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ORIGINAL PAPER

Mechanisms of Na^+ uptake, ammonia excretion, and their potential linkage in native Rio Negro tetras (*Paracheirodon axelrodi*, *Hemigrammus rhodostomus*, and *Moenkhausia diktyota*)

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Abstract Mechanisms of Na^+ uptake, ammonia excretion, and their potential linkage were investigated in three characids (cardinal, hemigrammus, moenkhausia tetras), using radiotracer flux techniques to study the unidirectional influx (J_{in}), efflux (J_{out}), and net flux rates (J_{net}) of Na^+ and Cl^-, and the net excretion rate of ammonia (J_{Amm}). The fish were collected directly from the Rio Negro, and studied in their native "blackwater" which is acidic (pH 4.5), ion-poor (Na^+, Cl^- ~20 μM), and rich in dissolved organic matter (DOM 11.5 mg C l^{-1}). J_{in}^{Na}, J_{in}^{Cl}, and J_{Amm} were higher than in previous reports on tetras obtained from the North America aquarium trade and/or studied in low DOM water. In all three species, J_{in}^{Na} was unaffected by amiloride (10^{-4} M, NHE and Na^+ channel blocker), but both J_{in}^{Na} and J_{in}^{Cl} were virtually eliminated (85–99 % blockade) by $AgNO_3$ (10^{-7} M). A time course study on cardinal tetras demonstrated that J_{in}^{Na} blockade by $AgNO_3$ was very rapid (≤5 min), suggesting inhibition of branchial carbonic anhydrase (CA), and exposure to the CA-blocker acetazolamide (10^{-4} M) caused a 50 % reduction in J_{in}^{Na}. Additionally, J_{in}^{Na} was unaffected by phenamil (10^{-5} M, Na^+ channel blocker), bumetanide (10^{-4} M, NKCC blocker), hydrochlorothiazide (5×10^{-3} M, NCC blocker), and exposure to an acute 3 unit increase in water pH. None of these treatments, including partial or complete elimination of J_{in}^{Na} (by acetazolamide and $AgNO_3$ respectively), had any inhibitory effect on J_{Amm}. Therefore, Na^+ uptake in Rio Negro tetras depends on an internal supply of H^+ from CA, but does not fit any of the currently accepted H^+-dependent models (NHE, Na^+ channel/V-type H^+-ATPase), or co-transport schemes (NCC, NKCC), and ammonia excretion does not fit the current "Na^+/NH_4^+ exchange metabolon" paradigm. Na^+, K^+-ATPase and V-type H^+-ATPase activities were present at similar levels in gill homogenates, Acute exposure to high environmental ammonia (NH_4Cl, 10^{-3} M) significantly increased J_{in}^{Na}, and NH_4^+ was equally or more effective than K^+ in activating branchial Na^+,(K^+) ATPase activity in vitro. We propose that ammonia excretion does not depend on Na^+ uptake, but that Na^+ uptake (by an as yet unknown H^+-dependent apical mechanism) depends on ammonia excretion, driven by active NH_4^+ entry via basolateral Na^+,(K^+)-ATPase.

Keywords Characids · Low pH water · Silver nitrate · Na^+/NH_4^+ exchange · Na^+ · NH_4^+ATPase

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Introduction

The acidic, ion-poor "blackwaters" of the Rio Negro and its tributaries support over 1,000 species of teleost fish.

The ambient conditions (major cations <30 μmol l^{-1}, pH's 3.5–5.5; Furch 1984; Val and Almeida-Val 1995) pose not only severe physiological challenges to the fish, but also severe theoretical challenges to our current understanding of how branchial Na^+ uptake mechanisms work (Randall et al. 1996; Gonzalez et al. 2005; Parks et al. 2008). In brief, thermodynamic considerations suggest that neither of the two dominant mechanisms seen in most freshwater fish [apical Na^+/H^+ exchange (NHE), or Na^+ uptake through an apical channel electrochemically coupled to H^+ extrusion via a V-type H^+-ATPase] should work under Rio Negro conditions. A third more recently proposed mechanism involving the apical co-transport of Na^+ and Cl^- driven by the electrochemical gradient for Na^+ (reviewed by Evans 2011) would appear to be subject to the same energetic challenges under Rio Negro conditions.

This problem has led to considerable research on ionoregulation in Rio Negro teleosts (reviewed by Gonzalez et al. 2005). Not surprisingly, general conclusions from these studies are that Na^+ uptake mechanisms are characterized by relatively high Na^+ affinity and high resistance to inhibition by low pH (high H^+) (e.g. Gonzalez et al. 1997, 2002; Gonzalez and Preest 1999; Gonzalez and Wilson 2001; Preest et al. 2005; Matsuo and Val 2007; Duarte et al. 2013). Amiloride has been tested in several species of tetra, small members of the Characiformes, the most abundant order in the Rio Negro. When applied at 10^{-4} M in the water, a concentration which should powerfully block both NHE's and epithelial Na^+ channels (Benos 1982; Kleyman and Cragoe 1988), amiloride proved to be only marginally effective (Gonzalez et al. 1997; Gonzalez and Preest 1999) in inhibiting unidirectional Na^+ uptake. More extensive pharmacological investigation (Preest et al. 2005) with a range of amiloride analogues (selective for NHEs or for Na^+ channels) confirmed this lack of effect. These findings have led to the suggestion that the uptake mechanism(s) might be unique, perhaps not involving reliance on H^+ as a counter-ion, and, therefore, very different from those in "standard" teleosts (Gonzalez and Preest 1999; Preest et al. 2005; Gonzalez et al. 2005).

The idea that Na^+ uptake might be coupled to NH_4^+ excretion was first presented by August Krogh (1938) and since then has had a long and chequered history (reviewed by Wilkie 2002, and Kirschner 2004). However, in the past few years, the discovery of ammonia-conductive Rh proteins in teleost gills (Nakada et al. 2007; Nawata et al. 2007, 2010) has led to a new paradigm that a "Na^+/NH_4^+ exchange complex" consisting of several apical membrane transporters (Rhcg, V-type H^+-ATPase, NHE, Na^+ channel, carbonic anhydrase) working together as a metabolon provides a loose coupling of Na^+ uptake with ammonia excretion (Wright and Wood 2009; Ito et al. 2013). Furthermore, evidence has started to accumulate (reviewed by Wright and Wood 2012, and Kwong et al. 2014) that this mechanism becomes prominent in several species (Osorezan dace—Hirata et al. 2003, zebrafish—Kumai and Perry 2011, Shih et al. 2012, and larval medaka—Lin et al. 2012) when chronically exposed to water of low pH and/or low Na^+ concentration. The theory proposes that the deprotonation of NH_4^+ as NH_3 enters the apical Rh channel creates a source of protons to fuel Na^+ uptake via NHEs and/or Na^+ channel/V-type H^+-ATPase while at the same time, the NH_3 leaving the Rh channel traps protons, thereby creating an external microenvironment in which $[H^+]$ is less concentrated. Earlier, Randall et al. (1996) had speculated that in Rio Negro fish, NH_4^+ might enter though the basolateral membrane by substituting for K^+ on the Na^+, K^+-ATPase, which would presumably give an energetic boost to the system, and provide an additional source of H^+ ions to exchange against Na^+ at the apical membrane. While the thermodynamics have not been worked out, essentially elevated ammonia excretion would drive Na^+ uptake under these conditions.

Ammonia excretion has been only sparsely studied in Rio Negro teleosts, with no clear picture emerging. In the oscar, a cichlid, there appeared to be a close coupling of Na^+ uptake and ammonia excretion at circumneutral pH, both co-varying in a Michaelis–Menten fashion with external $[Na^+]$ (Wood et al. 2007). When the tambaqui and neon tetra, both characids, were transferred to low pH, ammonia excretion increased (Wilson 1996; Wilson et al. 1999). However, there appeared to be no coupling of ammonia excretion to environmental $[Na^+]$ in either the neon tetra (Wilson 1996) or the blackskirt tetra (Gonzalez et al. 1997).

The Rio Negro blackwaters are rich in dissolved organic matter (DOM), and there is now considerable evidence that this aids ionoregulation in fish (Gonzalez et al. 1998, 2002; Wood et al. 2003, 2011; Matsuo and Val 2007; Galvez et al. 2008) by promoting Na^+ influx and ammonia excretion, and limiting Na^+ efflux, especially under low pH conditions. With a few exceptions (Gonzalez et al. 1998, 2002), most previous studies on Rio Negro teleosts have been performed in waters lacking DOM, which may confound the interpretation. Furthermore, in most cases, the "Rio Negro" fish had been obtained from the aquarium trade in North America, raising the possibility of acclimation-related or adaptation-related (i.e. genetic) differences from native fish.

With this background in mind, during a research expedition to the upper Rio Negro, we investigated Na^+ uptake, ammonia excretion, and their potential coupling in three species of native tetras freshly collected from the blackwater, and tested in this water. The major focus was on the cardinal tetra (*Paracheirodon axelrodi*), as this is a species which has been studied extensively to date (Gonzalez et al. 1998; Gonzalez and Wilson 2001; Matsuo and Val

2007). In vivo approaches included a range of pharmacological treatments, and changes in water pH and ammonia, together with appropriate controls, while the ability of NH_4^+ to activate gill Na^+, K^+-ATPase was evaluated in vitro. One pharmacological treatment was particularly effective at blocking Na^+ uptake, and its mechanism of action was investigated in greater detail.

Materials and methods

Experimental animals

All experiments were performed on board a research vessel (the Ana Clara, from Manaus) moored in the Rio Negro approximately 50 km northeast of Barcelos, AM, during May, 2012. All procedures were in compliance with Brazilian national and INPA animal care regulations. Cardinal tetra (*Paracheirodon axelrodi*), hemigrammus tetra (*Hemigrammus rhodostomus*), and moenkhausia tetra (*Moenkhausia diktyota*) of approximately 0.3–0.5 g were collected at the river's edge using baited minnow traps. On board the Ana Clara, they were held for 3–10 days in large aerated tanks under ambient conditions—a natural photoperiod of 12-h light:12-h dark, and water temperature ~30 to 35 °C. The holding and experimental water was pumped directly from the Rio Negro ($[Na^+]$ = 20 μM, $[Cl^-]$ = 20 μM, $[Ca^{2+}]$ = 10 μM, Mg = 5 μM, $[K^+]$ = 10 μM; pH 4.5; DOM 11.5 mg C l^{-1}, measured as dissolved organic carbon). The fish were not fed during the experiments, so the fasting period was 3–10 days prior to experimentation.

Flux measurements

Details on individual experiments are given in "Results". For all flux measurements, fish were transferred to individual 0.1-L plastic containers (shielded with black plastic) filled with 60 ml of fresh Rio Negro water. These flux containers were fitted with capillary aeration devices which kept water O_2 tension close to saturation, as well as lids containing sampling ports; they were placed in a water bath that maintained the ambient water temperature of 30–35 °C. While the experimental temperature varied on different days, it never varied by more than 1.5 °C in an individual experiment. The fish were allowed to settle for 0.25–0.5 h prior to experimentation. When radioisotopes (^{22}Na, ^{36}Cl) were used, this settling period was also used as a mixing period. Flux periods for specific treatments were 2 h, with water samples (5 ml) taken at 0, 1, and 2 h, yielding two 1-h flux estimates. These never differed significantly and so were averaged. Flux measurements included influx, efflux, and net flux rates of Na^+ and Cl^-, and net flux rates of ammonia and urea-N in various series.

When pharmacological treatments or environmental challenges (high environmental pH, high environmental ammonia) were imposed, these were allowed to act for 0.5 h before the 2-flux measurement was started. Rapid water replacement could be achieved using a 60-ml syringe to withdraw old water and add new water. At the end, the fish were terminally anaesthetized with MS-222 (0.2 g l^{-1}; Syndel Labs, Nanaimo, BC, Canada), blotted dry, and weighed individually.

This protocol was modified in one series which examined the time course of inhibition of Na^+ influx. In order to assay the onset and recovery of the inhibition with high resolution, the water volume was set to only 30 ml, and 1-ml water samples were taken at 5-min intervals for measurement of ^{22}Na radioactivity only. After the standard settling period, during which ^{22}Na was present, a 30-min control period was started, with sampling at 5-min intervals. At 30 min, the antagonist was added, and sampling continued for another 30 min so as to follow the onset of inhibition. In order to follow the recovery, in a separate series of fish, the agonist was allowed to act for 2 h to ensure complete blockade. The final 15-min was used as a ^{22}Na equilibration period, and then sampling commenced at 5-min intervals for 30 min to assess the extent of blockade. Immediately after the 30-min sample, the water was rapidly replaced with agonist-free water containing an identical level of ^{22}Na radioactivity. Sampling continued for another 30 min. In these experiments, due to sample volume constraints, only ^{22}Na radioactivity could be measured and not total water Na^+ concentration, so true flux rates could not be calculated.

The radioisotopes ^{22}Na (as NaCl) and ^{36}Cl (as HCl) were manufactured by New England Nuclear Dupont, Boston, MA, USA) and supplied by REM (Sao Paulo, SP, Brazil). For each unidirectional flux test, approximately 0.3 μCi (11.1 kBq) of radioisotope was added to the water.

Experimental treatments

The following potential antagonists were tested: (i) amiloride HCl (10^{-4} M) as a joint NHE and Na^+ channel blocker; (ii) phenamil methane sulfonate (10^{-5} M) as a more specific Na^+ channel blocker; (iii) bumetanide (10^{-4} M) as a blocker of Na^+, K^+, $2Cl^-$ co-transport; (iv) hydrochlorothiazide (5×10^{-3} M) as a blocker of Na^+, Cl^- co-transport (NCC); (v) acetazolamide (10^{-4} M) as a blocker of carbonic anhydrase (CA); and (vi) silver nitrate ($AgNO_3$, 10^{-7} M), a blocker with several postulated sites of action (see "Discussion"). All drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA) and with the exception of $AgNO_3$ (which is very soluble) were first dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich) and then diluted in river water such that the final DMSO

concentration was 0.1 %. Appropriate control experiments with 0.1 % DMSO only were performed (see "Results"). In addition, fish were exposed to (vii) high environmental ammonia (HEA; NH_4Cl, 10^{-3} M) at unchanged pH and to (viii) high pH (nominally 8.5, achieved by the addition of small volumes of 0.1 M KOH). Amiloride and $AgNO_3$ were evaluated in all species, whereas the other drugs and treatments were tested only in cardinal tetras.

Water pH was routinely monitored in all tests; pH started at 4.5 ± 0.1, and by the end of 2 h had risen to 4.9 ± 0.1. In the high pH tests, the measured starting pH of 8.2 ± 0.1 had fallen to 7.7 ± 0.1 by the end of the test.

Water analyses

Water pH was measured by a Radiometer GK2401C combination pH electrode and pHM 84 meter (Radiometer-Copenhagen, Denmark). Water DOM concentration, as dissolved organic carbon, was measured by combustion (Apollo 9000 TOC Analyzer, Teledyne Tekmar, Madison, OH, USA) on samples which had been first passed through a 0.45-μm filter (Acrodisc Supor Membrane, Pall Life Sciences, Port Washington, NY, USA).

Water total ammonia (Verdouw et al. 1978), total urea-N (Rahmatullah and Boyde 1980) and Cl^- concentrations (Zall et al. 1956) were measured by colorimetric assays. Both the 0.1 % DMSO and some of the drugs differentially interfered with the ammonia assay, so standards were made up in the actual test solutions for all drug experiments. Radioactivities of ^{22}Na and ^{36}Cl in water samples were measured by mixing 1 ml of water with 4 ml of Ultima Gold scintillation fluid (Perkin-Elmer, Waltham, MA, USA), then counting on a Triathler portable counter (Hidex, Helsinki, Finland). Tests showed that quench was constant so correction was unnecessary. Water total Na^+ concentrations were measured using a 910 Digital Flame Photometer (Instrumentação Analítica São Paulo, SP, Brazil).

Calculations

Net flux rates (J_{net}) in nmol g^{-1} h^{-1} of Na^+, Cl^-, total ammonia, and total urea-N were calculated from changes in water concentrations (in nmol ml^{-1}), factored by the known fish weight (in g), water volume (in ml), and experimental time (in h). By convention, fluxes into the fish are positive, while fluxes out of the fish are negative. Unidirectional flux rates of Na^+ and Cl^- were measured by monitoring the disappearance of radioactivity from the water (into the fish, so as to follow influx rate, J_{in}) as well as the change in total ion concentration in the water (so as to follow J_{net}), whereas efflux rate (J_{out}) was calculated by difference (c.f. Wood 1992). In this method, J_{in} (positive) is calculated as

$$J_{in} = \frac{([CPM_i] - [CPM_f])(V)}{(SA_{ext})(T)(W)}, \quad (1)$$

where CPM_i is the initial radioactivity in the water (in cpm ml^{-1}) at the start of the flux period, CPM_f is the final radioactivity in the water (in cpm ml^{-1}) at the end of the flux period, V is the volume of water (in ml), SA_{ext} is the mean external specific activity (radioactivity per total Na^+) in the water (in cpm $nmol^{-1}$), calculated from measurements of water radioactivity and total water $[ion]_{ext}$ at the start and end of the flux period, T is the time of flux period (in h), W is the weight of the fish (in g).

Conditions were such that estimated internal specific activity never exceeded 5 % of external specific activity, so correction for radioisotopic backflux was unnecessary (cf. Maetz 1956).

J_{out} (negative) was calculated by difference using the conservation equation:

$$J_{out} = J_{net} - J_{in} \quad (2)$$

Branchial ATPase activities

Cardinal tetras were terminally anaesthetized as above. The gills were quickly dissected out, flash-frozen in liquid N_2, and stored at −80 °C for later assay. Branchial Na^+, K^+-ATPase and V-type H^+-ATPase activities were measured using methods from McCormick (1993) and Lin and Randall (1993), respectively, as modified by Nawata et al. (2007). Protein concentrations were assayed with Bradford Reagent and BSA standards (Sigma-Aldrich). Na^+, K^+-ATPase and V-type H^+-ATPase assays were run simultaneously on the same samples. In order to test whether NH_4^+ could activate branchial Na^+, K^+-ATPase, and to evaluate its potency relative to K^+, various concentrations [1, 3, 10, and 20 mmol l^{-1} of NH_4^+ (as NH_4Cl) or K^+ (as KCl)] were added separately to the assay media (see "Results").

Statistics

Data have been expressed as the means ± 1 SEM (N). In experiments where each fish was used at its own control, effects were evaluated by a two-tailed paired Student's t test, whereas an unpaired two-tailed Student's t test was used for independent comparisons, with a Bonferroni correction when more than one comparison was made. A Mann–Whitney rank sum test was used in the case of failed normality or equal variance tests. For the branchial ATPase activities, a one-way repeated measures analysis of variance (ANOVA) followed by a Holm–Sidak post hoc test was used for multiple comparisons since the homogenates from the same fish were assayed at different ion concentrations. In all cases, differences were considered statistically significant at $P \leq 0.05$.

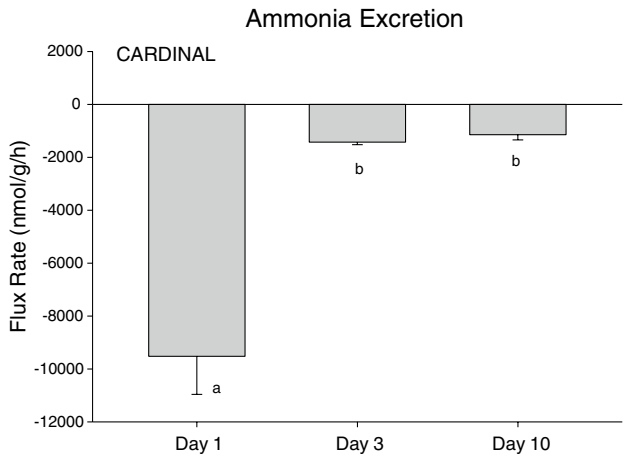

Fig. 1 Ammonia excretion rates (J_{Amm}) measured under control conditions in cardinal tetra immediately after capture, and after 3 and 10 days of holding, in the absence of feeding. Means ± 1 SEM ($N = 5$–6). Means sharing the *same letter* are not significantly different ($P > 0.05$)

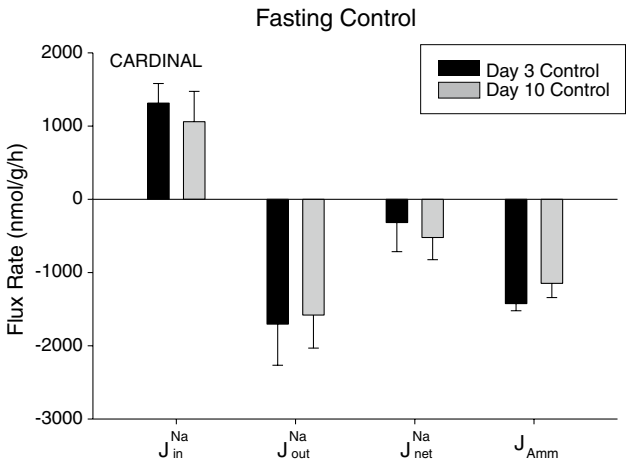

Fig. 2 The influence of 3 versus 10 days of fasting on unidirectional Na^+ influx (J_{in}^{Na}), efflux (J_{out}^{Na}), and net flux rates (J_{net}^{Na}), and on ammonia excretion rates (J_{Amm}) in cardinal tetra measured under control conditions in cardinal tetra. Means ± 1 SEM ($N = 5$–6). There were no significant differences ($P > 0.05$)

Results

Control experiments

As these fish were collected directly from the wild, we assessed how their physiology might change as a result of time since capture (during which they were fasted) and in response to experimental manipulations.

In cardinal tetra, net ammonia excretion (J_{Amm}) measured in the first few hours after capture was extremely high, almost −10,000 nmol g^{-1} h^{-1}, but by day 3 had fallen to about −1,500 nmol g^{-1} h^{-1}, and remained unchanged at day 10 (Fig. 1). A similar pattern was seen in moenkhausia tetra (day 0 = −10,665 ± 1,844, day 3 = −1,741 ± 148, day 7 = −1,659 ± 87 nmol g^{-1} h^{-1}, $N = 3$–5), whereas hemigrammus tetra were assessed only on days 3 and 7 (−2,375 ± 288, −2,473 ± 241 nmol g^{-1} h^{-1}, $N = 5$). Notably, urea-N excretion (J_{Urea}) was not elevated immediately post-capture, remaining low and essentially unchanged at about −200 to −400 nmol g^{-1} h^{-1} over the same time frames in all three species (data not shown).

In cardinals, the species which was studied most extensively, there were no significant changes in unidirectional Na^+ influx (J_{in}^{Na}), efflux (J_{out}^{Na}), or net flux rates (J_{net}^{Na}), or in ammonia excretion rates (J_{Amm}) between days 3 and 10 post-capture (Fig. 2). The same was true between days 3 and 7 for the other two species; flux rates were quantitatively similar to those in cardinal tetras (data not shown). Based on these results, all experiments were performed between days 3 and 10 post-capture.

The possible effects of 0.1 % DMSO, used as a vehicle in most of the drug trials, were assessed only in cardinal tetras. There were no significant effects on any measured parameter (J_{in}^{Na}, J_{out}^{Na}, J_{net}^{Na}, J_{Amm}; data not shown). Nevertheless, we ensured that 0.1 % DMSO was present in the control treatments for all tests in which this drug-solubilization vehicle was used.

A final concern was the effect of confinement and time in the flux measurement protocols. Our original intention was to follow a 2-h control treatment by a 2-h experimental treatment, so as to use each fish as its own control. However, experimental tests with all three species revealed significant changes in the second 2-h period relative to the first (Fig. 3). Specifically, in cardinal tetras, J_{out}^{Na} decreased greatly such that J_{net}^{Na} changed over to a positive value, with unchanged J_{in}^{Na} and J_{Amm} (Fig. 3a). In moenkhausia tetra, the same significant changes occurred in the second 2-h period, plus a decrease in J_{Amm} (Fig. 3b). In hemigrammus tetra, both J_{in}^{Na} and J_{Amm} decreased by 50 % or more in the second 2-h period, whereas J_{out}^{Na} and J_{net}^{Na} did not change significantly (Fig. 3c). Since it was unclear whether these time-dependent changes were due to progressive recovery from handling stress, or progressive effects of confinement stress in these wild-caught fish, we elected to use 2-h tests for all subsequent experimental treatments, with independent time-matched controls.

Experimental tests

Amiloride (10^{-4} M) had no significant effects on J_{Amm}, J_{in}^{Na}, J_{out}^{Na}, or J_{net}^{Na} in any of the three species, apart from an increase in J_{Amm} in cardinal tetras (Fig. 4a–c). Phenamil (10^{-5} M; Fig. 5a), bumetanide (10^{-4} M; Fig. 5b), and

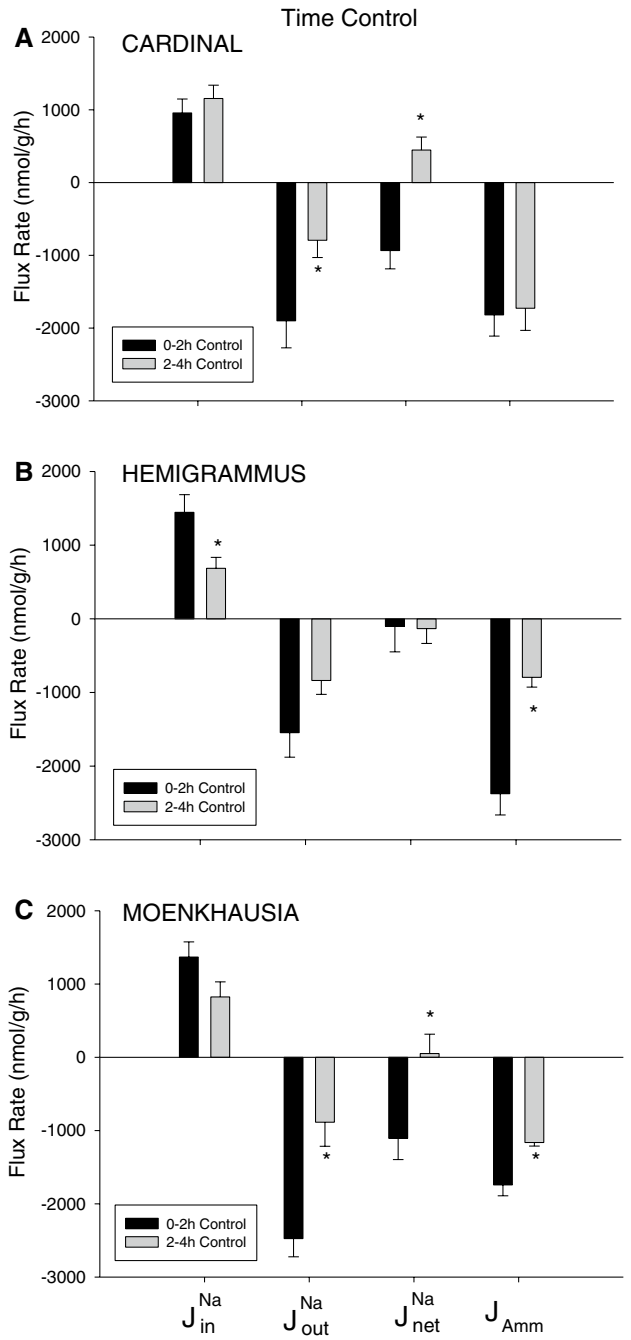

Fig. 3 The influence of time in the flux container (0–2 versus 2–4 h) on unidirectional Na^+ influx (J_{in}^{Na}), efflux (J_{out}^{Na}), and net flux rates (J_{net}^{Na}), and on ammonia excretion rates (J_{Amm}) measured under control conditions in **a** cardinal tetra, **b** hemigrammus tetra, and **c** moenkhausia tetra. Means ± 1 SEM ($N = 5$). *Asterisks* indicate significant differences ($P < 0.05$) between the first and second 2-h periods

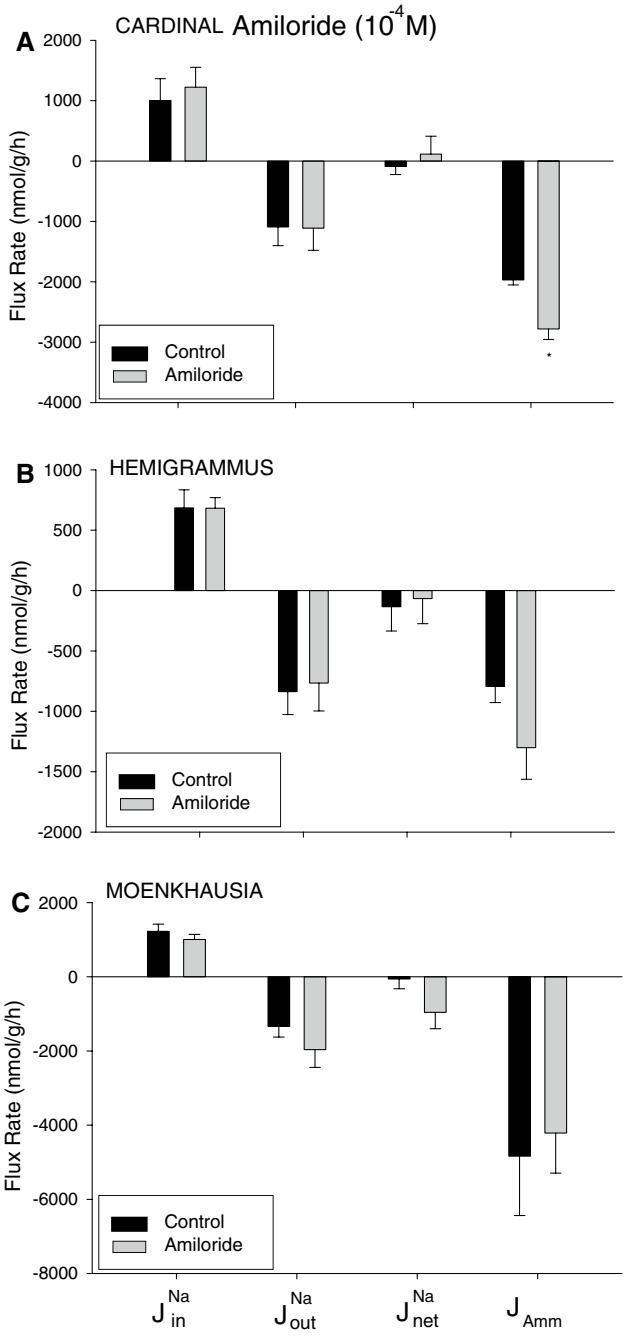

Fig. 4 The influence of amiloride (10^{-4} M) on unidirectional Na^+ influx (J_{in}^{Na}), efflux (J_{out}^{Na}), and net flux rates (J_{net}^{Na}), and on ammonia excretion rates (J_{Amm}) relative to simultaneous time-matched controls in **a** cardinal tetra, **b** hemigrammus tetra, and **c** moenkhausia tetra. Means ± 1 SEM ($N = 5$). There were no significant differences ($P > 0.05$)

hydrochlorothiazide (5×10^{-3} M; Fig. 5c) were tested only in cardinal tetras, and similarly had no significant effects on J_{Amm}, J_{in}^{Na}, J_{out}^{Na}, or J_{net}^{Na}. Overall, these results indicate that neither Na^+ uptake nor ammonia excretion are linked to "standard" NHE, Na^+ channel, NKCC, or NCC mechanisms of Na^+ transport.

Acetazolamide (10^{-4} M), which was tested only in cardinal tetras, caused a significant 50 % inhibition in J_{in}^{Na} with an accompanying switch to a negative J_{net}^{Na}, but no

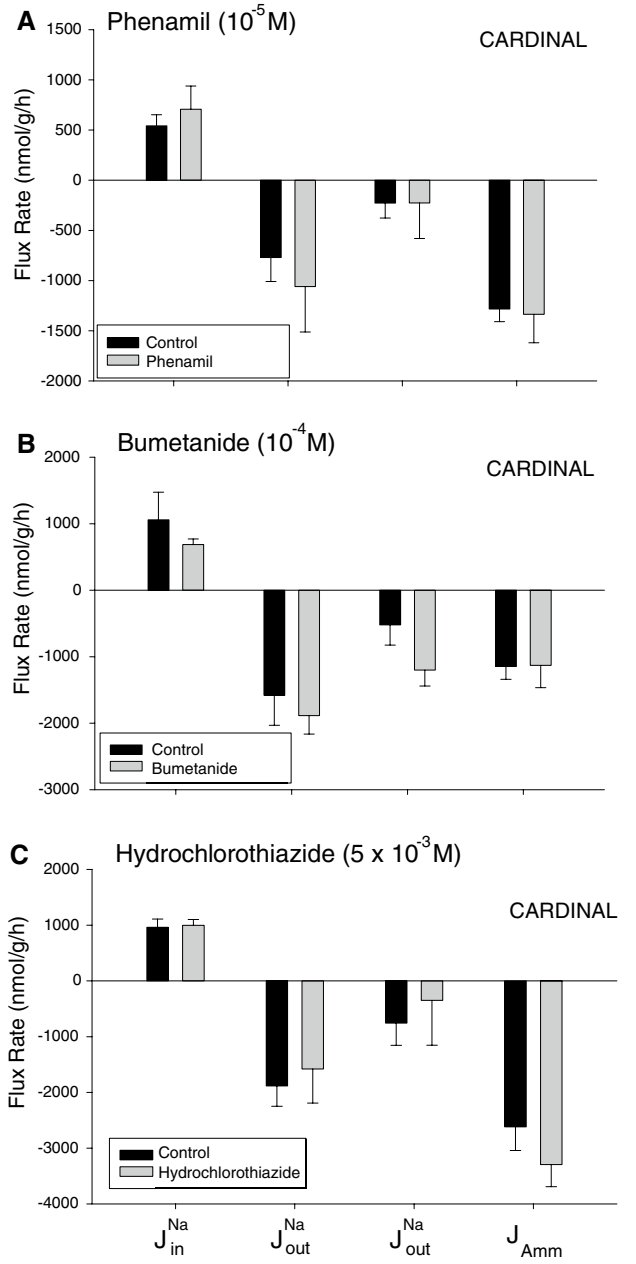

Fig. 5 The influence of **a** phenamil (10^{-5} M), **b** bumetanide (10^{-4} M), and **c** hydrochlorothiazide (5×10^{-3} M) on unidirectional Na^+ influx (J^{Na}_{in}), efflux (J^{Na}_{out}), and net flux rates (J^{Na}_{net}), and on ammonia excretion rates (J_{Amm}) relative to simultaneous time-matched controls in cardinal tetra. Means ± 1 SEM ($N = 6$). There were no significant differences ($P > 0.05$)

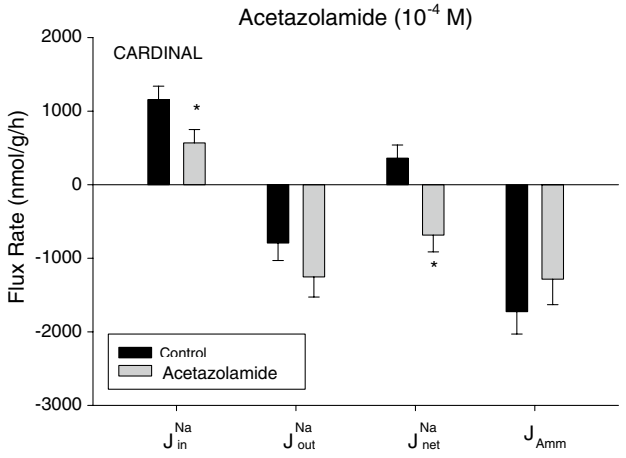

Fig. 6 The influence of acetazolamide (10^{-4} M) on unidirectional Na^+ influx (J^{Na}_{in}), efflux (J^{Na}_{out}), and net flux rates (J^{Na}_{net}), and on ammonia excretion rates (J_{Amm}) relative to simultaneous time-matched controls in cardinal tetra. Means ± 1 SEM ($N = 6$). *Asterisks* indicate significant differences ($P < 0.05$) between the acetazolamide and control treatments

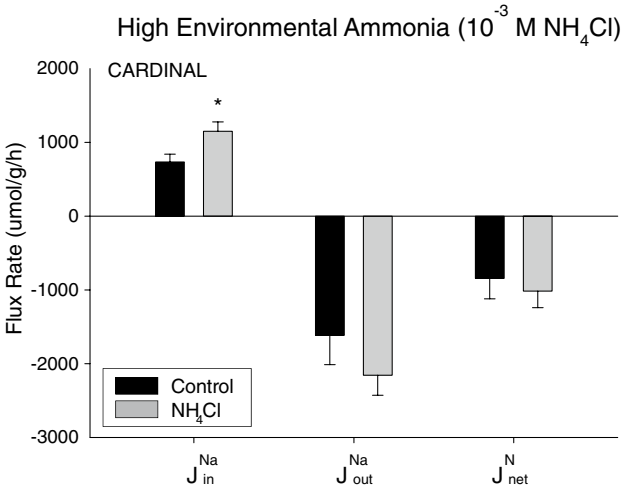

Fig. 7 The influence of high environmental ammonia (10^{-3} M NH_4Cl) on unidirectional Na^+ influx (J^{Na}_{in}), efflux (J^{Na}_{out}), and net flux rates (J^{Na}_{net}) relative to simultaneous time-matched controls in cardinal tetra. Means ± 1 SEM ($N = 6$). *Asterisks* indicate significant differences ($P < 0.05$) between the high environmental ammonia and control treatments

significant change in J^{Na}_{out} (Fig. 6). Notably, the decrease in J^{Na}_{in} was not accompanied by an inhibition of J_{Amm}, indicating that the two processes are not tightly coupled. Overall, this result suggests that Na^+ uptake is in some way dependent on the provision of H^+ ions from carbonic anhydrase function, whereas ammonia efflux is not.

Acute exposure to high environmental ammonia (HEA, 10^{-3} M NH_4Cl) at unchanged environmental pH caused a significant increase in J^{Na}_{in} with no significant change in J^{Na}_{out}, or J^{Na}_{net} (Fig. 7). This result suggests an interaction between Na^+ uptake and ammonia. However, in this experiment which was performed only on cardinal tetras, it was not possible to determine J_{Amm} during the HEA exposure

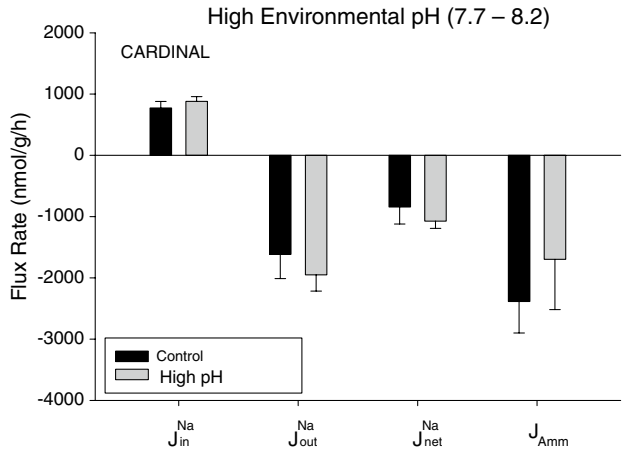

Fig. 8 The influence of high environmental pH (7.7–8.2) on unidirectional Na^+ influx (J_{in}^{Na}), efflux (J_{out}^{Na}), and net flux rates (J_{net}^{Na}), and on ammonia excretion rates (J_{Amm}) relative to simultaneous time-matched controls in cardinal tetra. The control pH was 4.5–4.9. Means ± 1 SEM ($N = 6$). There were no significant differences ($P > 0.05$)

because of the high background ammonia concentration in the water.

Acute exposure to more than a 3.0 unit rise in environmental pH (7.7–8.2 versus 4.5–4.9 in the controls) had no significant effect on J_{Amm}, J_{in}^{Na}, J_{out}^{Na}, or J_{net}^{Na} in cardinal tetras (Fig. 8). Therefore, neither Na^+ uptake nor ammonia excretion appears to be dependent on the bulk pH gradient across the gills.

$AgNO_3$ (10^{-7} M) was by far the most effective of all the treatments tested, causing a 90–97 % inhibition of J_{in}^{Na} in all three species, without changes in J_{out}^{Na}, such that J_{net}^{Na} became highly negative (Fig. 9). Notably, in all three, there was no significant change in J_{Amm}, reinforcing the conclusion from the acetazolamide experiment (Fig. 6) that J_{in}^{Na} and J_{Amm} are not tightly coupled.

To further explore the mechanism of action of $AgNO_3$ (10^{-7} M), its effects on unidirectional and net Cl^- fluxes were evaluated. This agent proved to be just as powerful in blocking J_{in}^{Cl} (85–99 % inhibition; Fig. 10) in the three species as it had been for J_{in}^{Na} (c.f. Fig. 9). However, J_{net}^{Cl} became significantly more negative only in hemigrammus tetras. There were no significant changes in J_{out}^{Cl}, similar to the lack of response in J_{out}^{Na}.

A further test focused on the time course of the inhibition of J_{in}^{Na}, and its potential reversibility when $AgNO_3$ was removed from the water. Inhibition was rapid. Upon addition of $AgNO_3$ (10^{-7} M), blockade of Na^+ uptake was complete within 5 min, as shown by the immediate cessation of the previous steady decrease in ^{22}Na cpm in the external water (Fig. 11a). In contrast, recovery was slow. After changeover to $AgNO_3$-free water, there was no significant restoration of Na^+ uptake in the following 30 min (Fig. 11b).

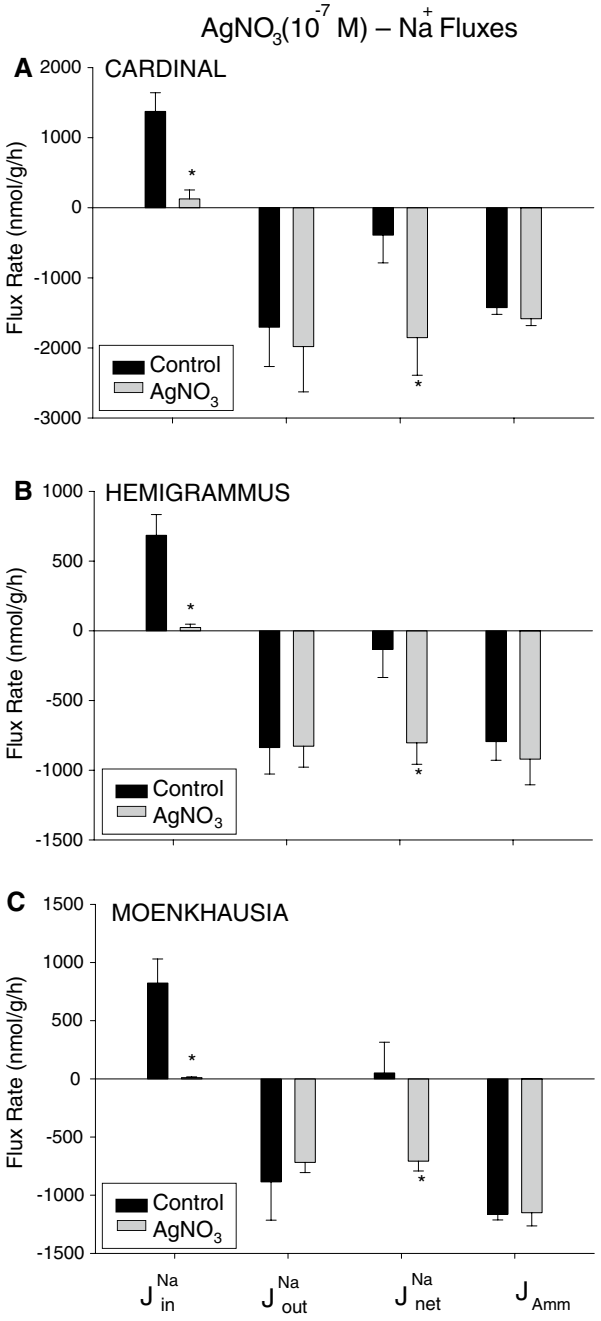

Fig. 9 The influence of $AgNO_3$ (10^{-7} M) on unidirectional Na^+ influx (J_{in}^{Na}), efflux (J_{out}^{Na}), and net flux rates (J_{net}^{Na}), and on ammonia excretion rates (J_{Amm}) relative to simultaneous time-matched controls in **a** cardinal tetra, **b** hemigrammus tetra, and **c** moenkhausia tetra. Means ± 1 SEM ($N = 5$). *Asterisks* indicate significant differences ($P < 0.05$) between the $AgNO_3$ and control treatments

Branchial ATPase activities

Na^+, K^+-ATPase and V-type H^+-ATPase exhibited comparable levels of activity (0.4–0.5 µmol ADP/mg protein/h) in gill homogenates from cardinal tetras. The activity of V-type H^+-ATPase was not affected by NH_4^+ versus K^+

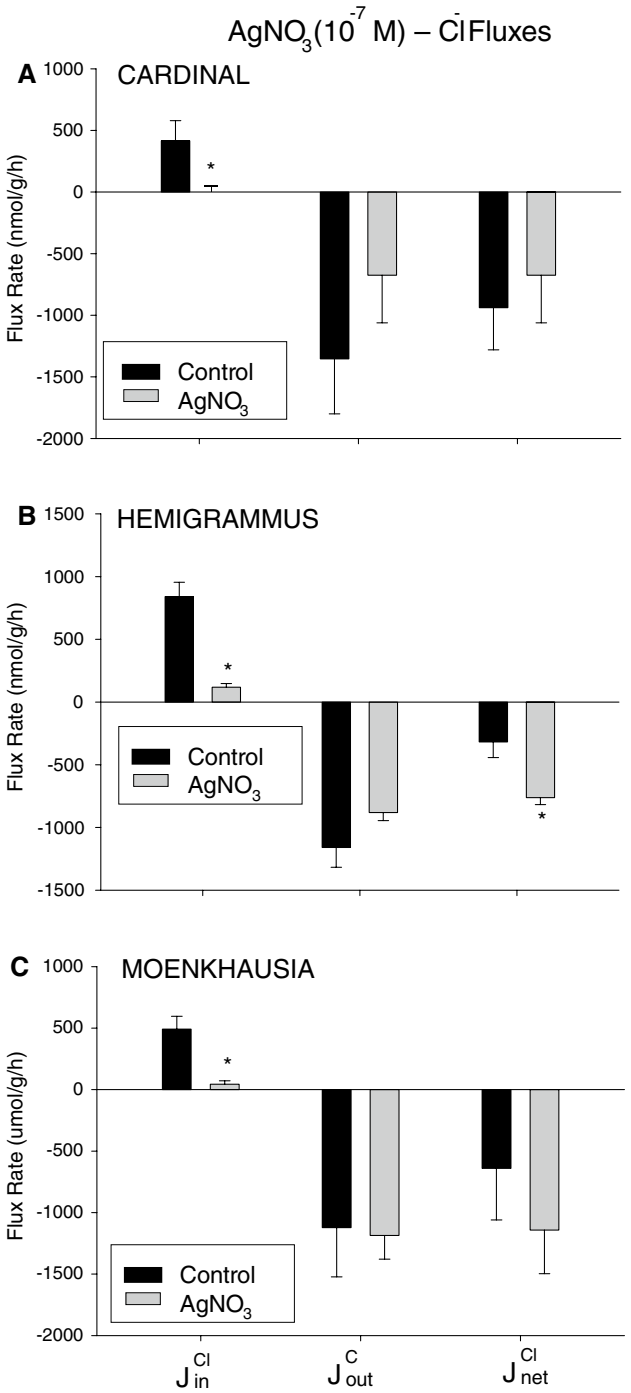

Fig. 10 The influence of $AgNO_3$ (10^{-7} M) on unidirectional Cl^- influx (J_{in}^{Cl}), efflux (J_{out}^{Cl}), and net flux rates (J_{net}^{Cl}) relative to simultaneous time-matched controls in **a** cardinal tetra, **b** hemigrammus tetra, and **c** moenkhausia tetra. Means ± 1 SEM ($N = 6$). *Asterisks* indicate significant differences ($P < 0.05$) between the $AgNO_3$ and control treatments

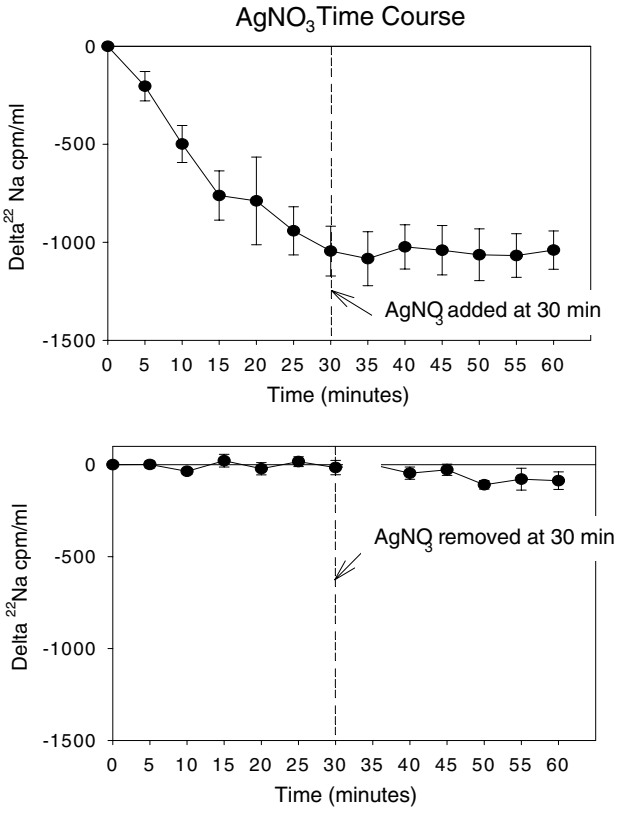

Fig. 11 The time course in cardinal tetra of **a** inhibition of unidirectional Na^+ influx rate (J_{in}^{Na}) by $AgNO_3$ (10^{-7} M) and **b** its recovery after removal of $AgNO_3$ from the external water. Na^+ influx is indicated by the change in ^{22}Na cpm concentration in the external water over 5 min intervals. A decline in cpm/ml represents Na^+ uptake by the fish. Means ± 1 SEM ($N = 6$)

concentration in the assay medium, except at 10 mM, where the presence of NH_4^+ provided higher activity (data not shown). For Na^+, K^+-ATPase, when NH_4^+ was present in the assay medium at a final concentration of 1 mM, it was equally effective to 1 mM K^+ in activating the enzyme in vitro (Fig. 12). However, at higher concentrations (3, 10, and 20 mM), NH_4^+ was significantly more effective than K^+ at the same concentrations, resulting in 60–100 % greater activity. Interestingly, activity fell significantly with increasing K^+ concentration, but this did not happen with increasing NH_4^+ concentration (Fig. 12). Overall, NH_4^+ was equally or more effective than K^+ in activating ouabain-sensitive Na^+-ATPase activity.

Discussion

Overview

Clearly, the mechanisms of branchial Na^+ uptake, ammonia excretion, and their potential linkages in Rio Negro tetras do not fit well with current models established in "standard" teleost fish. In this regard, our findings with respect to Na^+ uptake are in broad agreement with several previous

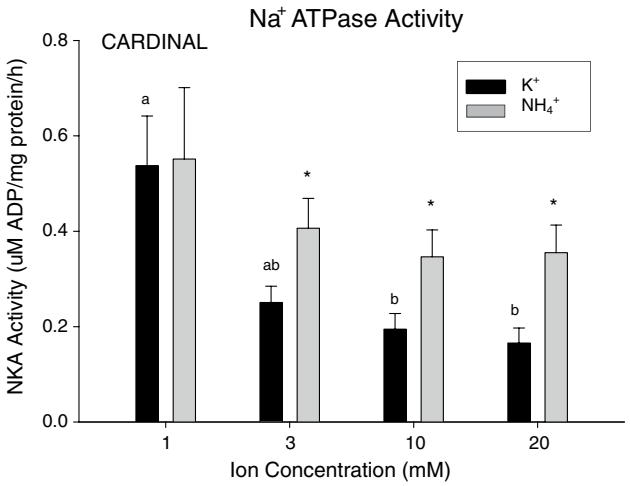

Fig. 12 A comparison of the ability of K^+ versus NH_4^+ (both in the form of chloride salts), added at various concentrations to the assay medium, to activate branchial Na^+-ATPase activity in vitro in cardinal tetra. Means ± 1 SEM ($N = 8$). *Asterisks* indicate significant differences ($P < 0.05$) between the K^+ and NH_4^+ means at the same concentrations. K^+ means sharing the *same letter* at different concentrations are not significantly different ($P > 0.05$). There were no significant differences among the NH_4^+ means

studies (Gonzalez et al. 1997; Gonzalez and Preest 1999), particularly those of Preest et al. (2005). The latter workers conducted a wide-scale pharmacological investigation on Na^+ influx in the neon tetra (*Paracheirodon innesi*) which is congeneric with the species which was the major focus of the present study, the cardinal tetra (*Paracheirodon axelrodi*). However, our study reports one important difference from Preest et al. (2005) and several new findings, especially with regard to ammonia excretion in these fish, about which there was previously little known (see "Introduction"). Most importantly it provides experimental evidence for a Na^+-ammonia linkage that was proposed by Randall et al. (1996) but for which there was previously no evidence in Rio Negro fish.

The importance of DOM

The present experiments were performed on native fish collected directly from the Rio Negro, in river water with a high, but typical concentration of DOM (11.5 mg C l^{-1}). There was broad qualitative agreement (with one important exception, the response to acetazolamide, discussed below) of the present pharmacological results with those of Preest et al. (2005) performed in the absence of Rio Negro DOM. Therefore, it seems unlikely that interactions of the drugs with the DOM in Rio Negro water rendered them ineffective, especially as several of these same drugs were effective in a previous study on freshwater stingrays studied in Rio Negro water with a similar DOM concentration (Wood et al. 2002). However, based on previous comparable data for these and other Rio Negro tetra species obtained from the aquarium trade in North America and/or tested in DOM-poor synthetic water, there were quantitative differences. In particular, rates of J_{in}^{Na}, J_{in}^{Cl}, and J_{Amm} were generally higher than in these previous studies, despite the fact that water Na^+ and Cl^- concentrations were generally lower (Wilson 1996; Gonzalez et al. 1997; Gonzalez and Preest 1999; Gonzalez and Wilson 2001; Preest et al. 2005; Matsuo and Val 2007). This suggests that Na^+ and Cl^- transporter affinities may be especially high under these "native" conditions. We cannot necessarily attribute these discrepancies to acclimation, adaptation, or the presence or absence of DOM, but the latter would seem to be a likely explanation in light of previous evidence that DOM aids in promoting J_{in}^{Na}, J_{in}^{Cl}, and J_{Amm} and limiting J_{out}^{Na}, especially under low pH conditions (Gonzalez et al. 1998, 2002; Wood et al. 2003, 2011; Matsuo and Val 2007; Galvez et al. 2008).

Na^+ uptake

In all three species, amiloride (10^{-4} M) had absolutely no effect on Na^+ uptake (Fig. 4). This finding is in accord with the wider pharmacological survey of Preest et al. (2005) on Na^+ uptake in the neon tetra using an array of amiloride analogues, and the lack of effect of phenamil (10^{-5} M), a more specific Na^+ channel blocker, on the cardinal tetra in the present study (Fig. 5a). Furthermore, amiloride (10^{-4} M) caused only 13 and 26 % drops in J_{in}^{Na} in neon tetras (Gonzalez and Preest 1999) and blackskirt tetras (Gonzalez et al. 1997), respectively. These results run counter to the pattern reported in virtually all freshwater teleosts where amiloride at this concentration inhibits Na^+ uptake to a substantial degree (e.g. Wright and Wood 1985; Wilson et al. 1994; Patrick and Wood 1999; Preest et al. 2005; Brix and Grosell 2012 and references therein), reflecting its ability to block both NHE and Na^+ channel mechanisms at this concentration (Benos 1982; Kleyman and Cragoe 1988). Indeed, even in the freshwater stingray, an elasmobranch which inhabits these same Rio Negro blackwaters, amiloride (10^{-4} M) as well as several of its analogues, strongly inhibited J_{in}^{Na} (Wood et al. 2002). However, an important difference is that in contrast to the tetras, the stingray does not operate a high-affinity, low pH resistant Na^+ uptake system, but rather relies on keeping a low rate of diffusive loss of Na^+ (Wood et al. 2002), a strategy which is also seen in some other Rio Negro teleosts (e.g. Gonzalez et al. 2002; Duarte et al. 2013). In contrast, cardinal, neon, and blackskirt tetras exhibit K_m values for Na^+ uptake which are more than an order of magnitude lower (i.e. much higher affinity) than those of the ray and some of these other Rio Negro teleosts, together with resistance

to inhibition by pH's as low as 3.25–3.5 (Gonzalez et al. 1997; Gonzalez and Preest 1999; Gonzalez and Wilson 2001; Preest et al. 2005; Matsuo and Val 2007). Hemigrammus tetras operate a similar high-affinity mechanism, though it may be more sensitive to inhibition by low pH (Gonzalez et al. 2002). There appears to be something fundamentally different about these high-affinity mechanisms of the tetras.

However, this difference does not seem to involve the participation of apical co-transport systems for Na^+ and Cl^-, either via an NCC or an NKCC. Recently, molecular and pharmacological evidence for the involvement of an apical NCC in the uptake of Na^+ from fresh water (reviewed by Evans 2011) has started to appear in both tilapia (e.g. Hiroi et al. 2008) and zebrafish (e.g. Wang et al. 2009). However, neither the NCC blocker hydrochlorothiazide (5×10^{-3} M; Stokes et al. 1984) nor the NKCC blocker bumetanide (10^{-4} M; Giménez 2006) inhibited J_{in}^{Na} in the cardinal tetra (Fig. 5b, c). The latter result is in agreement with the report of Preest et al. (2005) that furosemide (10^{-4} M), another NKCC blocker, was without effect in the neon tetra despite having a large inhibitory effect on both J_{in}^{Na} and J_{in}^{Cl} in the goldfish. Regardless, it would be difficult to understand how a system dependent on the trans-apical membrane electrochemical gradient for Na^+ could function in Na^+-poor Rio Negro water.

Na^+ uptake in cardinal tetras is clearly not limited by the high external concentration of H^+ ions at pH 4.5–4.9, as J_{in}^{Na} was not stimulated by an acute rise of >3.0 pH units (Fig. 8), in accord with other experiments on various tetra species where the pH was acutely changed in the opposite direction (Gonzalez et al. 1997; Gonzalez and Preest 1999; Gonzalez and Wilson 2001; Preest et al. 2005; Matsuo and Val 2007). This insensitivity to acute changes in environmental pH may be adaptive, because in fieldwork conducted on this same expedition, we routinely measured variations in pH of 0.4–0.6 units (maximum 1.0 unit) over short temporal and spatial scales in the poorly buffered Rio Negro. However, the same may not be true with respect to internal H^+ ions in the gill cells, as shown by the action of the CA blocker acetazolamide (10^{-4} M) in causing a significant 50 % inhibition of J_{in}^{Na} (Fig. 6).

This effect of acetazolamide represents an important difference between the present data on the cardinal tetra versus those of Preest et al. (2005) on the neon tetra. The lack of effectiveness of acetazolamide (5×10^{-4} M) in the neon tetra (Preest et al. 2005) may have been due to the different species, origin of the fish (North America), water chemistry (i.e. low DOM), or solubilization method (KOH versus 0.1 % DMSO in the present study). Indeed, since the first test by Maetz (1956) in goldfish, CA inhibition has proven to be almost uniformly effective in strongly reducing J_{in}^{Na} in a variety of teleosts (reviewed by Kirschner 2004, Gilmour and Perry 2009 and Evans 2011), though ethoxzolamide (10^{-4} M; an acetazolamide analogue) was ineffective in this regard in zebrafish acclimated to ion-poor softwater (Boisen et al. 2003). Regardless, the present result suggests that internally generated H^+ ions provided by the catalysed hydration of CO_2 are integral to the Na^+ uptake mechanism of the cardinal tetra. This evidence would be congruent with either the NHE or Na^+ channel/H^+-pump models, and indeed we report that V-type H^+-ATPase is present in the gills at about the same activity level as Na^+, K^+-ATPase. However, this does not preclude as yet unknown H^+-dependent mechanisms being involved in Na^+ uptake. For example, the NHE or Na^+ channel could be of a different type that is completely resistant to amiloride and its analogues.

The dramatic, essentially complete inhibition of both J_{in}^{Na} and J_{in}^{Cl} in all three species by a very low concentration of $AgNO_3$ (10^{-7} M) (Fig. 9) provides additional evidence, albeit indirect, for a key role of CA in the Na^+ uptake mechanism. We employed $AgNO_3$ at this concentration as it has earlier been shown to powerfully inhibit both Na^+ and Cl^- influx rates in rainbow trout (Wood et al. 1996; Morgan et al. 1997, 2004a, b), while Preest et al. (2005) demonstrated that it was even more effective in blocking J_{in}^{Na} in the neon tetra (>95 % reduction) than in the goldfish (50 % reduction). The present results corroborate Preest et al. (2005) and furthermore indicate that the gill transport system must be extremely sensitive to Ag^+ because a high proportion of the free silver ion is probably complexed by the Rio Negro DOM (Morgan et al. 2004b; Wood et al. 2011). The present results also demonstrate that $AgNO_3$ acts by a mechanism which links Na^+ and Cl^- influx together, blocking them equally (Figs. 9, 10). Originally the action of $AgNO_3$ was attributed to inhibition of basolateral Na^+, K^+-ATPase by the Ag^+ ion (Morgan et al. 1997), but later work showed that Na^+ ,K^+-ATPase blockade took 5–24 h to develop in vivo, whereas the blockade of J_{in}^{Na} and J_{in}^{Cl} developed within about 1 h, and that it occurred coincident with the blockade of CA activity (Morgan et al. 2004a, b). In trout gill cells in vitro, Goss et al. (2011) attributed the immediate inhibition of acid-stimulated Na^+ uptake by Ag^+ to an "apical process", likely associated with immediate inhibition of CA activity, whereas inhibition of Na^+, K^+-ATPase activity took 30 min. The time course experiments in the present study, showing complete blockade of J_{in}^{Na} within 5 min in cardinal tetras (Fig. 11), support the view of rapid inhibition of CA activity as the cause; a non-specific metabolic effect (e.g. inhibition of mitochondrial function) seems unlikely but cannot be entirely discounted. The classic work of Maetz and Garcia-Romeu (1964) and Garcia-Romeu and Maetz (1964) in the goldfish demonstrated that both Na^+ and Cl^- uptake were critically dependent on CA function in the gill, apparently by

supplying H^+ ions for Na^+ versus H^+ or NH_4^+ exchange, and HCO_3^- ions for Cl^- versus HCO_3^- exchange.

Ammonia efflux, and linkage to Na^+ influx

The complete lack of effect of all real or potential Na^+ uptake blockers on ammonia excretion demonstrates clearly that ammonia excretion does not depend on Na^+ influx in Rio Negro tetras. This contrasts with the majority of freshwater fish, including even the freshwater stingray which lives in these same blackwaters (Wood et al. 2002), where amiloride and its analogues have been almost invariably effective in blocking at least a portion of J_{Amm} (reviewed by Wilkie 2002, and Evans 2011). The $AgNO_3$ results are particularly convincing, because J_{Amm} remained unchanged despite 90–97 % blockade of J_{in}^{Na} (Fig. 9). Furthermore, in other Rio Negro tetra species, J_{Amm} did not change in response to acute changes in water Na^+ concentration (Wilson 1996; Gonzalez et al. 1997), in contrast to the Amazonian oscar (Wood et al. 2007). Thus the coupling mechanism envisaged in the "Na^+/NH_4^+ exchange complex" or metabolon (Wright and Wood 2009; Ito et al. 2013; see "Introduction") is not present in Rio Negro tetras.

It is unknown whether Rh proteins are involved in the tetras. However, it may be that the combination of low water pH and effective boundary layer acidification (important when pH is raised; Fig. 8) is all that is necessary to maintain the diffusive efflux of NH_3. Thus the function of Rh proteins, if present, could be redundant under normal circumstances.

However, while ammonia excretion does not depend on Na^+ uptake, we present two pieces of evidence that Na^+ uptake may well depend on ammonia excretion. The first is that J_{in}^{Na} was stimulated by acute exposure to high environmental ammonia (HEA) in the cardinal tetra (Fig. 7). This is unusual, inasmuch as HEA exposure in salmonids and cyprinids causes an initial inhibition of both J_{in}^{Na} and J_{Amm}, followed only somewhat later by either an increase or a return to control levels as the metabolon is upregulated (Twitchen and Eddy 1994; Wilson et al. 1994; Nawata et al. 2007; Zimmer et al. 2010; Liew et al. 2013; Sinha et al. 2013). Furthermore, in salmonids and cyprinids, HEA exposure routinely makes the transepithelial potential across the gills more positive relative to the environment, which would tend to reduce Na^+ uptake (reviewed by Wright and Wood 2012). The explanation for this dichotomy may be provided by the second piece of evidence, that NH_4^+ can activate the basolateral Na^+, K^+-ATPase equally or more effectively than K^+ in the cardinal tetra (Fig. 12); this does not occur in salmonids or cyprinids (Salama et al. 1999; Sinha et al. 2013). NH_4^+ often provides some low level of Na^+ ATPase activity, but only in two species of tilapia has preferential activation by NH_4^+ versus K^+ been reported previously (Balm et al. 1988; Wood et al. 2013). By this scheme, the rise in internal ammonia levels caused by HEA might give an energetic boost to the basolateral extrusion of Na^+, and, therefore, to the overall rate of J_{in}^{Na}, as well as greatly raising ammonia concentrations in the gill cells as NH_4^+ enters on the K^+ site of the enzyme. Whether or not Rh proteins are additionally involved is entirely unknown, but if they are, the deprotonation of NH_4^+ at the intracellular side of the apical Rh channel would provide more H^+ ions to additionally drive J_{in}^{Na}. Notably, this scheme was first proposed as a mechanism for driving Na^+ uptake in Rio Negro fish by Randall et al. (1996), but our results provide the first experimental evidence for this idea.

The present experiments were performed on fasted tetras. If ammonia excretion really does drive Na^+ uptake in these fish, it is interesting to speculate as to what levels of Na^+ uptake would occur in actively feeding tetras, when ammonia excretion rates are approximately sixfold higher (Fig. 1). Furthermore, would this in some way also stimulate Cl^- uptake? Feeding may not only be a source of ions from the diet, but also a source of protein nitrogen for ammonia excretion, thereby facilitating Na^+ uptake from the dilute, acidic Rio Negro water.

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