechanisms for Na^+ regulation, which is one of the main target for metals toxic action in freshwater fishes. Thus, we hypothesized that high Mg levels also reduce the deleterious effect of the most abundant metals (Fe, Mn, and Ba), in similar levels to 5% of formation water, on Na^+ regulation of tamoatá. Therefore, our study was designed to assess Na^+ accumulation in several internal compartments, branchial Na^+ unidirectional fluxes, and internal distribution of radio-labeled waterborne Na^+ uptake in juvenile tamoatá short-term exposed (3 h) to 5% of formation water, to high levels of Mg, Fe, Mn, and Ba separately, and in combination to Mg. In addition, we also evaluated the effect of short-term exposure to 5% of formation water, high Mg, and these metals separately and in

combination to Mg on gill and kidney Na^+/K^+ ATPase (NKA) and v-type H^+ATPase activities of tamoatá.

Material and methods

Experimental animals

Juvenile tamoatás (*Hoplosternum litoralle*) (mean weight 46.1 ± 3.1 g) were collected from earth tanks of Embrapa (Empresa Brasileira de Pesquisa Agropecuária) fish culture facility (Manaus, AM, Brazil). Fish were transported to the Laboratory of Ecophysiology and Molecular Evolution, Brazilian National Institute for Amazonian Research, and maintained in aerated 500 L tanks with ion-poor well water (Table 1) for at least 14 days. Fish were fed commercial food, 28% crude protein, until apparent satiety once a day, and fasted for a day prior to the experiments. All experimental procedures were performed in accordance with INPA's animal care guidelines and were previously approved by INPA's animal care committee (047/2012).

Na^+ flux experiments

A series of Na^+ unidirectional flux analysis were conducted to evaluate the effects on Na^+ regulation associated with short-term exposure to formation water or Mg contained therein. Fish were exposed to 5% formation water, as well as to well water spiked with Mg and major metals (Fe, Mn, Ba) separately and in combinations to Mg (i.e., Fe + Mg, Mn + Mg, and Ba + Mg) at concentrations consistent with those seen in 5% formation water, or unspiked well water (control). The concentrations of metals, major ions, and pH in formation water, diluted formation water, and well water were determined prior to starting the experiments and are presented in Table 1. This concentration of the formation water is the highest one that does not kill any tamoatá within 24 h (Baldisserotto et al. 2012).

Table 1 Concentration of major ions and metals (mg/L) of formation water and well water from the Brazilian National Institute for Amazonian Research (Manaus/Brazil)

	Formation water	Formation water 5%	INPA's well water
pH	6.43	5.35	5.5
Na	41,114.5	2056	2.530
K	982.8	45.58	1.560
Ca	17,336.0	866.80	0.440
Mg	1452.0	72.48	0.022
Cl	1917.0	95.85	1.952
Fe	1142.4	57.12	<0.001
Mn	264.0	13.20	<0.001
Ba	5.48	0.274	<0.001
DOC	n.d	n.d	<0.9

DOC dissoveld organica carbon, *n.d* not detected

Juvenile fish were weighed and transferred to 400 ml (one juvenile per chamber, $N = 8$ per group) flux chambers containing well water. After a 2 h settling period, the water in the chamber was completely removed and exchanged for the treatment water, which was previously prepared by adding appropriate aliquots of concentrate stock solutions ($MgCl_2$, $FeCl_3$, $MnCl_2$, and $BaCl_2$) in well water and kept under room temperature to achieve the chemical equilibrium of metals in solution. Then, 1.0 μCi/L ^{22}NaCl (GE Healthcare) was added to each chamber, and following 10 min of mixing by aeration, a 3 h flux measurement was started. At 0 and 3 h later, a 5-ml water samples were taken in replicate, one for measuring the total amount of Na^+ and metals in each treatment, and another 5 ml water sample for counting ^{22}Na radioactivity. All sampled water for metals analysis during the flux measurements were acidified with 100 μl of concentrated HNO_3. After the experiment, fish were anesthetized with buffered MS-222 (0.5 g/L of MS-222 and 2.0 g/L of $NaHCO_3$, Sigma Aldrich) and blood was collected from the caudal vein with 1 ml syringes treated with lithium heparin (Sigma-Aldrich, St. Louis, MO, USA). Blood samples were centrifuged at 10,000 *g* for 5 min to separate plasma. Juveniles were then euthanized by spinal cord section, and gills, kidney, and liver were dissected and weighed separately. The remaining structures were considered "carcass." Aliquots of all tissues were then separated for ^{22}Na radioactivity analysis (for newly accumulated Na^+).

For counting ^{22}Na, tissues (gill, carcass, plasma, liver, and kidney) were processed as described by Baldisserotto et al. (2012). Samples were counted on a liquid scintillation counter (LS 6500 Beckmann, Fullerton, CA). Counting efficiencies for ^{22}Na were determined by internal standardization, i.e., by addition/recovery of known amounts of ^{22}Na. Newly accumulated Na^+ and Na^+ unidirectional fluxes were calculated following procedures described in details by Baldisserotto et al. (2012). Briefly, newly accumulated Na^+ of each fish at each experimental condition were calculated as follows:

$$M_{new} = a/(b/c),$$

where M_{new} is the newly accumulated Na^+ (μmol/g tissue), a is the counting of ^{22}Na per minute (cpm) per gram of tissue or mililter of plasma as appropriate, b is the number of cpm per liter of water, and c is the total Na^+ concentration per liter of water (Grosell et al. 1997).

Unidirectional and net Na^+ flux rates (in μmol/kg/h) were measured following procedures described in detailed by Wood (1992), and Na^+ influx rates (J^{Na}_{in}) of fish at each experimental condition were calculated as follows:

$$J^{Na}_{in} = \left(cpm_i - cpm_f\right) * V \;/\; \left(SA^* T^* W\right),$$

where cpm_i is the counting of ^{22}Na (cpm/ml) at the start of flux

period, cpm_f is the counting of ^{22}Na (cpm/ml) after 3 h of exposure to each treatment, V is the volume of water in the experimental chamber (ml), T is the flux flux period (h), W is the wet mass of fish (kg), and SA is the mean specific activity of the radioisotope (cpm/μmol) in water samples, which was determined as the mean ratio between the counting of ^{22}Na (cpm/ml) and the concentration of total Na^+ in water (μmol/ml). The Na^+ net flux rates (J^{Na}_{net}) of fish at treatment were calculated as follows:

$$J^{Na}_{net} = (X_1 - X_2) * V / (T^* W),$$

where X_1 and X_2 were, respectively, the initial and final total Na^+ concentration (μmol/ml) in the water during the flux period. Unidirectional Na^+ efflux rates (J^{Na}_{out}) of fish at each treatment were then calculated as follows:

$$J^{Na}_{out} = J^{Na}_{net} - J^{Na}_{in}$$

Branchial and renal activity of Na^+/K^+-ATPase and v-type H^+-ATPase

The second gill arch and kidney of each fish were collected and stored at − 80 °C until the analysis of ATPase activities could be performed. NKA and v-type H^+-ATPase activities were measured simultaneously using the basic procedure described by Kültz and Somero (1995), in a assay based on the oxidation of reduced NADH by an enzymatic reaction coupled to the hydrolysis of ATP. Briefly, frozen gill and kidney samples were homogenized in ice-cold SEID buffer (150 mM sucrose, 50 mM imidazole, 10 mM EDTA, 0.5% Na-deoxycholate, pH 7.5) at 1:10 wet sample mass to buffer volume. Crude homogenates were then centrifuged (4 °C, 2000 g) for 10 min and the supernatant was collected for the enzymatic assay. The supernatant (5 μl) were added to 12 wells of a 96-well microplate and incubated with reaction solution (30 mM imidazole, 45 mM NaCl, 15 mM KCl, 3 mM $MgCl_2 \cdot 6H_2O$, 0.4 mM KCN, 1.0 mM ATP, 0.2 mM NADH, 0.1 mM fructose 1,6 diphosphate, 2 mM phosphoenolpyruvate, 3 IU/ml pyruvate kinase, and 2 IU/ml lactate dehydrogenase). Four out of 12 wells then received the reaction solution with 2 mM ouabain, while crude homogenate in another 4 wells received the reaction solution plus 2 mM *N*-ethylmaleimide. The rate of NADH oxidation was monitored every 10 s over 10 min at 340 nm, at room temperature. The slope difference in the rate of NADH oxidation versus time between reactions with solutions which were inhibitor-free versus inhibitor-enriched (ouabain and *N*-ethylmaleimide) were used to determine NKA and v-type H^+-ATPase activity, respectively. Both enzyme activities have been reported as μmol/h mg protein. Protein concentrations in crude homogenates of gills and kidney were determined using the Coomassie blue method, according to Bradford (1976).

Water and plasma analysis

Total levels of major cations (Na^+, K^+, Ca^{2+}, and Mg^{2+}) and metals (Fe, Ba, Mn) in the water samples were analyzed using atomic absorption spectrophotometry (AAnalyst 800, Perkin-Elmer, Wellesley, MA). Chloride concentrations in water samples were determined by the mercuric thiocynate colorimetric assay described by Zall et al. (1956). Standard solutions were made with analytical grade reagents (Merck) dissolved in deionized water, and each standard curve was made with five different concentrations.

Statistical analysis

Data were reported as mean ± standard error of mean (SEM). Homogeneity of variances among groups was tested with the Levene test. Data did not present homogeneous variances; consequently, comparisons among different treatments were made using Kruskal-Wallis one-way ANOVA test followed by the multiple comparison of mean ranks for all groups. Analyses were performed using the software Statistica (version 7.0), and the minimum significance level was set at $P < 0.05$.

Results

Unidirectional Na^+ fluxes

Juveniles tamoatás maintained in well water showed both high influx (J^{Na}_{in}) and efflux (J^{Na}_{out}) rates resulting in a slightly negative net flux (J^{Na}_{net}) (i.e., small Na^+ net loss). Fish exposed to 5% formation water presented an increase of 26-fold in J^{Na}_{in}. Fish exposed to both high waterborne Mg and Fe levels showed significant inhibition in J^{Na}_{in} compared to those animals maintained in well water; however, only fish exposed to high Fe levels presented significant stimulation of Na^+ net losses (J^{Na}_{net}) compared to fish maintained in well water. Interestingly, the exposure to high waterborne Fe + Mg levels reduced even more J^{Na}_{in} in comparison with fish at well water, but did not change significantly the J^{Na}_{out} resulting in J^{Na}_{net} similar to those seen in fish at high waterborne Fe levels, which were significantly higher than in control fish (Fig. 1). Exposure to high waterborne Mn levels decreased J^{Na}_{out}, and high waterborne Mn + Mg levels did not change this effect. High waterborne Ba levels reduced significantly J^{Na}_{out}, but did not alter J^{Na}_{net} compared to fish maintained in well water. The exposure to high Ba + Mg levels reduced J^{Na}_{in} compared to control fish, but did not alter significantly compared to those exposed to high waterborne Ba levels (Fig. 1).

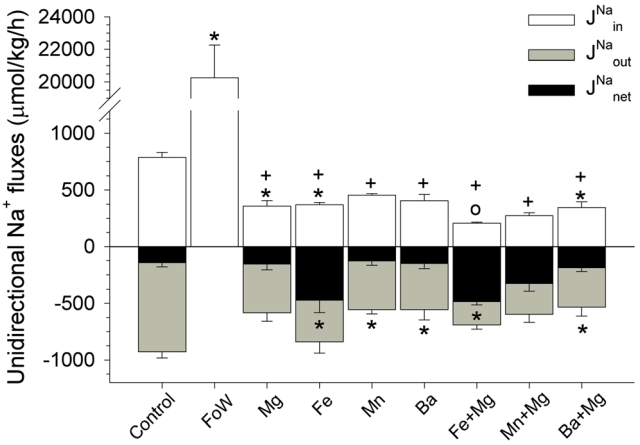

Fig. 1 Sodium influxes (J^{Na}_{in}), effluxes (J^{Na}_{out}), and net fluxes (J^{Na}_{net}) rates of *Hoplosternum littorale* acutely exposed (3 h) to well water (control), 5% formation water (FoW), and well water added with respective concentrations of 5% formation water of Mg, Fe, Mn, Ba separately and in combination to Mg (i.e., Fe + Mg, Mn + Mg, and Ba + Mg) ($N = 8$). (Asterisk) significantly different from control ($P < 0.05$). (Plus sign) significantly different from fish exposed to 5% formation water ($P < 0.05$). (White circle) significantly different from fish exposed to the same metal and low Mg ($P < 0.05$)

Newly accumulated Na^+

Newly accumulated waterborne Na^+ in fish maintained in well water was higher in the gills and plasma, followed by the liver, kidney, and carcass (i.e., gill>plasma>liver>kidney>carcass), evidencing a rapid transfer of this ion from the gills to the plasma and from the plasma to the tissues (Fig. 2). Specimens of tamoatá exposed to 5% formation water presented higher newly accumulated Na^+ in the plasma than those maintained in well water. Newly accumulated Na^+ increased 120-fold in the kidney and 10- to 27-fold in the gills, liver, plasma, and carcass of tamoatá exposed to 5% formation water (Fig. 2). Tamoatá exposed to high Mg showed lower newly accumulated Na^+ in the carcass compared to those maintained in well water. Exposure to high waterborne Fe + Mg significantly reduced newly accumulated Na^+ in the gills, plasma, and carcass compared to those maintained in well water. Tamoatá exposed to high waterborne Mn + Mg and Ba + Mg levels showed lower newly accumulated Na^+ in the gills, liver (only those exposed to Mn + Mg), and carcass compared to those maintained in well water. Fish exposed to high Fe + Mg and Mn + Mg levels also presented lower newly accumulated Na^+ in the plasma than specimens of tamoatá exposed to high waterborne Fe and Mn levels, respectively (Fig. 2).

When organ-specific newly accumulated Na^+ was expressed relative to total body load, in all groups carcass represented the greatest percentage, followed by the gills, liver, and kidney. Fish exposed to 5% formation water and high Ba + Mg presented a comparatively higher percentage of newly accumulated Na^+ in the gills and liver compared to the other treatments (Fig. 3).

Na^+/K^+-ATPase and v-type H^+-ATPase in gills and kidney

Specimens of tamoatá exposed to 5% formation water increased 1.5-fold and 1.8-fold of gill NKA and v-type H^+-ATPase activities, respectively, compared to fish in well water (Fig. 4). The exposure to both high waterborne Mg and Ba levels significantly reduced gill NKA activity of fish by 2.2-fold on average compared to animals maintained in well water (Fig. 4a). Fish exposed to high waterborne Ba + Mg levels increased 2.1-fold on average in both NKA and v-type H^+-ATPase activities in gills in relation to those exposed to high waterborne Ba levels (Fig. 4).

There is no significant effect of 5% formation water exposure on the activity of both NKA and v-type H^+-ATPase in kidney compared to fish in well water (Fig. 5). The exposure to both high waterborne Fe and Fe + Mg levels reduced in 3.0-fold and 6.7-fold the activity of NKA in kidney, respectively, in relation to fish maintained in well water (Fig. 5a). Interestingly, fish exposed to high waterborne Mg, major metals (Fe, Mn, Ba) individually, and in combination to Mg (Fe + Mg, Mn + Mg, and Ba + Mg) exhibited a significant inhibition of v-type H^+-ATPase activity in kidney compared to fish at well water (Fig. 5b). In this regards, the activity of v-type H^+-ATPase in kidney of tamoatá were lowered by 4.3-fold, 2.2-fold, 4.9-fold, and 3.3-fold in fish exposed to high waterborne Mg, Fe, Mn, and Ba levels, respectively. In addition, an inhibition of 6.7-fold, 2.6-fold, and 3.4-fold were seen in fish exposed to Fe + Mg, Mn + Mg, and Ba + Mg, respectively (Fig. 5b).

Discussion

Net fluxes of whole-body Na^+ observed in tamoatá in well water was slightly negative, indicating net losses of Na^+ that were close to the values of J^{Na}_{net} previously reported for this species at similar water quality conditions (Baldisserotto et al. 2012). Nevertheless, in the present study, Na^+ influx and efflux rates were somewhat higher in tamoatá at well water. The exposure to 5% formation water for 3 h caused an increase of 25-fold in J^{Na}_{in} in specimens of tamoatá and, as demonstrated by new Na^+ accumulation, was enough to cause an accumulation of Na^+ in the gills and a transfer of this ion to plasma and later to kidney and liver, but not to the carcass, as observed previously (Baldisserotto et al. 2012). Interestingly, the stimulation of Na^+ uptake displayed by tamoatá exposed to 5% formation water was associated to significant increases in gill NKA and v-type H^+-ATPase activities, suggesting that both transporters are directly involved in transcellular Na^+ uptake in gills of tamoatá.

It is widely proposed that changes in waterborne Mg, like Ca ions, might alter the fluidity of cellular membranes, the

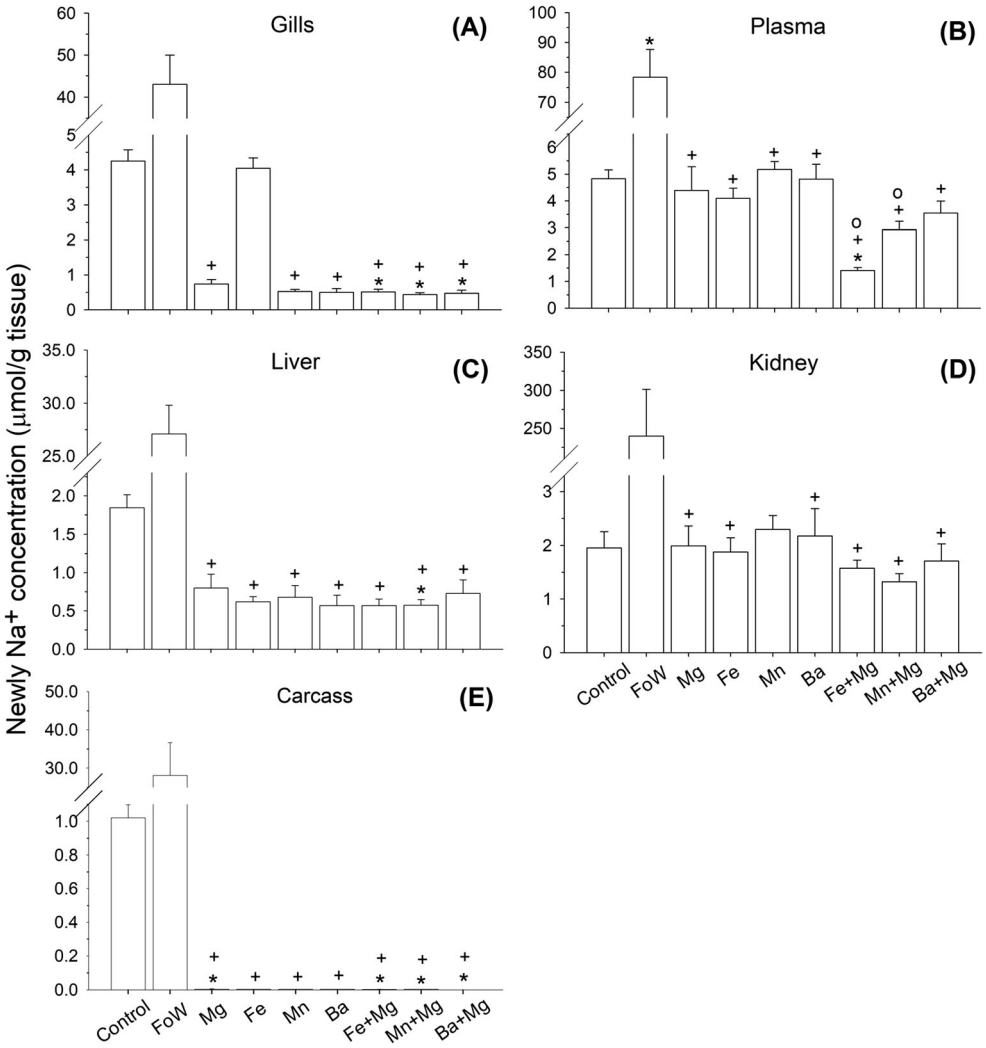

Fig. 2 Newly accumulated Na^+ concentrations in gills (**a**), plasma (**b**), liver (**c**), kidney (**d**), and carcass (**e**) of *Hoplosternum littorale* acutely exposed (3 h) to well water (control), 5% formation water (FoW), and well water added with respective concentrations of 5% formation water of Mg, Fe, Mn, Ba separately and in combination to Mg (i.e., Fe + Mg, Mn + Mg, and Ba + Mg) ($N = 8$). (Asterisk) significantly different from control ($P < 0.05$). (Plus sign) significantly different from fish exposed to 5% formation water ($P < 0.05$). (White circle) significantly different from fish exposed to the same metal and low Mg ($P < 0.05$)

permeability of branchial epithelium, and the diffusive ionic losses from fish to the freshwater environment (Bijvelds et al. 1998). In the present study, high waterborne Mg did not reduce the diffusive paracellular efflux of Na^+ in tamoatá, which was kept in similar rates than those seen in fish at well water. Further, high Mg levels also did not change J^{Na}_{net} in tamoatá. Similarly, high Mg levels had only minor effects on the control of Na^+ diffusive losses in brown trout (*Salmo trutta*) exposed to low pH conditions, in comparison to Ca effects (Brown 1981). Unlike Ca ions, our data do not evidence that high Mg levels exert a strong effect on controlling J^{Na}_{out} in tamoatá, which did not support our initial hypothesis that high Mg would lower the intrinsic permeability of gills, acting as a primary mechanism for the control of ionic diffusive efflux.

Tamoatá exposed to high Mg levels showed a substantial inhibition of J^{Na}_{in} that was accomplished by reduced gill NKA activity, resulting in lowered new Na^+ accumulation in gills of fish. Although Mg has been recognized to play an important role in ionic regulation of freshwater fishes (Cuthbert and Maetz 1972; Bijvelds et al. 1998), our data evidence that the high Mg levels, in similar levels of 5% of formation water, reduce the transcellular movements of Na^+ in gills of tamoatá, particularly through a mechanism mediated by the activity of NKA. However, the exact mechanism for disruption of Na^+ uptake mediated by Mg in tamoatá remains unclear. Increased Mg levels might directly affect the catalytic reactions of enzymes (Bijvelds et al. 1998) through the inactivation of phosphorylation via protein kinase A and cAMP, which has been demonstrated to be involved in the rapid activation of NKA activity (Tipsmark and Madsen 2001). Future studies evaluating species-specific effects of Mg on Na^+ uptake transport system might help understand the mechanisms for the inhibition of J^{Na}_{in} in tamoatás. Additionaly, specimens of tamoatá also exhibited a significant inhibition of H^+-ATPase activity in kidney following exposure to high Mg levels. In freshwater fish, kidney has an important role in acid-base regulation through the acid secretion that would appear to be driven by a mechanism coupling multiple transporters, as the v-type H^+-ATPase, carbonic anhydrase (CA), and Na^+/H^+ exchanger (NHE), which has been associated with increased HCO_3^- reabsorption through the Na^+/HCO_3^- co-transporter (NBC family) during acidosis (Perry and Fryer 1997; Perry et al. 2003).

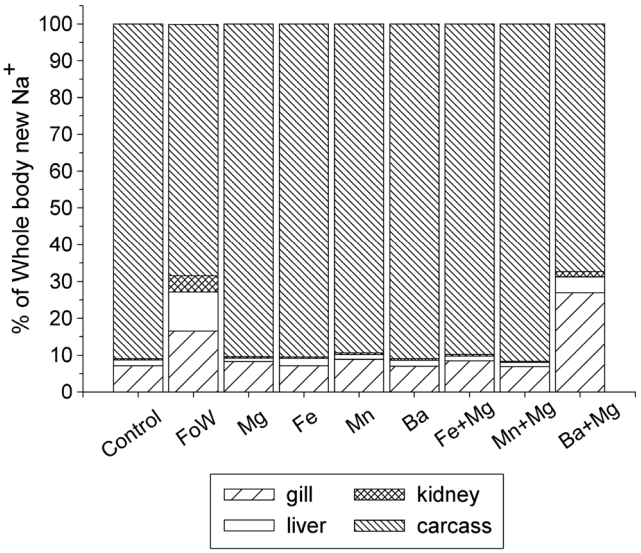

Fig. 3 Partitioning of whole body new Na^+ in *Hoplosternum littorale* acutely exposed (3 h) to well water (control), 5% formation water (FoW), and well water added with respective concentrations of 5% formation water of Mg, Fe, Mn, Ba separately and in combination to Mg (i.e., Fe + Mg, Mn + Mg, and Ba + Mg), expressed as the relative contribution (i.e., mass-weighted contribution of each organ) ($N = 8$)

In this model, intracellular CO_2 is hydrated to H^+ and HCO_3^- by CA, where the required H^+ to titrate HCO_3^- in renal tubular lumen is driven by either v-type H^+-ATPase and/or NHE, while intracellular HCO_3^- is moved to blood by NBC (Perry and Fryer 1997; Perry et al. 2003). Thus, although the present study did not evaluate the acid-base status of tamoatá, the inhibition of renal v-type H^+-ATPase might imbalance the mechanisms for acid-base regulation in kidney of fish.

In the present study, as previously reported by Baldisserotto et al. (2012), exposure to high Fe levels results in significant net losses of Na^+, which owes to significant inhibition of Na^+ uptake in tamoatá (around 53%), as J^{Na}_{out} is not stimulated (Fig. 1). However, in the previous study, the negative J^{Na}_{net} seen in fish was associated to a strong stimulation of J^{Na}_{out}, though Na^+ uptake was also up-regulated in tamoatá (Baldisserotto et al. 2012). It was already evidenced that the exposure of rainbow trout (*Oncorhynchus mykiss*) to 1–6 mg/l Fe for 1–2 days resulted in damages of gill epithelium, as epithelial lifting, hypertrophy, and tissue degeneration (Fish 2009). In addition, reduced whole body Na^+ levels have been reported to brook charr (*Salvelinus fontinalis*) exposed to sub-lethal concentrations of Fe (Gonzalez et al. 1990). Overall, our data corroborate these previous studies with temperate fish species, evidencing that a potential source of Fe toxicity in freshwater fishes is through the disruption of Na^+ balance in animals.

In gills of freshwater fishes, iron uptake appears to be closely linked to a v-type H^+-ATPase, which would generate a proton gradient that facilitate the apical ferrous iron uptake via a divalent metal transporter (DMT) (Bury and Grosell 2003a; Bury and Grosell 2003b). In zebrafish (*Danio rerio*), reduced whole-body Fe uptake was evidenced after exposure to bafilomycin A (Bury and Grosell 2003a; Bury and Grosell 2003b), a specific inhibitor of v-type H^+-ATPase that has been demonstrated to inhibit Na^+ uptake in gills of several freshwater fishes (Lin and Randall 1993; Fenwick et al. 1999; Boisen et al. 2003). Although the exposure to high Fe levels inhibit J^{Na}_{in} in tamoatá, there is no significant effect on gill NKA and v-type H^+-ATPase activities. In contrast, the activity of both NKA and v-type H^+-ATPase in kidney of tamoatá were significantly reduced following exposure to high Fe levels, which would likely result in both lowered Na^+ reabsorption and acid excretion in urine by fish. Nonetheless, the amount of newly accumulated Na^+ in various tissues of specimens of tamoatá exposed to high Fe levels was not significantly different from those seen in fish at well water, suggesting that high Fe levels did not disrupt the accumulation and distribution of Na^+ in tissues of tamoatá.

In the present study, the exposure of tamoatá to both high Mn and Ba significantly reduces J^{Na}_{out}, whereas values of both J^{Na}_{in} and J^{Na}_{net} remained similar to those seen in fish at well water. Additionally, the amount of newly accumulated Na^+ in gills was also reduced in tamoatá exposed to high Mn and Ba. Traditionally, the exposure to high Mn levels is recognized to cause an imbalance of Na^+ regulation in freshwater fishes, as *S. fontinalis* (Gonzalez et al. 1990) and brown trout (*S. trutta*) (Reader and Morris 1988), particularly through the stimulation of both J^{Na}_{in} and J^{Na}_{out} that results in lowered whole body and plasma Na^+ levels. Similarly, Baldisserotto et al. (2012) found that high Mn increase both J^{Na}_{in} and J^{Na}_{out} promoting an increase in newly Na^+ accumulation in gills, plasma, liver, and kidney of tamoatá. Instead, Ba ions has not been previously evidenced as disruptors of Na^+ regulation in gills of tamoatá (Baldisserotto et al. 2012). As pointed out above, it is widely reported that the intrinsec branchial permeability to ions is controlled by Ca waterborne levels, which is mainly dependent of the binding of Ca ions to the paracellular tight junctions (TJs) in gills (McDonald et al. 1983; Hunn 1985; Freda and McDonald 1988). Although it is expected that divalent metals displace Ca ions from binding sites in TJs of gills and stimulates the diffusive losses of Na^+ (Spry and Wood 1985; Reader and Morris 1988; Playle et al. 1993), our data indicate that, at least under short-term exposure, high Mn and Ba levels might render branchial epithelium of tamoatá less permeable to Na^+. To our knowledge, this is the first time that this phenomenon has been documented, and future studies are required to confirm the effects of high Mn and Ba on reducing Na^+ permeability in gills of tropical freshwater fishes.

Surprisingly, specimens of tamoatá exposed to high Ba displayed a significant inhibition of NKA activity in gills, though no inhibition of J^{Na}_{in} was seen in fish. In a previous study, Hoffmann et al. (2002) have evidenced that Ba can act

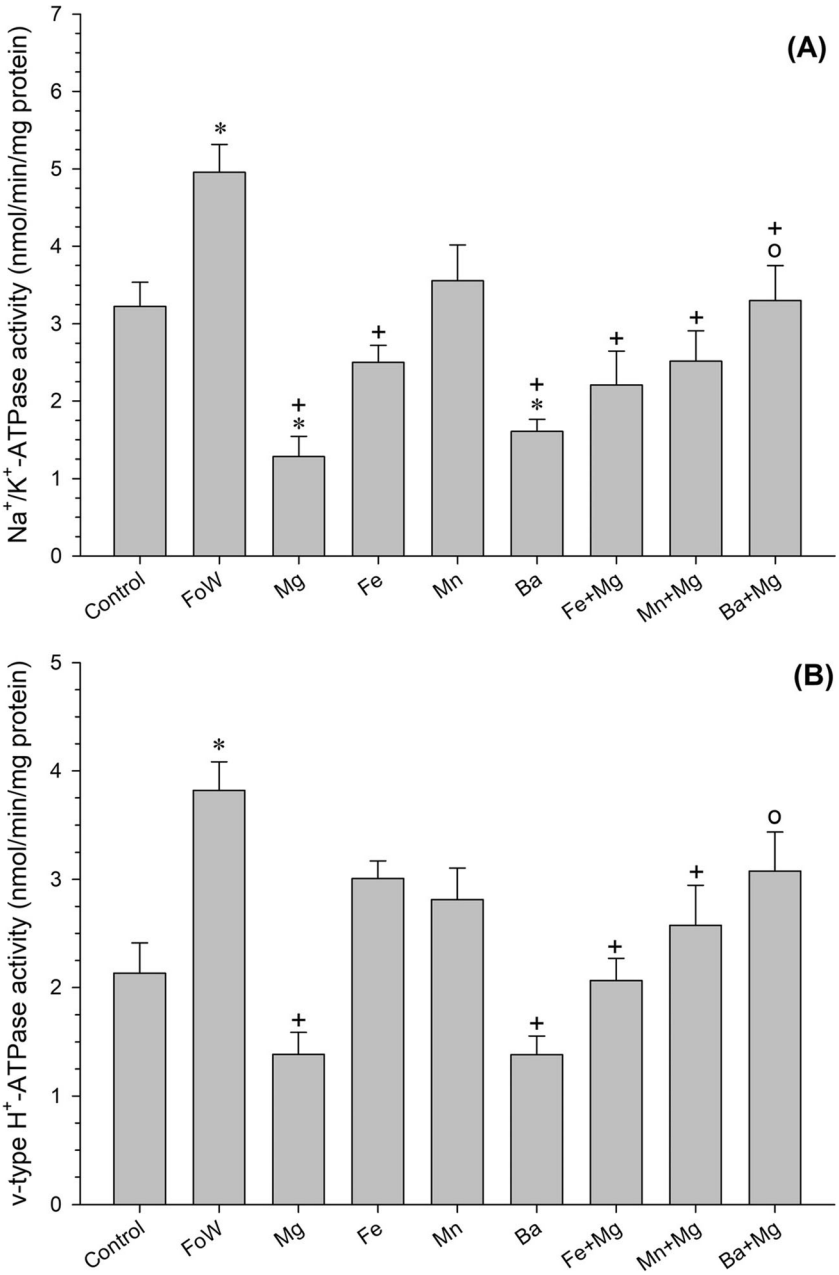

Fig. 4 Activity of branchial Na^+/K^+-ATPase (**a**) and v-type H^+-type ATPase (**b**) of *Hoplosternum littorale* acutely exposed (3 h) to well water (control), 5% formation water (FoW), and well water added with respective concentrations of 5% formation water of Mg, Fe, Mn, Ba separately and in combination to Mg (i.e., Fe + Mg, Mn + Mg, and Ba + Mg) ($N = 8$). (Asterisk) significantly different from control ($P < 0.05$). (Plus sign) significantly different from fish exposed to 5% formation water ($P < 0.05$). (White circle) significantly different from fish exposed to the same metal and low Mg ($P < 0.05$)

blocking K^+ channels in basolateral membrane of operculum tissue of fish. General models for ion uptake in freshwater fish point out that diffusive movements of K^+ via channels in basolateral membrane of ionocytes keep constant intracellular K^+ levels, once NKA actively pumps K^+ ions inside the cells (Marshall 2002). Thus, the effect of Ba ions in blocking K^+ channels is expected to decrease the activity of NKA in gills of fish, in order to maintain K^+ homeostasis and keep an intracellular negative potential in ionocytes. Additionally, the maintainace of the activity of v-type H^+-ATPase in gills of tamoatá exposed to Ba, in quite similar rates of control fish, is likely to have contributed for the no inhibition of J^{Na}_{in} seen in fish. On the other hand, a significant inhibition of v-type H^+-ATPase activity was also seen in kidney of tamotá exposed to both Mn and Ba, which might play a negative role on renal acid secretion of fish under this conditions.

Overall, the present study reinforces that exposure to 5% formation water, and to Fe, Mn, and Ba at similar levels found in 5% formation water, is harmful to tamoatá by promoting imbalances in Na^+ regulation of fish. Also, the presence of high Mg levels barely attenuated the toxic effect of metals, particularly Fe and Mn, on Na^+ regulation in tamoatá. For instance, the high concentration of Mg present in formation water does not lessen the toxic effect of Fe on J^{Na}_{net}; it reduces even more Na^+ uptake and the newly Na^+ accumulation in gills and plasma, and does not prevent the inhibition of both

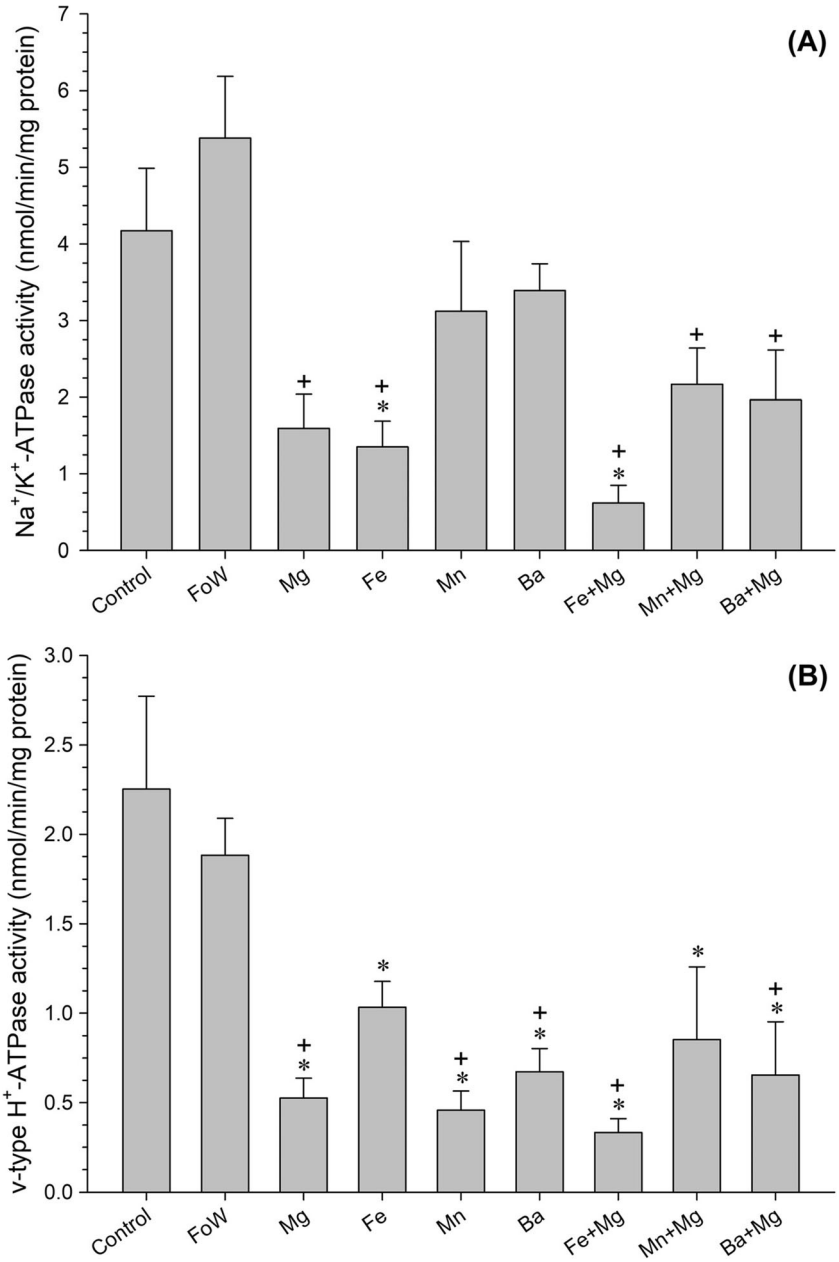

Fig. 5 Activity of Na^+/K^+-ATPase (**a**) and v-type H^+-type ATPase (**b**) in kidney of *Hoplosternum littorale* acutely (3 h) exposed to well water (control), 5% formation water (FoW), and well water added with respective concentrations of 5% formation water of Mg, Fe, Mn, Ba separately and in combination to Mg (i.e., Fe + Mg, Mn + Mg, and Ba + Mg) ($N = 8$). (Asterisk) significantly different from control ($P < 0.05$). (Plus sign) significantly different from fish exposed to 5% formation water ($P < 0.05$). (White circle) significantly different from fish exposed to the same metal and low Mg ($P < 0.05$)

NKA and v-type H^+-ATPases induced by Fe in kidney of tamoatá. Furthermore, in fish exposed to Mn + Mg, J^{Na}_{in} remained significantly reduced, in relation to control, while the new accumulation of Na^+ in plasma was reduced in comparison to exposure to high Mn. In these fish, the activity of v-type H^+-ATPase was kept at a significant lowered level relative to control. Despite the no recovery of J^{Na}_{in} in fish exposed to Ba + Mg, high Mg levels mildly attenuated the effects of Ba in Na^+ balance by increasing the new accumulation of Na^+ in liver, and restore the activity of both NKA and v-type H^+-ATPase in gills of tamoatá to levels close to control. However, high Mg levels do not soften the effects of Ba in inhibiting v-type H^+-ATPase activity in kidney of tamoatá.

Increases in waterborne Mg have been associated to reduction in toxicity (e.g., as LC_{50}–96 h) of metals (e.g., as Cu, Cd, and Zn) to freshwater organisms, by increasing competition of Mg with metals' ion for binding on the toxic action sites on biological membranes (Welsh et al. 2000; De Schamphelaere and Janssen 2002; Paquin et al. 2002; Santore et al. 2002). In contrast, our data indicate that high Mg levels, at similar level of 5% of formation water, might itself promote disruptions in Na^+ regulation of tamoatá, and does not seem to avoid the toxic action of Fe, Mn, and Ba on Na^+ balance of fish. In this regards, it is important to stress out that tamoatá has evolved in aquatic environments with reduced levels of Mg (Mg in Amazonian waters ranges from 0.5 to 2.2 mg/L in "white

waters," from 0.3 to 0.7 mg/L in "clear waters," and from 0.001 to 0.4 mg/L in "black waters") (Cunha and Pascoaloto 2009), and the high Mg concentration tested (around 13 mg/L) might be above the threshold causing deleterious effects on the ion regulation of this fish species. Thus, in case of formation water spill in the ion-poor waters of Amazon, the rise of metals (as Fe, Mn, and Ba) and micronutrients, as Mg, would adversely affect Na^+ homeostasis and would compromise the ion regulation of fish, which might turn into an additional challenge for fish of the Amazon.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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