Comparative Biochemistry and Physiology, Part A 166 (2013) 441–448

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Effect of low pH exposure on Na^+ regulation in two cichlid fish species of the Amazon

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ARTICLE INFO

Article history:
Received 7 June 2013
Received in revised form 21 July 2013
Accepted 21 July 2013
Available online 31 July 2013

Keywords:
Branchial H^+-ATPase activity
Intrinsic branchial permeability
Na^+ unidirectional fluxes
Na^+ uptake kinetics
Non-competitive inhibition

ABSTRACT

We evaluated the effects of acute exposure to low pH on Na^+ regulation in two Amazon cichlids collected from natural ion-poor "blackwaters", angelfish (*Pterophyllum scalare*) and discus (*Symphysodon discus*). Na^+ uptake kinetic parameters, unidirectional Na^+ fluxes, and net Cl^- fluxes were determined at pH 6.0 and 3.6. At pH 6.0, both species presented low unidirectional Na^+ flux rates, with kinetics showing a relatively low affinity for Na^+ (angelfish K_m = 79, discus K_m = 268 μmol L^{-1}), with similar maximum transport capacities (J_{max} ~ 535 nmol g^{-1} h^{-1}). Overall, there appeared to be high sensitivity to inhibition by low pH, yet low intrinsic branchial permeability limiting diffusive ion effluxes, resulting in relatively low net loss rates of Na^+, the same strategy as seen previously in other blackwater cichlids, and very different from the strategy of blackwater characids. At low pH, Na^+ uptake in angelfish was inhibited competitively (increased Km = 166 μmol L^{-1}) and non-competitively (decreased J_{max} = 106 nmol g^{-1} h^{-1}), whereas in discus, only a decrease in J_{max} (112 nmol g^{-1} h^{-1}) was statistically significant. An acute reduction in H^+-ATPase activity, but not in Na^+/K^+-ATPase activity, in the gills of angelfish suggests a possible mechanism for this non-competitive inhibition at low pH. Discus fish were more tolerant to low pH than angelfish, as seen by lesser effects of exposure to pH 3.6 on unidirectional Na^+ uptake and efflux rates and net Na^+ and Cl^- loss rates. Overall, discus are better than angelfish in maintaining ionic balance under acidic, ion-poor conditions.

1. Introduction

To maintain ion homeostasis, freshwater fishes have specialized branchial transport mechanisms to actively take up ions from the hypotonic surrounding media and a tight gill epithelium to control ionic diffusive losses through the paracellular tight junctions (Evans, 2011; Hwang et al., 2011). In freshwater fish gills, Na^+ transport is coupled to acid excretion and occurs either *via* an as yet uncharacterized apical Na^+ channel electrically linked to extrusion of H^+ by a V-ATPase (Avella and Bornancin, 1989; Lin and Randall, 1993), or through an electroneutral Na^+/H^+ (or Na^+/NH_4^+ or $H^+ + NH_3$) exchanger (NHE) (Kirschner, 2004; Hwang et al., 2011), or by a combination of these mechanisms. In both different pathways for Na^+ uptake, Na^+/K^+-ATPase in the basolateral membrane of gill ionocytes exports Na^+ into the blood and contributes to the electrochemical gradient for Na^+ movement across the apical membrane (Marshall, 2002; Kirschner, 2004; Evans, 2011). On other hand, diffusive losses of ions through paracellular pathways are commonly related to the tightness of the gill epithelium, which reflect the properties of gill tight junction protein complexes (Chasiotis et al., 2012), and the interaction of these tight junctions complexes with environmental factors, such as the external H^+ and Ca^{2+} concentrations (McWilliams, 1982; McDonald, 1983b; Freda and McDonald, 1988).

Acidic ion-poor waters are physiologically challenging to freshwater fish since the branchial mechanisms of Na^+ regulation are strongly modulated by the external conditions (Wood, 1989; Lin and Randall, 1991; Hwang et al., 2011). For example, inhibitions of the active uptake of Na^+, as well as stimulation of massive Na^+ diffusive losses, were previously reported in salmonid fish at low pH (Milligan and Wood, 1982; McDonald, 1983; Wood, 1992; Randall and Lin, 1993). More recent studies using zebrafish as a model for branchial Na^+ regulation have demonstrated that acidic and ion-poor conditions can modulate the pathways for Na^+ uptake, suggesting a differential involvement of Na^+ transporters (H^+-ATPase and/or NHE) in the maintenance of Na^+ balance during acclimation to these extreme environmental conditions (Boisen et al., 2003; Yan et al., 2007; Liao et al., 2009; Kumai and Perry, 2011; Kumai et al., 2011; Shih et al., 2012). With respect to paracellular Na^+ losses, low pH and ionic concentrations (particularly Ca^{2+} and Na^+) have a negative effect on gill permeability, since at high H^+ concentration, Ca^{2+} is displaced from the binding sites in paracellular tight junctions, resulting in increased Na^+ diffusive losses (McWilliams, 1982; McDonald et al., 1983b). However, high external Ca^{2+} concentrations limit the extent of stimulation of Na^+ diffusive losses, reducing

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1095-6433/$ – see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.cbpa.2013.07.022

the ionic and acid–base disturbances in freshwater fishes (McDonald et al., 1980; McWilliams, 1982; Gonzalez et al., 1998; Gonzalez and Wilson, 2001).

The adaptations to low pH of the fish endemic to the "blackwaters" of the Amazon basin are of particular interest. The Rio Negro and its tributaries drain an ancient alluvial flood plain; the waters here exhibit remarkably low ion concentrations (conductivity < 10 μS cm^{-1}), whereas the high concentration of aquatic humic substances (AHS), generated by the decomposition of allocthonous organic material from the surrounding forest, increases the water acidity (Junk, 1983; Furch, 1984; Val and Almeida-Val, 1995; Furch and Junk, 1997). Usually the pH in Amazon "blackwaters" ranges from 5.0–6.0, but in water bodies associated with flooded forest, as well as in the small streams from the uplands, pH can be as low as 3.0–4.0 (Walker, 1995). Despite the physiological challenges imposed by such extreme conditions, particularly with respect to maintenance of ionic balance, Amazon "blackwaters" possess unique fish diversity among tropical aquatic environments (De Pinna, 2006).

Previous studies (reviewed by Gonzalez et al., 2006) have demonstrated that Amazon teleost fish display several different strategies to maintain their Na^+ balance and thrive in these acidic ion-poor waters (Wood et al., 1998; Gonzalez and Preest, 1999; Wilson et al., 1999; Gonzalez et al., 2002; Matsuo and Val, 2007). For example, the acid tolerance seen in tambaqui (*Colossoma macropomum*) involves strong control of imbalances in branchial ion regulation mechanisms, particularly avoiding massive stimulation of net Na^+ and Cl^- losses with no significant declines in plasma Na^+ and Cl^- concentrations down to pH 4.0 (Wood et al., 1998; Wilson et al., 1999). Indeed, acid–base homeostasis was maintained even at pH 3.0. Furthermore, unidirectional fluxes of Na^+ and kinetic analyses of Na^+ uptake have revealed that the major component to maintain Na^+ balance in the characid fish *Paracheirodon axelrodi*, *Paracheirodon innesi*, *Gymnocorymbus ternetzi* and *Hemigrammus* sp. is an acid-insensitive Na^+ uptake system, which can maintain high Na^+ uptake rates in acidic ion poor conditions (high affinity for Na^+ uptake, *i.e.* low K_m). Notably, these same fish species display a weak control of intrinsic branchial permeability to ions, as seen as by the massive stimulation of Na^+ diffusive losses at low pH (Gonzalez et al., 1997; Gonzalez and Preest, 1999; Gonzalez and Wilson, 2001; Gonzalez et al., 2002). In contrast, an alternative pattern to maintain Na^+ balance under acidic conditions has been reported in the cichlid fishes angelfish (*Pterophyllum scalare*) and *Apistogramma* sp., which can maintain very low intrinsic branchial permeability, in order to reduce the overall ionic losses, yet their Na^+ uptake systems have much lower affinity (high K_m) and are extremely sensitive to inhibition by low pH exposure (Gonzalez and Wilson, 2001; Gonzalez et al., 2002).

The main goal of the present study was to analyze the patterns of Na^+ regulation, *i.e.*, Na^+ uptake kinetic parameters and unidirectional fluxes of Na^+, in the cichlid fish species angelfish (*P. scalare*) and discus (*Symphysodon discus*) under circumneutral conditions and during acute exposure to pH 3.5. We hypothesized that the same high K_m systems, with high sensitivity to inhibition by low pH, yet low intrinsic branchial permeability limiting diffusive ion losses would be seen, as in other cichlids. Net Cl^- fluxes were also measured as another indicator of overall ionoregulatory homeostasis. While Na^+ transport in *S. discus* has not been studied previously, *P. scalare* was investigated by Gonzalez and Wilson (2001). However, the authors noted that their experimental fish were obtained from commercial sources in North America, and may have been cultured there for many generations under unknown conditions. Therefore an additional objective of our study, which employed angelfish collected from natural "blackwaters", was to evaluate potential differences from this earlier study by comparing them with the strategies for Na^+ regulation in the gill of discus challenged by acute low pH exposure at ion-poor conditions. Furthermore, Na^+/K^+-ATPase and H^+-ATPase activities in the gills of angelfish were assessed during short-term (3 h) exposure to low pH, in order to test whether acid effects on the active transport of Na^+ could be explained by effects on these enzymes.

2. Materials and methods

2.1. Animals

Angelfish (*P. scalare*; 3.39 ± 0.11 g) and discus (*S. discus*; 40.47 ± 1.15 g), collected in the upper sector of the Rio Negro (00°309 S; 63°129 W), were donated by Turky's Aquarium (Manaus, Amazonas, Brazil), and held in plastic swimming pools in the laboratory of Ecophysiology and Molecular Evolution (LEEM, INPA) in local well water (in μmol L^{-1}: $Na^+ = 31$; $Cl^- = 49$; $K^+ = 10$, $Ca^{2+} = 9$; $Mg^{2+} = 4$; pH 6.0; temperature = 29 °C). All fish were maintained for one month under natural photoperiod and no mortalities were observed during the acclimation period. The fishes were fed dry food pellets (Nutripeixe, Purina) *ad libitum* but feeding was suspended for at least 48 h before starting the experimental period. All *in vivo* procedures followed INPA's animal care guidelines and were approved by INPA's animal care committee.

2.2. Na^+ uptake kinetics

In the first experimental series, the kinetic relationship between unidirectional Na^+ uptake rates and Na^+ concentrations in water was measured in angelfish and discus at neutral and low pH. Thus six fish of both species were transferred to individual aerated chambers (300 mL for angelfish and 1000 mL for discus) connected to a 150-L re-circulating system (flow = 0.15 L min^{-1} per chamber), and allowed to recover overnight in the same water as in the holding tanks (*i.e.* INPA's well water). To start the experiments, the reservoir was drained, refilled with INPA's well water and the pH was adjusted with 1% HNO_3. After 30 min of exposure to either pH 6.0 or pH 3.5, the flow was stopped, and the radioisotope ^{22}Na (as NaCl, PerkinElmer Life and Analytical Science, Boston, MA, USA) was added to each experimental chamber (0.27 and 0.45 μCi L^{-1} to angelfish and discus, respectively) in order to measure Na^+ influx over a 3-h period. Following a 15 min mixing period, two water samples (10 mL) were taken, and again at the end of the 3-h period. The same procedure was repeated with different animals in the other five higher external Na+ concentrations for angelfish (range of 81 to 764 μmol L^{-1}), and over four Na+ concentrations for discus (81 to 624 μmol L^{-1}) (N = 6 at each external Na^+ concentration). The external Na^+ concentration in the reservoir was adjusted to each desired level using a 1 M NaCl solution. At the highest external Na^+ concentration, the amount of ^{22}Na at each experiment was increased to 0.45 and 0.90 μCi L^{-1} for angelfish and discus, respectively, to increase precision and avoid large differences in the radioisotopic specific activity (SA) between the experimental flux periods.

Mean specific activity of the radioisotope (cpm μmol^{-1}) in water samples was determined as the ratio between concentration of ^{22}Na radioactivity (cpm mL^{-1}), and the concentration of total Na^+ in the water (nmol mL^{-1}) during a flux period. Influx rates (J_{in}; nmol g^{-1} h^{-1}) were based on the amount of ^{22}Na isotope incorporated by the fish during the experimental periods at each pH level, and calculated as:

$$J_{in} = (cpm_i - cpm_f)*V(SA*T*W)^{-1}. \quad (1)$$

where cpm_i = radioisotope cpm mL^{-1} at the beginning of flux period, cpm_f = radioisotope cpm mL^{-1} at the end of flux period, V = volume of water in the experimental chamber (mL), SA = mean specific activity of the isotope, T = flux period (h) and W = the wet mass of fish (g). Non-linear regression (Sigma Plot 11.0) was used to derive the kinetic parameters for Na^+ uptake — J_{max} (maximum Na^+ uptake rate) and K_m (the water Na^+ concentration

that yields an uptake rate of 50% of J_{max}) in gills of both angelfish and discus at neutral and low pH, using the Michaelis–Menten equation:

$$J^{Na}_{in} = \left[J_{max*}\left(Na^+\right)\right]\left[K_m + \left(Na^+\right)\right]^{-1}. \quad (2)$$

2.3. Unidirectional and net Na^+ fluxes, and net Cl^- flux rates

In the second experimental series, changes of unidirectional and net Na^+ flux rates (J_{in}, J_{out} and J_{net}) and net Cl^- flux rates were evaluated during acute exposure to pH 3.5 at the acclimation Na^+ concentration. The same experimental set-up as for Na^+ kinetic analysis was used, but the flux measurements were performed only in the external Na^+ concentration of INPA's well water (31 μmol L^{-1}). Thus, after the overnight recovery in the experimental chambers, the pH was adjusted with addition of 1% HNO_3 to the re-circulating system. After 30 min of exposure to either pH 6.0 or pH 3.5, the flow was stopped, and the radioisotope ^{22}Na was added to each experimental chamber (0.27 and 0.45 μCi L^{-1} to angelfish and discus, respectively). Two water samples (10 mL) were taken at the beginning of the flux period and after 3 h and 6 h of exposure to pH 6.0 and 3.5. The pH in each experimental chamber was monitored throughout the exposure period, and corrected when necessary with 0.1% HNO_3. Radioactivities and total Na^+ and Cl^- concentrations were measured in water samples, and the mean specific activity of the radioisotope and Na^+ J_{in} rates over the 0–3 h and 3–6 h periods were calculated as described in the previous section. Na^+ and Cl^- net fluxes rates (J_{net}) were assessed by measuring the gain or loss of Na^+ and Cl^- from the fish to the water, and were calculated as:

$$J_{net} = (ion_1 - ion_2)*V(T*W)^{-1} \quad (3)$$

where ion_1 and ion_2 were, respectively, the initial and final Na^+ or Cl^- concentrations (nmol mL^{-1}) in the experimental solution during the flux period. Efflux rates of Na^+ (J_{out}) were estimated by the difference between Na^+ net flux and influx rates:

$$J_{out} = J_{net} - J_{in}. \quad (4)$$

All calculations followed the equations described by Wood (1992).

2.4. Na^+/K^+-ATPase and H^+-ATPase activities

The third series was designed to assess the effects of low pH exposure on Na^+/K^+-ATPase and H^+-ATPase activities in gills of angelfish. Thus, 24 fish were transferred to individual aerated chambers (300 mL) connected to a 150-L re-circulating system, and allowed to recover overnight in INPA's well water. After 24 h, separate groups of six fish were exposed to pH 3.5 over 1, 2 and 3 h, while an additional group of six fish were sampled in INPA's well water with pH 6.0. All fish were killed with an overdose of buffered anesthetic (1 g L^{-1} MS-222 and 2 g L^{-1} $NaHCO_3$, Sigma Aldrich), and gills (whole gill baskets) were excised, frozen in liquid nitrogen, and stored at −80 °C prior to the analyses of Na^+/K^+-ATPase and H^+-ATPase activities using the basic procedure described by Kültz and Somero (1995). The assay is based on the oxidation of reduced NADH by an enzymatic reaction coupled to the hydrolysis of ATP. Briefly, frozen gill baskets 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 to run the enzymatic assay. The supernatant (5 μL) was 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^{-1} pyruvate kinase and 2 IU mL^{-1} lactate dehydrogenase). Four out of twelve wells then received the reaction solution with 2 mM ouabain, while crude homogenate in another four 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 that were inhibitor-free *versus* inhibitor-enriched (ouabain and N-ethylmaleimide) was used to determine Na^+/K^+-ATPase and H^+-ATPase activities, respectively. Both enzyme activities have been reported as μmol h^{-1} mg $protein^{-1}$. Protein concentrations in crude homogenates of gills were determined using the Bradford method (Bradford, 1976).

2.5. Analytical techniques

Radioactivity in water samples was analyzed in 5-mL aliquots mixed with 10 mL Ultima Gold scintillation counting cocktail (PerkinElmer Life and Analytical Sciences, Boston, MA, USA), and determined using a liquid scintillation counting (LS6500; Beckman and Coulter, Fullerton, CA, USA). Tests showed that quench was constant, so no correction was necessary. Ionic concentrations (Na^+, K^+, Ca^{2+} and Mg^{2+}) of INPA's well water and Na^+ concentrations in the experimental solutions were determined using atomic absorption spectrophotometry, flame mode (AAnalyst 800, PerkinElmer, Singapore). Cl^- concentrations were determined by the colorimetric assay of Zall et al. (1956).

2.6. Statistical analysis

All data are reported as means ± 1 SEM (N = 6). The kinetic parameters for Na^+ uptake (J_{max} and K_m) in both fish species at pH 6.0 and pH 3.5 were determined using non-linear regressions, and the calculated mean ± SEM of each parameter and the number of fish at each experimental condition (N) were used for posterior comparative analysis between both pH levels tested using a paired Student's *t*-test. Statistically significant differences of unidirectional Na^+ fluxes (J_{in}, J_{out} and J_{net}) and of Cl^- net fluxes were determined by a two-way ANOVA (fish species and pH levels were used as factors), followed by *a posteriori* Holm-Sidak test. A one-way ANOVA, followed by the *a posteriori* Dunnett's test, was used to determine the significance of differences in Na^+/K^+-ATPase and H^+-ATPase activities in gills of angelfish at pH 6.0 and over various times of exposure to pH 3.5. In all analyses, statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Na^+ uptake kinetics

In both angelfish and discus, unidirectional Na^+ uptake showed saturation kinetics with increasing Na^+ concentration in water, at both neutral and low pH (Fig. 1). This kinetic characterization of branchial Na^+ uptake revealed that both angelfish and discus had relatively low and virtually identical Na^+ uptake capacities (J_{max}) at pH 6.0 (approximately 535 nmol g^{-1} h^{-1}, respectively) (Table 1). Furthermore, during short-term (3 h) exposure to pH 3.5, the estimated Na^+ J_{max} was almost 80% inhibited in both angelfish and discus. Regarding the affinity for Na^+ uptake, both species exhibited relatively high K_m values, well above the Na^+ concentration (31.0 μ 1.2 mol L^{-1}) in the INPA's well water to which they were acclimated. Discus showed a 3.3-fold higher K_m (*i.e.* much lower affinity for Na^+ uptake) than the angelfish at both pH 6.0 and 3.5 (Table 1). At low pH, both angelfish and discus showed a reduction of branchial affinity for Na^+ uptake (almost 2-fold increase in K_m), though this change was statistically significant only in angelfish. Thus, Na^+ uptake in the gills of angelfish was competitively (increased K_m) and non-competitively (decreased J_{max}) inhibited (Fig. 1A). While the same trends were seen in discus,

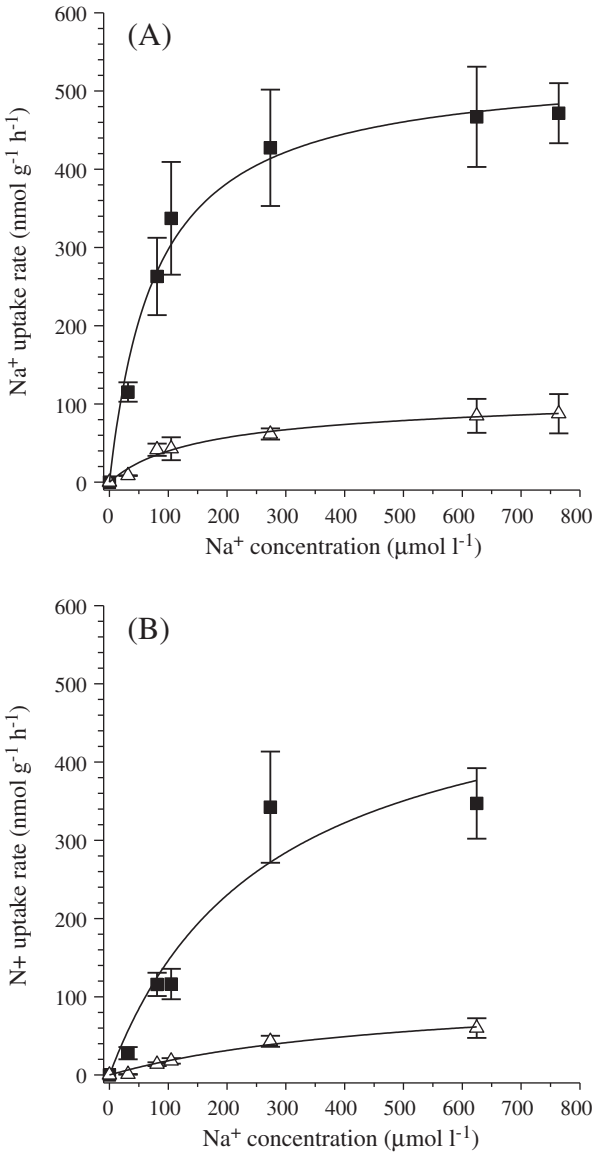

Fig. 1. Relationship between unidirectional Na^+ uptake rates (nmol g^{-1} h^{-1}) and Na^+ concentration in water for angelfish (A) and discus (B). Na^+ uptake rates in fish were measured at neutral pH (■) and at low pH (Δ). Means ± 1 SEM (N = 6).

only the non-competitive inhibition (decreased J_{max}) was significant (Fig. 1B).

3.2. Unidirectional and net Na^+ fluxes, and net Cl^- flux rates

These measurements revealed that overall angelfish were significantly less tolerant to low pH effects than discus at the acclimation water Na^+ (31.0 ± 1.2 μmol L^{-1}) and Cl^- (49.5 ± 0.7 μmol L^{-1}) concentrations (Figs. 2, 3, and 4). Although angelfish exhibited Na^+ J_{in} rates (on average 61.6 nmol g^{-1} h^{-1}) approximately 3-fold higher than discus at pH 6.0, Na^+ J_{in} in angelfish was on average 70% inhibited (on average18.7 nmol g^{-1} h^{-1}) after acute exposure to pH 3.5 (Fig. 2), similar to what would be predicted (89% inhibition) by the Michaelis–Menten relationship (2) using the kinetic constants reported in Table 1. Surprisingly, no inhibition in Na^+ J_{in} rates was observed in discus after 6 h in pH 3.5 (Fig. 3; on average 19.0 nmol g^{-1} h^{-1}), whereas the Michaelis–Menten constants of Table 1 would have predicted an 88% inhibition. In addition, Na^+ J_{in} was significantly higher in angelfish over 6 h in pH 6.0 than in discus, while at pH 3.5 Na^+ J_{in} presented similar low rates for both fish species. In neutral pH, Na^+ J_{out} rates were almost 1.5 times higher in angelfish than in discus, but were not statistically different. Moreover, the effects of low pH exposure on Na^+ efflux rates were less apparent in discus. The exposure for 3 and 6 h to pH 3.5 significantly stimulated Na^+ J_{out} in angelfish over 2.3 and 2.7 fold, respectively, relative to rates at the same time at pH 6.0 (Fig. 2B). However in discus, Na^+ J_{out} was not changed at 0–3 h, and increased by 1.8 fold only at 3–6 h of low pH exposure (Fig. 3B). Note that the Na^+ J_{out} rates in angelfish were 3.4 and 2.2 fold higher than those in discus after exposure for 3 and 6 h to pH 3.5, respectively. Consequently, Na^+ J_{net} was significantly elevated in angelfish at pH 3.5, with increased Na^+ net losses of 4.0 and 6.2-fold at 0–3 h and 3–6 h, respectively (Fig. 2B). In contrast, as seen for Na^+ J_{out}, only at 3–6 h of exposure to low pH was there a significant 1.9-fold increase of Na^+ J_{net} in discus. The net losses of Na^+ in angelfish were also higher: 4.1 and 2.1 fold than those in discus after exposure for 3 and 6 h to pH 3.5, respectively. Net Cl^- flux rates exhibited a qualitatively similar trend, increasing by up to 3.7- and 2.1-fold in angelfish after 3 and 6 h of exposure to low pH, respectively, while the increases were about 1.5-fold in discus, and smaller on an absolute basis (Fig. 4). In addition, Cl^- J_{net} rates in angelfish were higher: 2.5 fold than those in discus after exposure for 3 h to pH 3.5.

Table 1
Kinetic parameters for Na^+ uptake (J_{max} and K_m, mean ± SEM, N = 6) in angelfish and discus at neutral and low pH. * represents significant differences in Na^+ kinetic parameters between the same species in neutral and low pH.

		Na^+ kinetics constants			
Species	Treatment	pH	J_{max} (nmol g^{-1} h^{-1})	K_m (μmol L^{-1})	R^2
Angelfish	Neutral pH	5.9 ± 0.2	533.6 ± 23.6	79.3 ± 12.9	0.98
	Low pH	3.6 ± 0.04	106.1 ± 7.8*	166.4 ± 35.9*	0.98
Discus	Neutral pH	6.0 ± 0.14	537.9 ± 131.2	267.6 ± 141.9	0.93
	Low pH	3.6 ± 0.02	112.3 ± 22.2*	516.5 ± 181.3	0.98

3.3. Branchial Na^+/K^+-ATPase and H^+-ATPase activities

Despite the marked acute inhibitory effects of low pH exposure on branchial unidirectional and net Na^+ flux rates in angelfish, short-term (3 h) exposure to pH 3.5 had no significant effects on Na^+/K^+-ATPase activity (Fig. 4A). In contrast, H^+-ATPase activity in gills of angelfish was significantly inhibited by 67% and 53% after 2 h and 3 h of exposure to pH 3.5, respectively, with a non-significant decline at 1 h (Fig. 4B).

4. Discussion

4.1. Overview

Our study has five major findings. Firstly, in accord with our original hypothesis, both angelfish and discus exhibited relatively high K_m systems (*i.e.* low affinities) for Na^+ uptake, with high sensitivity to inhibition by low pH, yet low intrinsic branchial permeability limiting diffusive ion losses, the same strategy as seen previously in other cichlids, and very different from the strategy of characids (Gonzalez and Wilson, 2001; Gonzalez et al., 2002, 2006). Thus, both angelfish and discus defend their Na^+ balance under acidic ion-poor conditions mostly through a strong control of the Na^+ efflux component. Secondly, for the first time we have shown that the Na^+ influx inhibition at low pH in cichlids is due to both competitive (increased K_m) and non-competitive inhibitions (decreased J_{max}). Thirdly, the observed acute reduction in H^+-ATPase activity, but not in Na^+/K^+-ATPase activity, in the gills of angelfish suggests a possible mechanism for this non-competitive inhibition at low pH. Fourthly, at circumneutral pH, the kinetic parameters for angelfish collected directly from Rio Negro “blackwaters” were remarkably similar to those previously reported in angelfish obtained

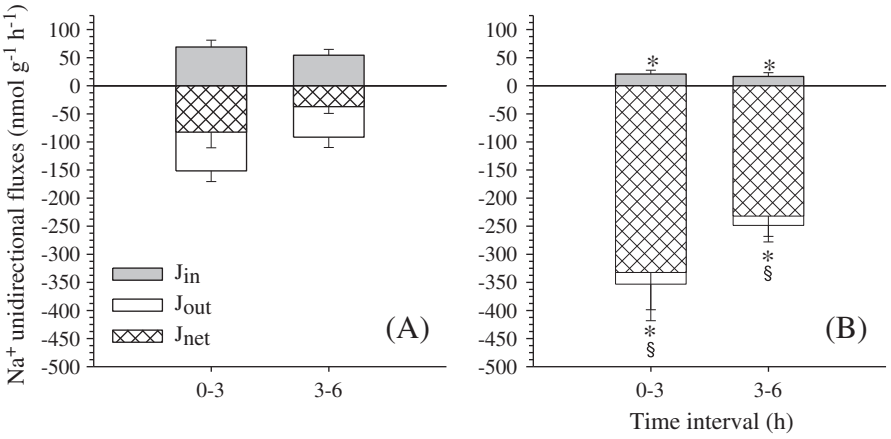

Fig. 2. Unidirectional and net Na^+ flux rates of angelfish over 0–3 h and 3–6 h of exposure to neutral pH (A) and low pH (B). (*) symbol represents significant differences in sodium influx (J_{in}) and net flux (J_{net}) between animals submitted to neutral and low pH at the related interval of exposure. (§) symbol represents significant differences in sodium outflux (J_{out}) between animals submitted to neutral and low pH at the related interval of exposure. Means ± 1 SEM (N = 6).

from the North American aquarium trade (Gonzalez and Wilson, 2001; Gonzalez et al., 2002). Finally, there were marked differences between these two cichlids. In angelfish, the inhibitory effects of low pH exposure on Na^+ uptake were more pronounced, in addition to greater stimulatory effects on branchial Na^+ and Cl^- permeability. In contrast, discus showed lesser impairment of Na^+ transport, at low pH presenting low Na^+ uptake rates that were very similar to those observed at neutral pH, in addition to better control of branchial Na^+ and Cl^- permeability.

4.2. Na^+ uptake kinetics

Gonzalez et al. (2002, 2006) have summarized kinetic parameters (K_m, J_{max}) of Na^+ uptake for a range of Amazonian teleosts under circumneutral conditions. The present results for angelfish and discus (Table 1) show K_m values in the range of other cichlids (*e.g. Apistogramma*, *Geophagus*, *Satanoperca*), and considerably higher than most other "blackwater" teleosts, particularly the characids. The angelfish K_m however appears to be at the lower end of the cichlid range, whereas the discus value is very typical. On the other hand, J_{max} values for both species were in the range of both characids and other cichlids. The lower K_m in angelfish allows it to maintain higher Na^+ uptake rates than those of discus in natural ion-poor conditions, at least at circumneutral pH, but it also exhibits higher efflux rates (Figs. 2, 3). Thus, even within the cichlids, there is a range of variation between a more "characid-like" and a more "cichlid-like" strategy. Very similar K_m (74 μmol L^{-1}) and high efflux rates were also reported in the cyprinid zebrafish acclimated to soft water, a condition that greatly enhances the capacity and affinity for Na^+ uptake in this species (Boisen et al., 2003).

Notably the K_m and J_{max} values for angelfish were both very close to those reported by Gonzalez and Wilson (2001) and Gonzalez et al. (2002) for the same species cultured in North America. The present responses of unidirectional and net Na^+ fluxes to low pH were also very similar. Thus, an unknown period of time and/or generations under unnatural conditions seems to have had little impact on the branchial Na^+ transport physiology of *P. scalare*.

Unique to the present study is the finding that the inhibition of unidirectional Na^+ influx by low pH is due to both competitive and non-competitive inhibitions in the angelfish (Table 1). Model Michaelis–Menten calculations using the K_m and J_{max} values from Table 1 demonstrate that at the very low Na^+ concentration of the acclimation water (31 μmol L^{-1}), it is the non-competitive inhibition (decreased J_{max}) which has by far the larger impact in reducing unidirectional Na^+ influx rate, as seen in Fig. 2. In discus, a similar large decrease in J_{max} (Table 1) should have comparably reduced unidirectional Na^+ influx in the acclimation water, but it did not (Fig. 3). The reason for this discrepancy is unclear, but one explanation comes to mind. The observed K_m (Table 1) was 9–17-fold higher than the Na^+ concentration in the acclimation water. Possibly, there may be a second, very high affinity (*i.e.* very low K_m) Na^+ transport system

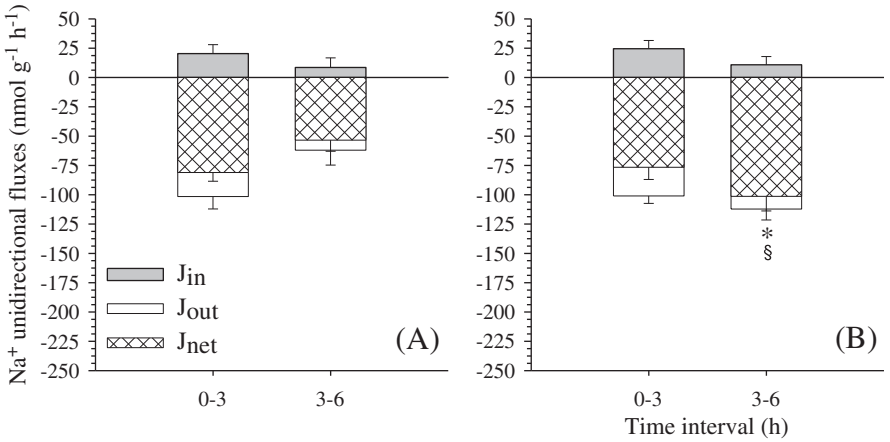

Fig. 3. Unidirectional and net Na^+ flux rates of discus over 0–3 h and 3–6 h of exposure to neutral pH (A) and low pH (B). (*) symbol represents significant differences in sodium net flux (J_{net}) between animals submitted to neutral and low pH at the related interval of exposure. (§) symbol represents significant differences in sodium outflux (J_{out}) between animals submitted to neutral and low pH at the related interval of exposure. Means ± 1 SEM (N = 6).

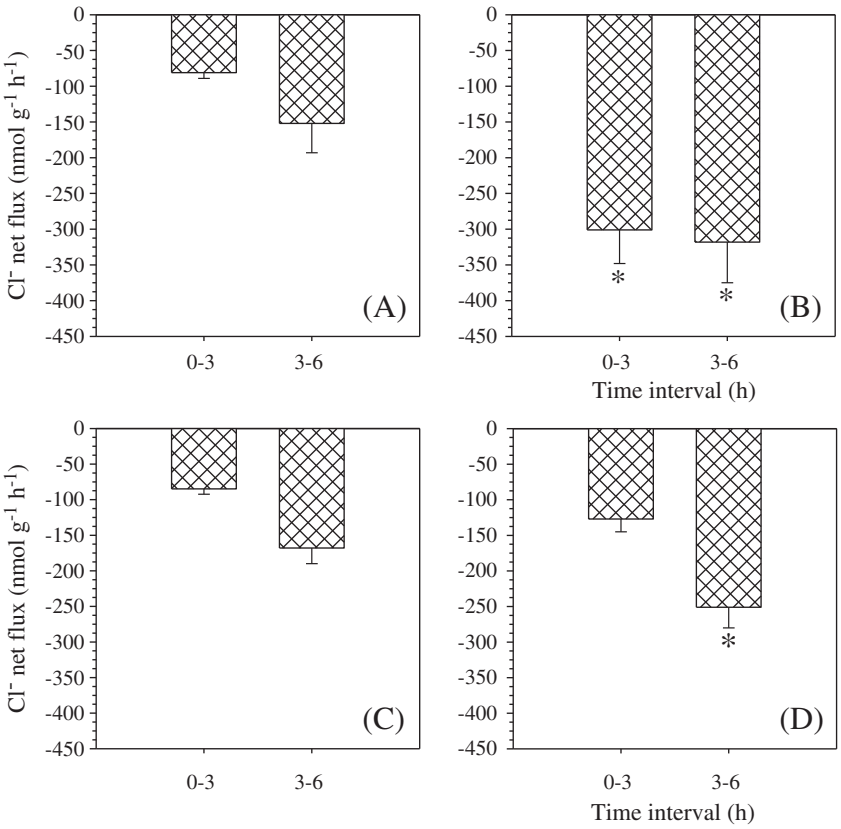

Fig. 4. Net Cl^- flux rates of angelfish over 0–3 h and 3–6 h of exposure to neutral pH (A) and low pH (B), and of discus over 0–3 h and 3–6 h of exposure to neutral pH (C) and low pH (D). (*) symbol represents significant differences in net flux (J_{net}) between animals submitted to neutral and low pH at the related interval of exposure. Means ± 1 SEM (N = 6).

in discus that operates at very low water Na^+ concentrations and which is acid-resistant. This would have been missed by our kinetic analysis which started only at the acclimation Na^+ concentration. As all the other five points in the kinetic analysis were above this value, the error would be greatest in the low range. Such a system was reported by Frain (1987) in the salt-depleted minnow *Phoxinus phoxinus*. In addition, Gonzalez (1996) hypothesized that a high affinity exchanger for Na^+ in the apical membrane of ionocytes would counter for the competitive inhibitory effects of increased H^+ concentration at low pH.

Inhibitory effects of low pH exposure on Na^+ uptake of freshwater fish are well documented (McDonald and Wood, 1981; Wright and Wood, 1985; Wood, 1992; Randall and Lin, 1993), and are usually explained on the basis of a competitive inhibition of Na^+ transport by increased H^+ concentration (Wood, 1989), and non-competitive inhibition through the reduction/reversal of the H^+ gradient across the apical membranes of ionocytes, consequently decreasing H^+ secretion, and promoting impairments of the Na^+ uptake mechanisms (Randall and Lin, 1993; Lin and Randall, 1995). This effect was clearly illustrated by Parks et al. (2008), that showed that under low external Na^+ concentration and acidic conditions, Na^+ uptake through an apical Na^+/H^+ exchanger driven by a basolateral Na^+/K^+-ATPase would function in the forward direction. The authors argued that another primary active transport is required for Na^+ uptake at low external Na^+ and pH, which probably occurs through a Na^+ channel electrically coupled H^+-ATPase. Thus, the present results reveal another potential mechanism for the non-competitive inhibition — a rapid reduction in H^+-ATPase activity (Fig. 5B), but not in Na^+/K^+-ATPase activity (Fig. 5A), in the gills of angelfish. Notably, Randall et al. (1996) speculated that inhibition of H^+-ATPase at low pH would promote a reversal in the apical membrane potential, making the inside of the cells more positive, thereby reducing Na^+ uptake through electrically coupled apical Na^+ channels. Perhaps related to this, a previous study on another Amazonian fish, the acid-tolerant tambaqui (*C. macropomum*) revealed a persistent reversal of the whole gill transepithelial potential (TEP) – *i.e.* electrical gradient from blood to water – at pHs below 4.0 and low external Ca^{2+} concentration (20 μmol L^{-1}); this was associated with increased net Na^+ losses as the blood side became more positive (Wood et al., 1998).

4.3. Branchial H^+-ATPase activity

To our knowledge, the rapid reduction of branchial H^+-ATPase activity (measured *in vitro*) upon acute exposure to low pH seen in angelfish (Fig. 5B) has not been reported previously in any other teleost fish. However, inhibition of H^+-ATPase activity has also been seen in another Amazonian cichlid fish *Mesonauta insignis* acutely exposed (1 h) to pH 4.0 under naturally acidic ion-poor conditions (R. M. Duarte and A.L.Val, unpublished data). Previous studies on salmonids have shown that this enzyme is sensitive to modulation by other environmental factors such as Na^+, Ca^{2+}, salinity, ammonia, and PCO_2 levels (Lin and Randall, 1993; Nawata et al., 2007; Wood and Nawata, 2011). The time course of the response to low pH (2–3 h; Fig. 5B) could reflect either a genomic effect or post-translational modifications as the internal milieu of the gill ionocytes is presumably altered at low pH. Unfortunately, we were not able to estimate such changes in internal ionocyte pH, as well as the integrity of gill epithelium during low pH exposure. However, considering the natural history of this fish species at episodic acid conditions and the unchanged Na^+/K^+-ATPase activity (Fig. 5A), the reduction seen in H^+-ATPase activity unlikely involves gill damage or loss of ionocytes. According to Randall and Lin (1993), the negative potential in the inner apical membrane of ionocytes should build up when Na^+ uptake is inhibited, which eventually would stop the operation of H^+-ATPase on fish gills. Presumably it serves as a cost-saving strategy to reduce energy expenditure under conditions

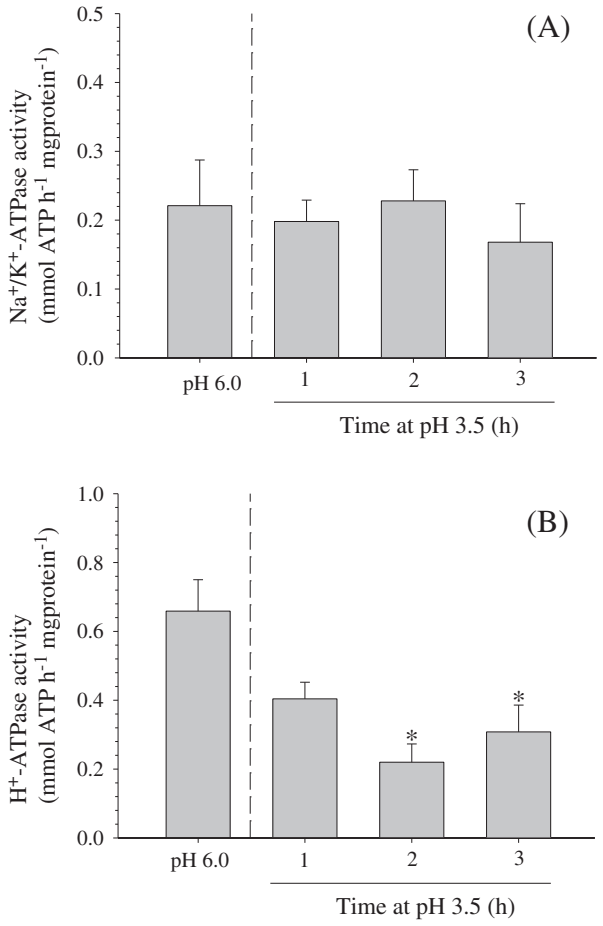

Fig. 5. Branchial Na^+/K^+-ATPase (A) and H^+-ATPase (B) activities in angelfish acclimated to pH 6.0 and exposed for 3 h to low pH (3.5). (*) symbol represents significant differences in ATPase activity from the pH 6.0 group. Means ± 1 SEM (N = 6).

(very low external pH) where the H^+-pump can no longer function effectively.

4.4. Permeability

Responses of increased Na^+ efflux to acute low pH exposure were greater in angelfish than in discus, and occurred more rapidly. In contrast to the 2.5-fold increase of Na^+ J_{out} throughout the exposure to pH 3.5 in angelfish (Fig. 2), discus showed no stimulation in Na^+ J_{out} over the first 3 h, and almost 3-fold lower Na^+ efflux rates than angelfish at low pH (Fig. 3). Indeed the significantly greater Na^+ efflux at 6 h in discus was in fact very similar to that observed at 3 h in pH 3.5, and probably reflects the reduced Na^+ J_{out} measured at 6 h in pH 6.0 rather than an increase in branchial permeability in this species. The magnitude of Na^+ J_{out} in angelfish at low pH was very close to values previously reported in the North American cultured angelfish (Gonzalez and Wilson, 2001), and lower than that in two other Amazonian cichlids *Apistogramma* and *Geophagus* at low pH in natural Rio Negro water (Gonzalez et al., 2002). In addition, Na^+ J_{out} seen in both angelfish and discus was on average threefold and eightfold lower than that in acidic non-tolerant fish species as trout and common shiner at pH 4.0, respectively (Freda and McDonald, 1988). Therefore, it is likely that differences in the tolerance to low pH observed for Amazon fish and acidic non-tolerant fish species, as well as among the cichlid fish of the Amazon, are dependent on the ability to limit the increase in branchial permeability at low pH.

Although freshwater fish display a very tight gill epithelium to minimize passive ion losses (Evans et al., 2005; Hwang, 2009), at low pH, the increased external H^+ concentration is thought to compete with Ca^{2+} ions for binding to paracellular tight junctions, thereby rendering the branchial epithelium more permeable and resulting in stimulation of diffusive ion losses (McWilliams, 1982; McDonald et al., 1983b; Gonzalez et al., 2006). Thus, it is likely that discus have a tighter epithelium, which is more resistant to low pH effects on Na^+ efflux in ion-poor water than do angelfish. Interestingly, the net Cl^- flux results (Fig. 4) suggest that the permeability difference applies to anions as well as cations. In most teleosts, increases in water Ca^{2+} concentration generally protect against increased Na^+ effluxes at low pH (reviewed by Gonzalez et al., 2006), but Gonzalez et al. (1998) reported that 10-fold elevations of water Ca^{2+} had no protective action in three Rio Negro characids. In contrast, elevated Ca^{2+} levels reduced Na^+ efflux in both Amazon blackskirt and neon tetras at neutral pH, but the magnitude of this effect was lower in fish at pH 3.5 (Gonzalez et al., 1997; Gonzalez and Preest, 1999). Thus, the picture that emerges is that native fish from Rio Negro posses gills with extremely low intrinsic permeability, which in turn would be strongly regulated by a high branchial affinity for Ca^{2+}, or alternatively displaying a Ca^{2+}-independent mechanism to control ion efflux at low pH. Future studies should address whether the apparent difference between angelfish and discus reflects the differences in Ca^{2+} dependency of permeability and/or in the binding affinity of the gills for Ca^{2+}.

5. Future perspectives

In future studies, it would also be of interest to examine longer-term effects of low pH exposure in these fish. Do H^+ ATPase activity and Na^+ influx recover? Does gill permeability decrease? Notably, zebrafish acclimated to acidic conditions for five days upregulated Na^+ uptake through an increased Na^+ J_{max} and K_m, and Na^+ balance was achieved by increased influx rather than reduced efflux (Kumai et al., 2011). The increased Na^+ uptake appeared to reflect an increased reliance on NHE (Kumai and Perry, 2011). Indeed such a mechanism appears to be particularly prominent not only for Na^+ uptake in the zebrafish (Kumai and Perry, 2011; Shih et al., 2012), but also for the Osorezan dace (Hirata et al., 2003) and larval medaka (Lin et al., 2012) when chronically exposed to low water pH in fresh water of relatively low Na^+ concentration. Strong thermodynamics arguments against operating an NHE at low water Na^+ at an external pH 3–4 units below that of the gill ionocyte have been raised (Randall et al., 1996; Parks et al., 2008). Nevertheless, evidence is accumulating that such a system can be driven by increased ammonia efflux through an Rh-protein/Na^+ uptake metabolon (summarized by Wright and Wood, 2012). Clearly future studies should investigate the role of ammonia excretion and Rh proteins in longer-term adaptation to low pH in these "blackwater" species.

Acknowledgments

This study was funded by FAPEAM and CNPq through the INCT-ADAPTA grant to ALV, and by an NSERC Discovery grant to CMW. ALV is a recipient of a research fellowship from CNPq. RMD was supported by the CAPES PhD fellowship. MSF is supported by the CNPq PhD fellowship. CMW is supported by the Canada Research Chair Program and a recipient of a fellowship to the Science without Borders program (CNPq-Brazil).

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