

## Acclimation to humic substances prevents whole body sodium loss and stimulates branchial calcium uptake capacity in cardinal tetras *Paracheirodon axelrodi* (Schultz) subjected to extremely low pH

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Kinetics of sodium ( $\text{Na}^+$ ) and calcium ( $\text{Ca}^{2+}$ ) uptake were studied in cardinal tetras *Paracheirodon axelrodi* acclimated to humic substances (HS,  $35 \text{ mg C l}^{-1}$ ) and low pH (pH 3.72), parallel to analysis of whole body  $\text{Na}^+$  and  $\text{Ca}^{2+}$  content. This species had a high uptake capacity ( $J_{\text{max}}$ ) for both  $\text{Na}^+$  and  $\text{Ca}^{2+}$  in soft, ion-poor water. The affinity constant ( $K_{\text{m}}$ ) did not vary significantly among treatments for either  $\text{Na}^+$  or  $\text{Ca}^{2+}$ .  $J_{\text{max}} \text{ Na}^+$  increased 30% in fish acclimated to HS for 5 weeks. Acclimation to low pH had no effect on  $J_{\text{max}} \text{ Na}^+$  but this treatment was associated with a 32% decrease on whole body  $\text{Na}^+$  content, suggesting that fish were unable to compensate for the increased  $\text{Na}^+$  loss induced by extreme acidity. Exposure of fish to HS + low pH, the treatment most closely approximating to the conditions experienced by the species in its native environment, resulted in an increase in whole body  $\text{Na}^+$  by 31% relative to acclimation to low pH alone.  $J_{\text{max}} \text{ Ca}^{2+}$  in cardinal tetras was high relative to that documented in other freshwater species acclimated to soft water ( $J_{\text{max}} = 30 \text{ nmol g}^{-1} \text{ h}^{-1}$ ). Prolonged exposure of fish to pH 3.72 inhibited  $J_{\text{max}} \text{ Ca}^{2+}$  by 53%, although whole body  $\text{Ca}^{2+}$  content remained unchanged relative to control. Acclimation of fish to HS + low pH resulted in an increase of  $J_{\text{max}} \text{ Ca}^{2+}$  by 166% relative to low pH alone. Collectively, these results suggest that HS protect cardinal tetras acclimated to soft, acidic waters by preventing excessive  $\text{Na}^+$  loss (as indicated by whole body  $\text{Na}^+$  content) and by stimulating  $\text{Ca}^{2+}$  uptake (as indicated by increased  $J_{\text{max}} \text{ Ca}^{2+}$ ) to ensure proper homeostasis.

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### INTRODUCTION

In the Amazon basin, the term 'blackwater' applies to dark coloured, transparent waters that typically have remarkably low ion content, high acidity and high concentrations of dissolved humic material (Matsuo & Val, 2003). Ion concentrations in these waters are so low that they were once referred to as

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'slightly contaminated distilled water' (Sioli, 1968). The low mineral content of Amazonian blackwaters (conductivity  $<10 \mu\text{S cm}^{-1}$ ) results from the geochemical poverty of their associated drainage basin (Leenheer, 1980; Stallard & Edmond, 1983). The acidity of blackwaters is in the range of pH 5.0–6.0 in most of the Rio Negro, the major blackwater tributary in the Amazon basin. In slow-moving blackwaters associated with flooded forest (locally known as 'igapós'), however, the pH can be as low as 3.0–4.0 (Walker, 1995). The acidity of the Rio Negro is, in part, attributed to the high concentrations of humic material (Leenheer, 1980). Humic substances (HS), organic compounds generated by the decay of both plant and animal debris, contain acidic functional groups such as carboxylic and hydroxyl-phenolic groups (Thurman, 1985). It is therefore not surprising that HS greatly influence the pH of blackwaters. For example, extremely acidic blackwaters (pH 3.0–3.5) are usually associated with a very high content of HS ( $>35 \text{ mg C l}^{-1}$ ; Walker, 1995).

Waters with low ion content and low pH are physiologically challenging for fishes, primarily because of their known effects on branchial ion regulation (McDonald & Rogano, 1986; Wood, 1989). In particular, the extremely soft nature of blackwaters, attributed to the low concentrations of hardness cations (especially  $\text{Ca}^{2+}$ ), is virtually intolerable for typical freshwater fishes such as rainbow trout *Oncorhynchus mykiss* (Walbaum). Despite the physiological constraints imposed by blackwaters, the Rio Negro sustains an impressive diversity of fishes, with over a 1000 species reported (Chao, 2001).

Fishes living in the Rio Negro are highly specialized to survive in very dilute, acidic waters. Studies have suggested that the basis for this tolerance is an increased branchial affinity for  $\text{Ca}^{2+}$  at the paracellular tight junctions in the gills, which in turn, limits branchial permeability and prevents ion leakage under dilute, acidic conditions (Gonzalez *et al.*, 1998; Gonzalez & Preest, 1999; Gonzalez & Wilson, 2001). In addition, these species are able to take up ions at high rates, even at very low environmental ion concentrations, when experiencing high diffusive ion loss (Gonzalez & Wilson, 2001; Gonzalez *et al.*, 2002).

There is growing recognition that HS can interact with biological membranes and that they can directly affect organisms (Campbell *et al.*, 1997; Steinberg, 2003), probably as a result of hydrophobic interactions between membrane lipids and the hydrophobic domains of the HS. Circumstantial evidence further suggests that HS, ubiquitous in the Rio Negro, influence ion transport processes in fishes native to these waters (Gonzalez *et al.*, 1998, 2002, 2005; Wood *et al.*, 2002, 2003). These authors reported that fishes tested in natural blackwater had lower ion efflux rates following exposure to low pH than did fishes tested in ion-poor water with no humic material, suggesting that HS help limit the branchial permeability of the gills to diffusive ion loss. This seems reasonable, given that  $\text{Ca}^{2+}$ , a major element controlling the permeability of biological membranes, is available in negligible amounts in the Rio Negro. For example,  $\text{Ca}^{2+}$  concentrations in blackwaters associated with extreme acidity (pH 3.0–3.5) are typically *c.*  $\leq 10 \mu\text{mol l}^{-1}$  (Walker, 1995). Moreover, in most Rio Negro fishes exposed to low pH, ion losses are unaffected by increased  $\text{Ca}^{2+}$  concentrations in the water, which may indicate that  $\text{Ca}^{2+}$  is not involved in the control of branchial permeability (Gonzalez *et al.*, 1998; Gonzalez & Preest, 1999; Gonzalez & Wilson, 2001).

Although  $\text{Ca}^{2+}$  is not a major electrolyte in blood plasma (c.  $2 \text{ mmol l}^{-1}$ ) relative to  $\text{Na}^+$  (c.  $150 \text{ mmol l}^{-1}$ ) in freshwater fishes,  $\text{Ca}^{2+}$  is essential in a number of physiological processes (e.g. control of membrane permeability, activation of muscular contraction, transmission of nervous impulses and bone formation).  $\text{Ca}^{2+}$  uptake occurs predominantly through the gills, and it is under a tight hormonal control because freshwater fishes are often exposed to a constant supply of  $\text{Ca}^{2+}$  in the water (Flik & Verbost, 1993). Studies on  $\text{Ca}^{2+}$  uptake rates in Amazonian fishes living in extremely low  $[\text{Ca}^{2+}]$  waters, however, is poorly known. The only work on  $\text{Ca}^{2+}$  transport in a native Rio Negro fishes appears to be that of Wood *et al.* (2002), in which unidirectional  $\text{Ca}^{2+}$  fluxes in freshwater stingrays *Potamotrygon* sp. were studied. Information on  $\text{Ca}^{2+}$  uptake kinetics in Amazonian fishes is not available in the literature.

To assess the involvement of HS on ion regulation in fishes, the kinetic relationships of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  uptake were investigated in cardinal tetras *Paracheirodon axelrodi* (Schultz), a species native to the dilute, acidic waters of the Rio Negro. Fish were acclimated to soft water with a high concentration of HS and in low pH to simulate the blackwaters to which this species is native. Whole body  $\text{Na}^+$  and  $\text{Ca}^{2+}$  concentrations were analysed in parallel with kinetics experiments to assess overall net ion balance following extended acclimation to the treatments.

## MATERIALS AND METHODS

### EXPERIMENTAL ANIMALS

Cardinal tetras ( $0.063 \pm 0.001 \text{ g}$ ), collected from the upper Rio Negro ( $00^{\circ}30' \text{ S}$ ;  $63^{\circ}12' \text{ W}$ ), were donated by Turky's Aquarium (Manaus, Amazonas, Brazil). Once in the laboratory, fish were acclimated for 3 weeks to soft well water of the following composition:  $\text{Na}^+ = 34$ ,  $\text{Ca}^{2+} = 11$ ,  $\text{Cl}^- = 28$ ,  $\text{Mg}^{2+} = 0.8$  and  $\text{K}^+ = 15 \text{ } \mu\text{mol l}^{-1}$ ; pH 6.35; dissolved humic material  $< 1 \text{ mg C l}^{-1}$ ; temperature =  $28^{\circ} \text{ C}$ . Fish were fed Tetramin flakes daily and at least 90% of the water in the holding tanks was renewed on alternate days.

### ACCLIMATION REGIME

Following acclimation to soft well water, fish were randomly assigned to four treatments (two replicates each) to which they were acclimated for five more weeks. These treatments were: 1) control (well water), 2) HS ( $35 \text{ mg C l}^{-1}$ ), 3) low pH (pH 3.72) and 4) HS + low pH (see Table I for additional details). Base water for all treatments

TABLE I. Characteristics of treatments (means  $\pm$  S.E.,  $n = 35$ ) to which cardinal tetras were acclimated for 5 weeks

Treatment	Nominal HS ( $\text{mg C l}^{-1}$ )	Water pH	$\text{Na}^+$ ( $\mu\text{mol l}^{-1}$ )	$\text{Ca}^{2+}$ ( $\mu\text{mol l}^{-1}$ )
Control	1	$6.35 \pm 0.04$	$36 \pm 2$	$12 \pm 1$
HS	35	$6.37 \pm 0.03$	$78 \pm 3$	$42 \pm 2$
Low pH	1	$3.72 \pm 0.04$	$52 \pm 2$	$12 \pm 3$
HS + low pH	35	$3.75 \pm 0.05$	$86 \pm 3$	$42 \pm 2$

HS, humic substances.

consisted of soft well water in which humic content and pH were adjusted accordingly. Acclimation solution in holding tanks was renewed by 90% daily for all treatments. There was no fish mortality during acclimation to any of the treatments.

Aldrich humic acid (Sigma-Aldrich, St Louis, MO, U.S.A.) was used to adjust the concentrations of HS in the acclimation tanks. Humic solution was reconstituted by dissolving Aldrich humic acid in distilled water in a darkened beaker and agitating for 3 h using a magnetic stirrer. This solution was then added to well water in the acclimation tanks. Nominal concentrations of dissolved organic carbon (DOC) were set at *c.* 35 mg C l<sup>-1</sup> (or 87.5 mg Aldrich humic acid l<sup>-1</sup>) similar to what cardinal tetras experience in their natural habitat in the flooded forests of the Rio Negro basin. DOC concentrations were estimated based on the observation that the Aldrich humic acid is *c.* 40% DOC (McGeer *et al.*, 2002). Background Na<sup>+</sup> and Ca<sup>2+</sup> concentrations were unavoidably raised slightly with the addition of Aldrich humic acid in the acclimation water (Table I).

Water pH in treatments involving chronic exposure to low pH was adjusted by adding concentrated acid (1 N HNO<sub>3</sub>). During acclimation, water pH remained relatively constant in the replicated treatments (Table I), given the large volume of water in the tanks (40 l) in relation to the small biomass of the fish (*c.* 16 g: 150 fish × 0.065 g).

## EXPERIMENTAL PROTOCOL

### *Kinetics of Na<sup>+</sup> and Ca<sup>2+</sup> uptake*

The kinetics of the relationship between ion uptake rates at various environmental ion concentrations in cardinal tetras was based on clean ('fresh') acclimation water from each treatment. This procedure was used to prevent osmotic and ionic stress or artefacts on uptake rates upon transfer to artificially reconstituted solutions with low concentrations of the ion. The respective concentrations of Na<sup>+</sup> or Ca<sup>2+</sup> from each acclimation treatment were therefore used as the starting points for ion uptake measurements. The original aspects of each treatment (humic content or pH level) were maintained throughout ion uptake experiments. Feeding was suspended 48 h prior to the onset of the ion uptake experiments.

Fish were transferred to aerated polyethylene chambers containing freshly renewed acclimation solution (100 ml), and allowed to recover for 1 h prior to the onset of experiments. Ion uptake was determined over five or six external ion concentrations. Influx measurements were based on the amount of isotope incorporated by the fish during a 2–3 h period using terminal analysis. Each fish yielded only one uptake rate measurement at one ion concentration, with seven to 10 fish tested at each concentration. Na<sup>+</sup> concentrations were adjusted sequentially from 45 to 600 μmol l<sup>-1</sup> using a concentrated stock of NaCl, and isotope (<sup>22</sup>Na; Amersham Pharmacia, Cardiff, Wales, U.K.) was added proportionally to yield from 18.5 to 148 kBq. Ca<sup>2+</sup> concentrations were adjusted sequentially from 10 to 600 μmol l<sup>-1</sup> using a concentrated stock of Ca(NO<sub>3</sub>)<sub>2</sub> 4H<sub>2</sub>O, and isotope (<sup>45</sup>Ca; Amersham Pharmacia) was added proportionally to yield from 370 to 11 840 kBq (freshwater fishes take up Ca<sup>2+</sup> at much lower rates relative to Na<sup>+</sup>, hence more isotope is needed to measure Ca<sup>2+</sup> uptake rates). Water samples (5 ml) were taken for later analysis both at the beginning and at the end of kinetics experiments. There was no significant variation in water pH during kinetics uptake in the low pH treatments.

Ion uptake was based on the total radioactivity incorporated into the fish, following Perry & Wood (1985). At the end of the exposure, fish were rinsed for 30 s in 500 mmol l<sup>-1</sup> NaCl or Ca(NO<sub>3</sub>)<sub>2</sub> 4H<sub>2</sub>O to displace surface bound isotope ('cold displacement'). Fish were then killed with an overdose of anaesthetic (1 g l<sup>-1</sup> MS-222, Sigma-Aldrich), blotted dry in paper towel and transferred to pre-weighed vials for analysis of incorporated <sup>22</sup>Na or <sup>45</sup>Ca.

### *Whole body Na<sup>+</sup> and Ca<sup>2+</sup> content*

Whole body Na<sup>+</sup> concentration is a useful estimate of the net ion loss experienced by fishes, particularly following chronic exposure to low pH (Grippio & Dunson, 1991;

Gonzalez *et al.*, 1998). Total body  $\text{Ca}^{2+}$  concentration is not typically used as an indicator of overall  $\text{Ca}^{2+}$  loss, but it was addressed in this study for comparative purposes.

To determine whole body ion content in cardinal tetras following acclimation to the various treatments, fish ( $n = 10$ ) were quickly rinsed in deionised water and instantly killed upon exposure to liquid  $\text{N}_2$  on the same day the kinetics experiments were performed. Fish were transferred to pre-weighed vials, the mass of the fish was recorded, and samples were set for acid digestion.

## ANALYTICAL TECHNIQUES

### *Kinetics of $\text{Na}^+$ and $\text{Ca}^{2+}$ uptake*

Specific activity of the isotope in the water samples was determined based on analysis of  $\text{Na}^+$  or  $\text{Ca}^{2+}$  concentration and  $^{22}\text{Na}$  or  $^{45}\text{Ca}$  radioactivity. Ion concentrations were determined using flame atomic absorption spectrophotometry (AAAnalyst 800, Perkin Elmer, Singapore). Lanthanum chloride (0.3%  $\text{LaCl}_3$ ) was added to prevent interference of other cations in  $\text{Ca}^{2+}$  analysis. Radioactivity of the water samples was analysed in 1 ml aliquots mixed with scintillation cocktail for aqueous samples (ACS; Amersham Pharmacia) and determined using liquid scintillation counting (LS6500; Beckman & Coulter, Fullerton, CA, U.S.A.).

Fish samples were digested in tissue solubilizer (NCS-II; Amersham Pharmacia) at 60° C for 24 h under steady agitation. Tissue digests were then neutralized with glacial  $\text{CH}_3\text{COOH}$ , to which scintillation cocktail for organic samples (OCS; Amersham Pharmacia) was added. Incorporated radioactivity in digested fish samples was analysed by liquid scintillation counting, corrected for 'quenching' effects, and ion uptake was determined based on the following equation (Perry & Wood, 1985):  $J_{\text{in}} = R_{\text{WB}}(A_sMT)^{-1}$ , where  $R_{\text{WB}}$  is the radioactivity in counts per minute of the whole body (cpm),  $A_s$  is the mean specific activity of the isotope during the exposure ( $\text{cpm } \mu\text{mol}^{-1}$  ion),  $M$  is the wet mass of the fish (g) and  $T$  is the duration of the ion uptake experiment (h).

The kinetic estimates,  $J_{\text{max}}$  (maximum ion uptake rate) and  $K_m$  (the ion concentration that yields an uptake rate of 50% of  $J_{\text{max}}$ ), in each treatment were obtained using a non-linear curve-fitting programme (Sigma Plot version 2000).

### *Whole body $\text{Na}^+$ and $\text{Ca}^{2+}$ content*

For analysis of total  $\text{Na}^+$  and  $\text{Ca}^{2+}$  content, fish samples were digested in 2 ml of 1 N  $\text{HNO}_3$  in tightly capped vials for 24 h at 70° C. Following acid digestion, fish samples were cooled to room temperature and an aliquot of supernatant of each sample was diluted in deionised water for determination of ion concentrations according to techniques described in the previous section. Ion concentrations in tissue digests were corrected for dilution factors. Whole body ion concentrations in cardinal tetras were expressed in  $\mu\text{mol g}^{-1}$  (wet mass).

## STATISTICAL ANALYSIS

Bootstrap randomization was employed to test the reliability of the results observed in the seven to 10 pseudoreplicates of each treatment.  $J_{\text{max}}$  and  $K_m$  was determined using non-linear regression in 500 bootstrap replications of the original data, and the mean  $\pm$  s.e. were compiled in Table II. Statistical differences in  $J_{\text{max}}$  and  $K_m$  values were analysed using paired *t*-tests, comparing the mean  $\pm$  s.e. of the control group (or low pH treatment) with the mean  $\pm$  s.e. of each treatment, one at a time. The mean  $\pm$  s.e. of whole body ion content in the treatments was analysed using ANOVA, followed by multiple comparison tests (Dunnett's test) when the differences proved to be significant. All comparisons were made relative to the control group, and also relative to the low pH group when specified. Significance of the tests was  $\alpha < 0.05$  throughout.

TABLE II. Kinetic parameters ( $J_{\max}$  and  $K_m$ , means  $\pm$  S.E.) for  $\text{Na}^+$  and  $\text{Ca}^{2+}$  uptake, for cardinal tetras acclimated to low pH and humic substances (HS). Whole body  $\text{Na}^+$  and  $\text{Ca}^{2+}$  concentrations (means  $\pm$  S.E.) for fish in each treatment are also given

Treatment	$J_{\max} \text{Na}^+$ ( $\text{nmol g}^{-1} \text{h}^{-1}$ )	$K_m \text{Na}^+$ ( $\mu\text{mol l}^{-1}$ )	Whole body $\text{Na}^+$ ( $\mu\text{mol g}^{-1}$ wet mass)
Control	2076 $\pm$ 108	19 $\pm$ 7	71 $\pm$ 3
HS	2692 $\pm$ 196*	33 $\pm$ 15	74 $\pm$ 2
Low pH	2279 $\pm$ 139	38 $\pm$ 11	48 $\pm$ 3*
HS + low pH	2492 $\pm$ 109*	23 $\pm$ 8	63 $\pm$ 4 <sup>†</sup>
Treatment	$J_{\max} \text{Ca}^{2+}$ ( $\text{nmol g}^{-1} \text{h}^{-1}$ )	$K_m \text{Ca}^{2+}$ ( $\mu\text{mol l}^{-1}$ )	Whole body $\text{Ca}^{2+}$ ( $\mu\text{mol g}^{-1}$ wet mass)
Control	30 $\pm$ 4	102 $\pm$ 39	168 $\pm$ 6
HS	34 $\pm$ 3 <sup>†</sup>	39 $\pm$ 12	202 $\pm$ 13*
Low pH	14 $\pm$ 2*	125 $\pm$ 49	158 $\pm$ 24
HS + low pH	39 $\pm$ 4 <sup>†</sup>	81 $\pm$ 25	184 $\pm$ 17

\*Significant differences relative to control group ( $\alpha < 0.05$ ).

<sup>†</sup>A significant difference relative to low pH treatment.

## RESULTS

### $\text{Na}^+$ TRANSPORT

The kinetic analysis of  $\text{Na}^+$  uptake indicated a very high  $\text{Na}^+$  uptake capacity (high  $J_{\max}$ ) in cardinal tetras chronically exposed to soft, ion-poor waters (Fig. 1 and Table II). A 5 week exposure of cardinal tetras to HS (35 mg C l<sup>-1</sup>) resulted in an increase of  $J_{\max} \text{Na}^+$  by 30%, suggesting a stimulatory effect of HS on ion regulation.

Prolonged exposure of fish to low pH (3.72) had no effect on  $J_{\max} \text{Na}^+$ , but it was associated with a 32% decrease in whole body  $\text{Na}^+$  concentration (Table II).  $J_{\max} \text{Na}^+$  in fish exposed to HS + low pH was 20% higher than the control. More importantly, this treatment was associated with a 31% increase in whole body  $\text{Na}^+$  concentration relative to fish acclimated to low pH alone (Table II).

$K_m$  estimates for  $\text{Na}^+$  uptake in cardinal tetras did not differ among treatments. The values were typically low (Table II). Estimation of  $K_m$  values for  $\text{Na}^+$  uptake in this study, however, may have been compromised by the lack of data on uptake rates at lower water  $\text{Na}^+$  concentrations, given that at the concentrations tested, most treatments had already reached a plateau (Fig. 1 and Table II).

### $\text{Ca}^{2+}$ TRANSPORT

The saturation kinetics for  $\text{Ca}^{2+}$  uptake in cardinal tetras displayed a typical Michaelis-Menten relationship. Kinetic analysis of  $\text{Ca}^{2+}$  transport revealed a relatively high  $J_{\max} \text{Ca}^{2+}$  in this species following acclimation to soft, ion-poor water (Fig. 2 and Table II). A 5 week exposure of cardinal tetras to HS did not significantly affected  $J_{\max} \text{Ca}^{2+}$  relative to control values (Fig. 2

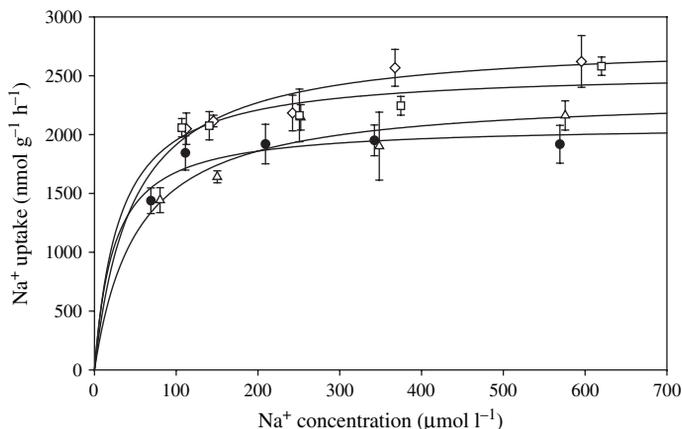


FIG. 1. Kinetics of  $\text{Na}^+$  uptake in cardinal tetras acclimated to various treatments for 5 weeks [●, control; ◇, humic substances (HS,  $35 \text{ mg C l}^{-1}$ ); △, low pH (3.72); □, HS + low pH]. Value are means  $\pm$  s.e. ( $n = 7\text{--}10$  fish per concentration tested).

and Table II), as opposed to the stimulation seen in  $J_{\max} \text{Na}^+$ . Exposure to HS, however, was associated with a 20% increase in whole body  $\text{Ca}^{2+}$  content (Table II).

Extended exposure of fish to low pH (pH 3.72) had a marked effect on the  $\text{Ca}^{2+}$  uptake capacity, resulting in a 51% inhibition in  $J_{\max}$ . Whole body  $\text{Ca}^{2+}$  concentration in this treatment, however, remained unchanged relative to controls (Table II). Interestingly, acclimation of fish to HS + low pH increased  $J_{\max} \text{Ca}^{2+}$  by 166% relative to low pH alone (Fig. 2 and Table II), suggesting that HS protected cardinal tetras against the inhibitory effect on  $\text{Ca}^{2+}$  uptake induced by extreme acidity.  $K_m \text{Ca}^{2+}$  did not vary among treatments (Table II).

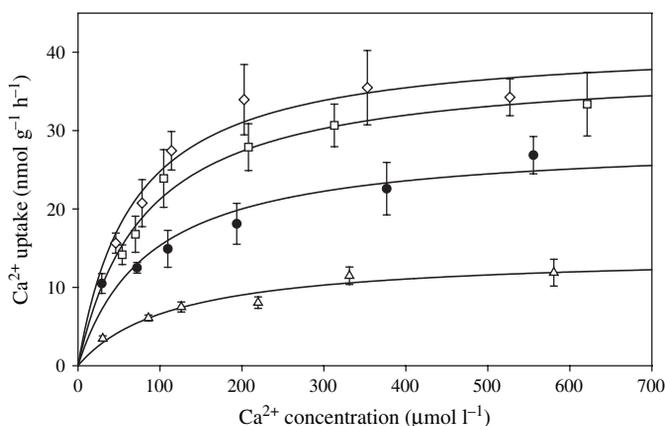


FIG. 2. Kinetics of  $\text{Ca}^{2+}$  uptake in cardinal tetras acclimated to various treatments for 5 weeks [●, control; ◇, humic substances (HS,  $35 \text{ mg C l}^{-1}$ ); △, low pH (3.72); □, HS + low pH]. Value are means  $\pm$  s.e. ( $n = 7\text{--}10$  fish per concentration tested).

## DISCUSSION

Humic substances, once assumed to be inert compounds in freshwater bodies, can be very reactive because of their functional groups, mainly carboxylic and hydroxyl-phenolic groups. In a recent review, Steinberg *et al.* (2006) summarized some of the biological effects HS play on living organisms, particularly organisms from aquatic habitats. This growing recognition that HS interacts directly or indirectly with biological membranes in native organisms from HS-enriched habitats such as the Rio Negro represents a whole new way of looking at HS, not only considering the effects from the geochemical stand point (*e.g.* adsorption, chelation, or complexation of elements) but also from the biological stand point (*e.g.* physiological, biochemical and molecular). The major finding of this work is in agreement with the concept that HS can biologically affect aquatic organisms, as HS significantly affected  $\text{Na}^+$  and  $\text{Ca}^{2+}$  transport in cardinal tetras, especially under extremely low pH conditions, as discussed below.

### $\text{Na}^+$ TRANSPORT

Kinetic analysis of  $\text{Na}^+$  transport in control fish (soft water only) revealed a very high  $\text{Na}^+$  uptake capacity in cardinal tetras ( $J_{\max} = 2076 \text{ nmol g}^{-1} \text{ h}^{-1}$ ; Fig. 1 and Table II), which was 2.7 times greater than that previously documented for the same species by Gonzalez & Wilson (2001). Whole body  $\text{Na}^+$  content of cardinal tetras ( $71 \text{ } \mu\text{mol g}^{-1}$ ; Table II) was similar to that reported by Gonzalez *et al.* (1998) ( $64 \text{ } \mu\text{mol g}^{-1}$ ).

Acclimation of cardinal tetras to HS stimulated  $J_{\max} \text{ Na}^+$  relative to control fish (Table II). In a previous study, rainbow trout acutely exposed to HS in soft water did not show a significant increase in  $J_{\max} \text{ Na}^+$  (Matsuo *et al.*, 2004), probably because acclimation to low concentration of HS (*c.*  $8 \text{ mg C l}^{-1}$ ) had not been established, or because rainbow trout may have different gill physiology relative to cardinal tetras that renders them insensitive to HS, given that they are not native to humic-enriched waters.

Chronic exposure to pH 3.72 confirmed the complete insensitivity of  $\text{Na}^+$  uptake to low pH in cardinal tetras previously reported by Gonzalez & Wilson (2001). This 5 week exposure of fish to low pH resulted in similar  $J_{\max}$  relative to control fish at pH 6.4 (Fig. 1 and Table II). Inhibition of  $\text{Na}^+$  influx is a typical response of freshwater fishes to low pH, in addition to the stimulation of  $\text{Na}^+$  efflux (Wood, 1989). Gonzalez & Wilson (2001) documented that, despite the lack of inhibition on  $\text{Na}^+$  influx at pH 3.5, cardinal tetras still experienced significant diffusive  $\text{Na}^+$  efflux. This inability of the species to prevent excessive  $\text{Na}^+$  loss in extremely low pH helps explain the 32% decrease in whole body  $\text{Na}^+$  content found in the present study (Table II). Thus, despite the great ability of cardinal tetras to sustain high  $\text{Na}^+$  uptake rates in extremely acidic conditions, the species is unable to completely compensate for the diffusive  $\text{Na}^+$  loss induced by low pH. Gonzalez *et al.* (1998) found no decrease in whole body  $\text{Na}^+$  content in cardinal tetras exposed to pH 3.5 over 5 days, suggesting that either a longer exposure to low pH may be needed to reflect such differences in whole body  $\text{Na}^+$  concentration, or that size-effects may play a role in the ability of cardinal tetras

to control ion loss better than smaller fish. Fish used in the work of Gonzalez *et al.* (1998) were 2.5 times heavier than the cardinal tetras used in this study.

Exposure of fish to HS + low pH, the treatment most likely to simulate the conditions of the species' native habitat, resulted in a 20% increase in  $J_{\max}$   $\text{Na}^+$  relative to controls (Fig. 1 and Table II). More importantly, the presence of HS in the acclimation media prevented the decrease in whole body  $\text{Na}^+$  content compared to acclimation to low pH alone (Table II), suggesting that the presence of HS helped prevent increased diffusive  $\text{Na}^+$  loss. Protection of the  $\text{Na}^+$  efflux component in fishes exposed to natural HS has also been reported for freshwater stingrays (Wood *et al.*, 2003) as well as other teleosts from the Rio Negro (Gonzalez *et al.*, 2002).

It is worth noting that the higher  $J_{\max}$   $\text{Na}^+$  reported in this study relative to that of Gonzalez & Wilson (2001) may be attributed to either acclimation effects or mass-specific gill area. As pointed out by Gonzalez *et al.* (2002), fishes native to ion-poor waters of Amazonian tributaries that are acquired outside this region (*e.g.* in North America or Europe) have been exposed to variable water chemistries, often with higher ion concentrations than those that these fishes experience in the wild, which ultimately may affect the rates of ion transport. That cardinal tetras used by Gonzalez & Wilson (2001) were much larger (0.545 g) than specimens used in the present study (0.063 g) could also potentially explain the differences in  $J_{\max}$  between the two studies. Because the mass-specific gill area is inversely correlated with body mass in fishes (*i.e.* larger branchial area relative to the body; Hughes & Morgan, 1973), smaller fishes may have higher ion uptake rates (Grosell *et al.*, 2002).

The determination of  $K_m$  was complicated by a lack of data for uptake rates at lower  $\text{Na}^+$  concentrations ( $<100 \mu\text{mol l}^{-1}$ ). Uptake rates at these concentrations were difficult to assess, particularly in 'HS' and 'HS + low pH' treatments, because Aldrich humic acid unavoidably raised  $\text{Na}^+$  concentration in the water (Table I) and because experiments were based in water from the holding aquaria as a starting point. It should be kept in mind that because  $\text{Na}^+$  uptake rates in fish at the lowest  $\text{Na}^+$  concentrations tested in this study were already near the plateau point (Fig. 1), it was difficult to accurately determine  $K_m$  using non-linear regression. Yet, the results for  $\text{Na}^+$  uptake rates at the concentrations that were tested yielded results quantitatively similar to those for  $J_{\max}$   $\text{Na}^+$  calculated for each treatment (Fig. 1 and Table II), which suggests that the lack of lower uptake rates in the kinetics curve had a minimal effect on the computation of  $J_{\max}$ .

## $\text{Ca}^{2+}$ TRANSPORT

Fishes native to the Rio Negro face not only an extremely low water  $\text{Ca}^{2+}$  availability, but also an extremely low pH in their habitat.  $\text{Ca}^{2+}$  uptake in most freshwater fishes occurs predominantly through the gills, at apical  $\text{Ca}^{2+}$  channels located in the chloride cells.  $\text{Ca}^{2+}$  enters the cell passively, based on favourable electrochemical conditions, and is then transported to the blood by the basolateral  $\text{Ca}^{2+}$ -ATPase and a  $\text{Na}^+$ -dependent  $\text{Ca}^{2+}$  pump (Flik & Verbost, 1993; Flik *et al.*, 1996).

Exposure to low environmental  $\text{Ca}^{2+}$  concentrations has been suggested to induce higher  $\text{Ca}^{2+}$  uptake rates in fishes (Flik *et al.*, 1986). Cardinal tetras acclimated

to low  $\text{Ca}^{2+}$  concentration sustained high  $\text{Ca}^{2+}$  uptake rates. For example, when water  $\text{Ca}^{2+}$  concentrations were *c.* 30–50  $\mu\text{mol l}^{-1}$ ,  $\text{Ca}^{2+}$  uptake occurred at rates as high as 10–16  $\text{nmol g}^{-1} \text{h}^{-1}$ , except in those fish acclimated to low pH (Fig. 2). Comparison of  $J_{\text{max}} \text{Ca}^{2+}$  in cardinal tetras was likewise higher ( $J_{\text{max}} \text{Ca}^{2+} = 30 \text{ nmol g}^{-1} \text{h}^{-1}$ ) relative to *Fundulus heteroclitus* (L.) acclimated to 100  $\mu\text{mol l}^{-1} \text{Ca}^{2+}$  ( $J_{\text{max}} \text{Ca}^{2+} = 15.5 \text{ nmol g}^{-1} \text{h}^{-1}$ ; Patrick *et al.*, 1997) or rainbow trout acclimated to 25  $\mu\text{mol l}^{-1} \text{Ca}^{2+}$  and tested *in vitro* ( $J_{\text{max}} \text{Ca}^{2+} = 16 \text{ nmol g}^{-1} \text{h}^{-1}$ ; Perry & Wood, 1985). The results of this study suggest that cardinal tetras take up  $\text{Ca}^{2+}$  at relatively high rates, probably in response to the low environmental  $\text{Ca}^{2+}$  concentrations or, again, possibly because of the small size of the fish (which are still actively growing and presumably experiencing a higher demand for  $\text{Ca}^{2+}$ ).

Exposure to HS increased  $\text{Ca}^{2+}$  uptake capacity ( $J_{\text{max}}$ ) of cardinal tetras only slightly (*i.e.* not statistically significant), as opposed to the differences seen in  $J_{\text{max}} \text{Na}^{+}$  (Fig. 2 and Table II). Interestingly, the 20% increase in whole body  $\text{Ca}^{2+}$  content associated with this treatment presumably resulted from this mild stimulation on  $J_{\text{max}} \text{Ca}^{2+}$  by HS. The dynamics of the body  $\text{Ca}^{2+}$  reserve, however, may not accurately reflect the conditions fishes usually experience in a given water chemistry, so comparisons are somewhat limited.

Chronic exposure of fish to low pH had a severe impact on  $J_{\text{max}} \text{Ca}^{2+}$ , which was reduced by 51% relative to the control (Fig. 2 and Table II). Inhibition of  $\text{Ca}^{2+}$  uptake in fishes is known to occur at low pH (Höbe *et al.*, 1984, Wood *et al.*, 2002), however the exact mechanism by which low pH inhibits  $\text{Ca}^{2+}$  uptake remains unclear. Despite the marked inhibition on  $J_{\text{max}} \text{Ca}^{2+}$  in fish exposed to low pH, whole body  $\text{Ca}^{2+}$  content was unaffected (Table II). Thus, to sustain normal  $\text{Ca}^{2+}$  homeostasis, cardinal tetras probably compensated for the decreased  $\text{Ca}^{2+}$  uptake capacity either by reducing the rate of  $\text{Ca}^{2+}$  loss or by obtaining  $\text{Ca}^{2+}$  through the diet.

Exposure of fish to HS + low pH stimulated  $J_{\text{max}} \text{Ca}^{2+}$  by a marked percentage (166%) relative to acclimation to low pH alone (Fig. 2 and Table II). This supports the hypothesis that HS indeed have a protective and stimulatory effect on ion regulation, especially under low pH conditions (Gonzalez *et al.*, 1998, 2002; Wood *et al.*, 2003).

It is noteworthy to point out that, in the treatments involving Aldrich humic acid ('HS' and 'HS + low pH'), cardinal tetras were exposed to initially higher  $\text{Ca}^{2+}$  concentrations during acclimation (Table I), the possibility that this influenced the results cannot be discarded. Prolonged exposure to environmental  $\text{Ca}^{2+}$  concentrations, however, has been reported to result in a gradual return to control  $\text{Ca}^{2+}$  uptake rates (Perry & Wood, 1985), which was not seen in this study.

The estimated  $K_m$  values, *i.e.* the  $\text{Ca}^{2+}$  concentration that yields an uptake rate of 50% of  $J_{\text{max}}$ , were between 39 and 125  $\mu\text{mol l}^{-1} \text{Ca}^{2+}$ , and they did not differ significantly among treatments (Table II).  $K_m \text{Ca}^{2+}$  in cardinal tetras acclimated to 10  $\mu\text{mol l}^{-1} \text{Ca}^{2+}$  ( $K_m \text{Ca}^{2+} = 102 \mu\text{mol l}^{-1}$ ) was higher relative to that of *F. heteroclitus* acclimated to 100  $\mu\text{mol l}^{-1} \text{Ca}^{2+}$  ( $K_m \text{Ca}^{2+} = 62.5 \mu\text{mol l}^{-1}$ ; Patrick *et al.*, 1997), but it was similar to that of rainbow trout acclimated to 1000  $\mu\text{mol l}^{-1} \text{Ca}^{2+}$  ( $K_m \text{Ca}^{2+} = 92 \mu\text{mol l}^{-1}$ ; Hogstrand *et al.*, 1994).

Overall, the results of this study support the hypothesis that the presence of HS in acidic waters provides protection against ionoregulatory disturbances in fishes, either by preventing excessive efflux rates of ions or by stimulating ion

uptake capacity (Gonzalez *et al.*, 2002, 2005; Wood *et al.*, 2003). HS are therefore environmentally relevant and physiologically beneficial to fishes living in naturally acidified waters such as those found in the Rio Negro basin, because they probably improve  $\text{Na}^+$  and  $\text{Ca}^{2+}$  homeostasis. Yet, additional studies using natural HS are necessary to corroborate these results because the use of Aldrich humic acid as a surrogate for natural HS remains controversial (Richards *et al.*, 1999; Wood *et al.*, 2003; Matsuo *et al.*, 2004).

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