Effects of Water pH and Calcium Concentration on Ion Balance in Fish of the Rio Negro, Amazon

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ABSTRACT

We examined the effects of acute low-pH exposure on ion balance (Na^+, Cl^-, K^+) in several species of fish captured from the Rio Negro, a dilute, acidic tributary of the Amazon. At pH 5.5 (untreated Rio Negro water), the four Rio Negro species tested (piranha preta, *Serrasalmus rhombeus;* piranha branca, *Serrasalmus* cf. *holandi;* aracu, *Leporinus fasciatus;* and pacu, *Myleus* sp.) were at or near ion balance; upon exposure to pH 3.5, while Na^+ and Cl^- loss rates became significant, they were relatively mild. In comparison, tambaqui (*Colossoma macropomum*), which were obtained from aquaculture and held and tested under the same conditions as the other fish, had loss rates seven times higher than all the Rio Negro species. At pH 3.0, rates of Na^+ and Cl^- loss for the Rio Negro fish increased three- to fivefold but were again much less than those observed

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in tambaqui. Raising water Ca^{2+} concentration from 10 μmol L^{-1} to 100 μmol L^{-1} during exposure to the same low pH's had no effect on rates of ion loss in the three species tested (piranha preta, piranha branca, aracu), which suggests that either they have such a high branchial affinity for Ca^{2+} that all sites are saturated at 10 μmol L^{-1} and additional Ca^{2+} had no effect, or that Ca^{2+} may not be involved in regulation of branchial ion permeability. For a final Rio Negro species, the cardinal tetra (*Paracheirodon axelrodi*), we monitored body Na^+ concentration during 5 d of exposure to pH 6.0, 4.0, or 3.5. These pH's had no effect on body Na^+ concentration. These data together suggest that exceptional acid tolerance is a general characteristic of fish that inhabit the dilute acidic Rio Negro and raise questions about the role of Ca^{2+} in regulation of branchial ion permeability in these fish.

Introduction

The waters of the Rio Negro, a major tributary of the Amazon River, drain nutrient-poor jungle soils and are extremely dilute. Just above the city of Manaus, for example, Furch (1984) found ion concentrations (mean ± SD) for the river to be (in μmol L^{-1}): Na^+, 16.5 ± 5.3; K^+, 8.2 ± 2.7; Ca^{2+}, 5.3 ± 1.6; Mg^{2+}, 4.7 ± 1.4; and Cl^-, 47.9 ± 19.7. Individual forest streams that drain into the Rio Negro can be even more dilute. A forest stream approximately 60 km northwest of Manaus has Na^+ and Ca^{2+} concentrations of only 9.4 ± 2.5 and 1.0 ± 0.9 μmol L^{-1}, respectively (Furch 1984). Because of the very low ion levels, the waters have virtually no buffering capacity and are susceptible to acidification from organic acids released by decomposing plants. While the main river channel typically has a pH around 5.5 (Furch 1984), forest streams and flooded forest areas can be at least 2 pH units lower (Goulding 1980; Val and Almeida-Val 1995; Walker and Henderson 1996).

Dilute, acidic waters, such as those found in the Rio Negro, have been shown to disrupt ion balance in fish by inhibiting active salt uptake and stimulating diffusive losses (McDonald and Wood 1981; McDonald et al. 1983; Gonzalez and Dunson 1987, 1989). Ion uptake, which is dependent on external concentration, may be reduced simply because of the extreme paucity of ions in the bulk medium (Kirschner 1988; Potts 1994). Alternatively, it may be directly inhibited by low pH, although the exact mechanism is not yet fully understood (Lin and Randall 1991, 1993; Potts 1994). At the same time, the control of diffusive ion efflux requires a low ion permeability

of the paracellular tight junctions, which is dependent on Ca^{2+} binding to the membrane-bound junctional proteins (Hunn 1985; Madara 1988). When ambient Ca^{2+} levels and/or pH are low, Ca^{2+} is leached from tight junctions and branchial ion permeability and ion loss rates rise. Together, the inhibition of ion uptake and stimulation of efflux results in a net loss of ions, and if the rate of loss is too high or the total amount lost too great (around 50%), then serious, potentially fatal internal ionic and coupled fluid balance disturbances result (Milligan and Wood 1982).

Despite these many challenges to ion regulation, the dilute, acidic waters of the Rio Negro still support an incredible diversity of fish. Recent estimates indicate that over 1,000 different species inhabit the Rio Negro (Val and Almeida-Val 1995). To better understand how these fish are able to maintain ion balance (Na^+, Cl^-, K^+) and inhabit the waters of the Rio Negro, we examined the ionoregulatory ability during acute low-pH exposure of four species collected directly from the Rio Negro in the region of the Anavilhanas archipelago: piranha preta (*Serrasalmus rhombeus*), piranha branca (*Serrasalmus* cf. *holandi*), pacu (*Myleus* sp.), and aracu (*Leporinus fasciatus*), and, for comparison, a fifth species obtained from aquaculture in Manaus (tambaqui, *Colossoma macropomum*). These species were chosen because they were of good size, collected in adequate number, and represented some of the diversity of the species-rich Rio Negro. In addition, we examined the role that water Ca^{2+} concentration played in the severity of the ion disturbances experienced during low-pH exposure by these species. Finally, we examined the cardinal tetra (*Paracheirodon axelrodi*), which was collected from the upper reaches of the Rio Negro, for low-pH tolerance by exposing it to pH 4.0 or 3.5 for 5 d and measuring body Na^+ concentration.

Material and Methods

Experimental Animals

Piranha preta ($n = 9$, mean wet mass $\pm$ SE = 231.1 $\pm$ 32.1 g), piranha branca ($n = 11$, mean wet mass = 176.3 $\pm$ 16.5 g), pacu ($n = 6$, mean wet mass = 205.2 $\pm$ 21.6 g), and aracu ($n = 10$, mean wet mass = 236.3 $\pm$ 14.8 g) were collected by seine net from the Rio Negro in the Anavilhanas archipelago and held on board the research vessel *Amanai II*. The fish were allowed to recover overnight in large fiberglass tanks supplied on a flow-through basis with water pumped directly from the river. The river water was analyzed with a flame photometer and found to have the following ion concentrations (in μmol L^{-1}): Na^+, 52; K^+, 27; Ca^{2+}, 10, and Cl^-, 55 (pH = 5.5, temperature = 30°C). Tambaqui ($n = 6$, mean wet mass = 160.8 $\pm$ 9.9 g) were supplied by the National Institute for Amazon Research aquaculture station in Manaus and held in Manaus groundwater (in μmol L^{-1}: Na^+, 15; K^+, 9; Ca^{2+}, 10; and Cl^-, 16; pH = 6.0, temperature = 30°C) for several weeks before

testing. They were then transported up the river to the experimental site on board the *Amanai II*. They were kept in fiberglass tanks filled with Rio Negro water during transport up the river and at the experimental site for a total of about 48 h before the start of experiments. The fish were not fed during the time they were held. Cardinal tetras ($n = 44$, mean wet mass = 0.157 $\pm$ 0.008 g) that had been collected from the upper reaches of the Rio Negro were obtained from a commercial dealer in Manaus and were held in Manaus groundwater for a few days before the start of the test.

Experimental Protocol

Net Na^+ flux, net Cl^- flux, and net K^+ flux were measured in unmodified water from the river (pH 5.5) as a control and during serial 1-h exposures to river water acidified to pH 3.5 and 3.0. In a second series, on a separate group of fish, the same protocol was used except that the water Ca^{2+} concentration was raised to 100 μmol L^{-1} (from 10 μmol L^{-1}) after the initial control flux in unmodified water. At the end of the flux measurements, the water Ca^{2+} concentrations were confirmed with the flame photometer, and in all cases the actual concentration was within 3–5 μmol L^{-1} of the nominal 100 μmol L^{-1} concentration.

To make the measurements, six fish of each species were placed into individual 3.5-L chambers (water in each chamber was aerated) connected to a 100-L recirculating system filled with river water and allowed to recover overnight. Flow rate into each chamber was approximately 500 mL min^{-1}. At the beginning of a measurement period, flow was stopped to all containers, and a 20-mL water sample was removed. One hour later another 20-mL water sample was removed and water flow was restored. The pH was lowered to 3.5 with concentrated H_2SO_4, and after a 30-min exposure another measurement period was started. After the completion of the second measurement period, the water flow was restored, the pH was lowered to 3.0, and after 30 min the final measurements were made. At the conclusion of the third flux period the fish were removed, weighed, and released to the river. The second series (on a new group of fish) was performed exactly as the first, except that as the pH was lowered to 3.5, the water Ca^{2+} concentration was raised to 100 μmol L^{-1} by addition of $CaSO_4$. During the low-pH exposures, water pH was monitored continuously with an Orion model 250A pH meter and adjusted as needed. The pH of the bathwater was kept within 0.05 units of the nominal pH. At the end of the flux periods, the pH of the water in each test chamber was checked and found to have risen less than 0.05 units in virtually all cases.

The water samples were analyzed for Na^+, K^+, and Ca^{2+} concentrations with a flame photometer. Water Cl^- concentration was determined using a colorimetric assay (Zall et al. 1956). Net ion fluxes (J_{net}^{Ion}) were calculated from the changes in the ion concentration of the bathwater over the 1-h period
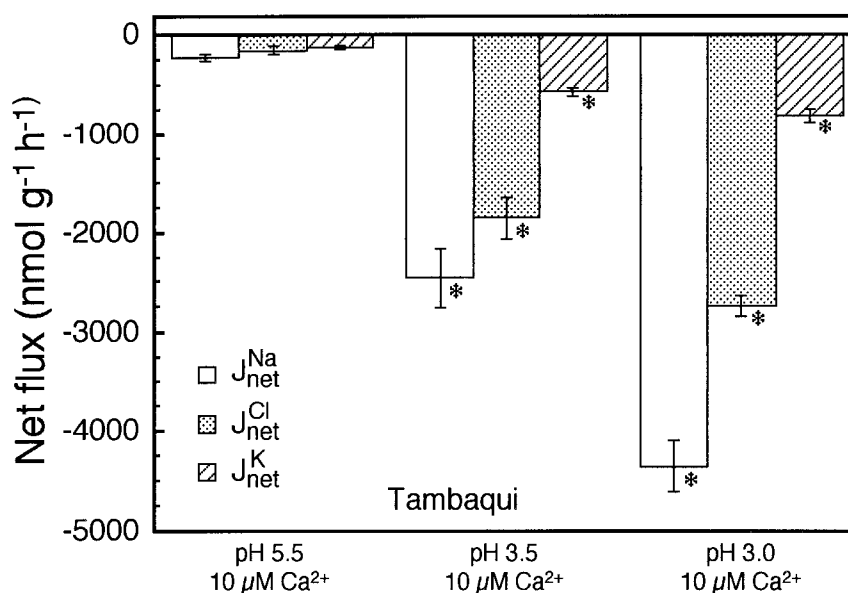
Figure 1. Effects of water pH on net Na$^+$ flux (J_{net}^{Na}), net Cl$^-$ flux (J_{net}^{Cl}), and net K$^+$ flux (J_{net}^{K}) of tambaqui (*Colossoma macropomum*) from aquaculture. Fluxes were measured during the first hour of exposure to each low pH. Values are means ± SE. Asterisks indicate significant differences from corresponding fluxes at pH 5.5.

using the following equation: $J_{net}^{Ion} = V([Ion]_1 - [Ion]_2)/(Mt)$, where $[Ion]_1$ and $[Ion]_2$ are the bath ion concentrations at the beginning and end of the flux period, respectively, V is the bath volume in liters, M is the mass of the fish in grams, and t is the duration of the flux period in hours.

In Manaus, the cardinal tetras were divided among three 7-L containers filled with aerated Manaus groundwater. After a day to recover from the transfer, two fish were removed from each of the three containers, weighed, placed in individual beakers, and dried in an oven at 90°C. While the pH of one container was maintained at 6.0, the pH of one of the remaining two was lowered to 4.0 and the other to 3.5 with dilute H_2SO_4. After 1 and 5 d, five to seven fish were removed from each container and processed as before. When the exposure was completed, the dried fish were dissolved in concentrated analytical grade HNO_3, and the resulting liquid was diluted and analyzed for whole body Na$^+$ concentration with the flame photometer.

Statistical Analyses

All data are reported as means ± 1 SE. Means were compared using paired *t*-tests or ANOVA (overall $P \leq 0.05$) with multiple comparisons (Scheffé test) if the ANOVA proved significant.

Results

At the beginning of each of the two series of exposures for each species, net ion fluxes were measured in untreated Rio Negro water at pH 5.5 as controls. For each species, there were no significant differences between the control measurements from the two separate series, and they were combined. Under control conditions, all five species were in or near equilibrium for all ions (Figs. 1–5), which indicates that they had recovered from any stress induced by placement into the test chambers.

Upon exposure to pH 3.5, tambaqui obtained from aquaculture experienced rates of Na$^+$ and Cl$^-$ loss 12–13 times greater than at pH 5.5; rates of K$^+$ loss were five times greater (Fig. 1). In sharp contrast, at pH 3.5, pacu, piranha preta, piranha branca, and aracu from the Rio Negro all experienced only mildly elevated net losses of Na$^+$ and Cl$^-$, averaging about 300 nmol g^{-1} h^{-1} (Figs. 2–5; drawn to the same scale as tambaqui to facilitate comparisons). Further, while net K$^+$ flux was negative in all four Rio Negro species at pH 3.5, it was unchanged relative to pH 5.5 measurements. For the three species tested (piranha preta, piranha branca, and aracu), raising the water Ca^{2+} concentration from 10 to 100 μmol L^{-1} with exposure to pH 3.5 had no significant effect on any ion loss rates (Figs. 3–5).

When the pH was dropped to pH 3.0, rates of Na$^+$ and Cl$^-$ loss for tambaqui rose 78% and 48%, respectively, relative to rates at pH 5.5 (Fig. 1). In the Rio Negro species, ion loss rates rose as well (Figs. 2–5), but they were still much lower than rates for tambaqui. However, some differences among the four other species became apparent. Piranha preta experienced the smallest net loss of Na$^+$ and Cl$^-$ (net Na$^+$ and Cl$^-$ fluxes were three and two times greater than at pH 3.5, respectively). The other three Rio Negro species all had net Na$^+$ and Cl$^-$ loss rates about 60%–70% higher than piranha preta. Along with the greatly stimulated Na$^+$ and Cl$^-$ losses at pH 3.0, all four species from the Rio Negro experienced increased rates of K$^+$ loss. In these cases, the magnitude of net K$^+$ flux rose by about 50%. As at pH 3.5, increased water Ca^{2+} concentration had
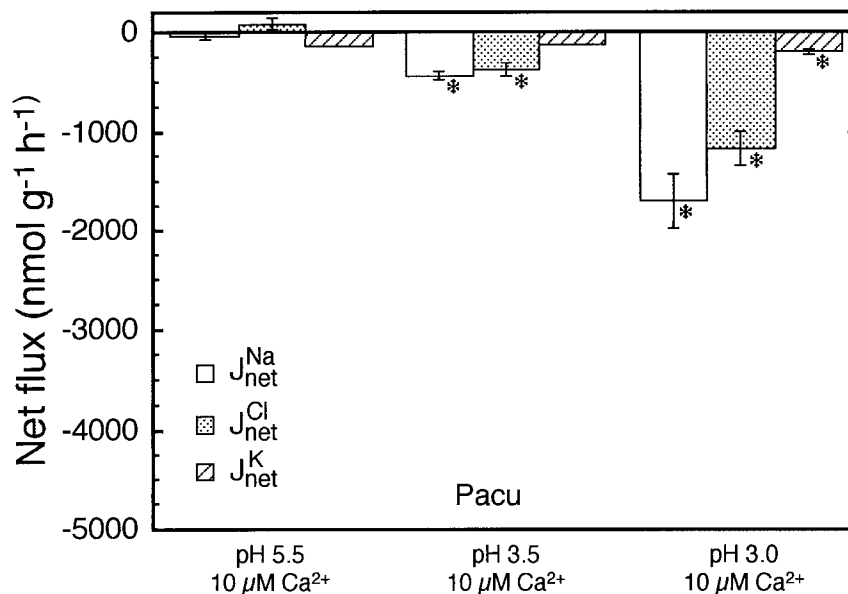
Figure 2. Effects of water pH on net Na$^+$ flux (J_{net}^{Na}), net Cl$^-$ flux (J_{net}^{Cl}), and net K$^+$ flux (J_{net}^{K}) of pacu (*Myleus* sp.) from the Rio Negro. Fluxes were measured during the first hour of exposure to each low pH. Values are means ± SE. The y-axis is drawn with the same scale as Figure 1 to facilitate comparisons. Asterisks indicate significant differences from corresponding fluxes at pH 5.5.

little effect on rates of Na$^+$ and Cl$^-$ loss at pH 3.0. Only aracu exhibited a significant reduction in ion losses when water Ca^{2+} concentration was raised to 100 μmol L^{-1}; rates of Na$^+$ and Cl$^-$ loss dropped by 50% (Fig. 5). Interestingly, when the water Ca^{2+} concentration was raised, piranha preta appeared to lose even more K$^+$ (Fig. 3).

Cardinal tetras that were held at pH 4.0 or 3.5 for 5 d appeared to be unaffected by the exposure (Fig. 6). Body Na$^+$ concentration of fish held at the two low pH's did not change significantly over the 5 d relative to those held at pH 6.0.

Discussion

Our flux measurements clearly show that the five species from the Rio Negro tested here were exceptionally tolerant of low pH. All four large species experienced only mild disruptions of Na$^+$ and Cl$^-$ balance during the first hour of exposure (when the disturbance is usually greatest) to pH 3.5 and no disruption of K$^+$ balance, an indicator of serious internal osmotic disturbances (McDonald et al. 1980; Milligan and Wood 1982; Audet and Wood 1988). The magnitude of the ion disturbances observed in the Rio Negro fish at pH 3.5 was similar to that observed in the banded sunfish (*Enneacanthus obesus*), an acid-tolerant species native to North America that can survive indefinitely at this pH (Gonzalez and Dunson 1987, 1989). In contrast, tambaqui, which were cultured in Manaus, experi-

enced much larger disturbances of Na$^+$ and Cl$^-$ balance and a sizable K$^+$ disturbance. In the wild, tambaqui occur naturally in both the dilute waters of the Rio Negro and the more ion-rich waters of the main Amazon (Val and Almeida-Val 1995), so it is unclear whether the greater sensitivity in the present study was due to the aquacultural origin of the stock tested or reflected a general characteristic of the species.

At pH 3.0, although all species experienced significant ion disturbances, the results still indicate great acid tolerance in the Rio Negro species. On exposure to pH 3.0, the Rio Negro fish experienced a three- to fivefold increase in net Na$^+$ and Cl$^-$ losses relative to fluxes at pH 3.5. Further, the doubling of K$^+$ loss rates in each species indicated that they were experiencing an internal osmotic imbalance, since K$^+$ is lost primarily from intracellular pools. However, the rates of Na$^+$ and Cl$^-$ loss in these species were only one-half to one-fourth as great as that observed in tambaqui and were also less than those observed in other species at less severe pH's. For example, pacu at pH 3.0 (the species from the Rio Negro with the highest rates of ion loss at that pH) lost Na$^+$ at only one-third the rate of common shiners (*Notropis cornutus*) and two-thirds the rate of rainbow trout (*Oncorhynchus mykiss*) at pH 4.0 (Freda and McDonald 1988).

The fifth Rio Negro species examined, the cardinal tetra, was also very tolerant of low pH. During 5 d of exposure to pH 4.0 or 3.5, they did not experience any drop of body Na$^+$ concentration, which indicates that they were able to maintain ion balance at those pH's. Similarly, the acid-tolerant banded sunfish does not experience a depression of body Na$^+$ concentration at pH 4.0 (Gonzalez and Dunson 1987). However, while sunfish can survive extended periods at pH 3.5, they lose about 30% of their body Na$^+$ concentration during the first 2 wk of exposure (Gonzalez and Dunson 1987). It would seem, then,
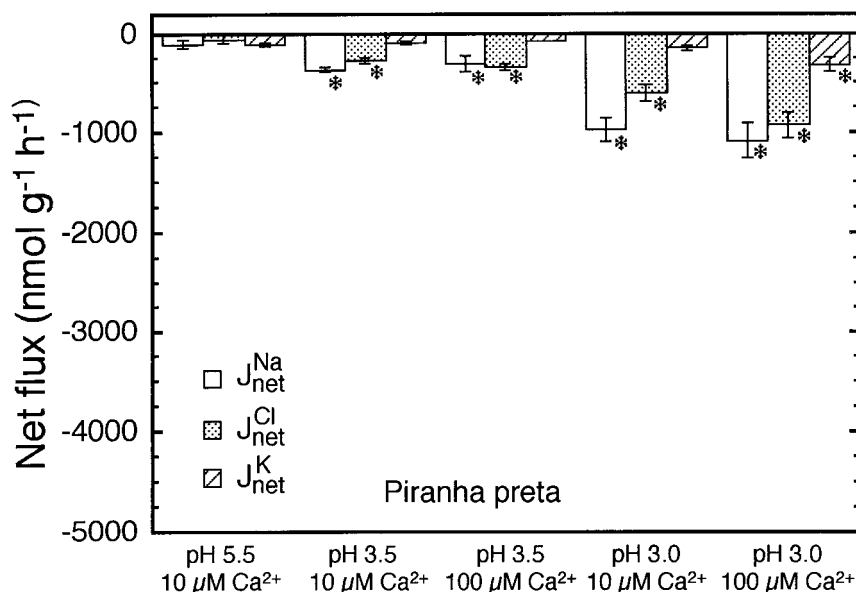
Figure 3. Effects of water pH and Ca^{2+} concentration on net Na^+ flux (J_{net}^{Na}), net Cl^- flux (J_{net}^{Cl}), and net K^+ flux (J_{net}^{K}) of piranha preta (*Serrasalmus rhombeus*) from the Rio Negro. Fluxes were measured during the first hour of exposure to each low pH. Values are means ± SE. The y-axis is drawn with the same scale as Figure 1 to facilitate comparisons. Asterisks indicate significant differences from corresponding fluxes at pH 5.5.

that the cardinal tetra's ability to maintain ion balance at low pH is superior to that of the banded sunfish.

It should not be surprising that these fish exhibit such a high degree of tolerance to low pH given the extremely ion-poor nature of their native waters and the extremely low pH found in some locations. The question is, How do they do it? Numerous studies on several North American species have shown that the key to tolerance of dilute waters of low pH is the ability to avoid increased branchial ion permeability. Elevated gill permeability, which, evidence suggests, is caused by displacement by H^+ of Ca^{2+} from paracellular tight junction proteins (see review by Wood [1988]), leads to increased diffusive ion losses. This claim is bolstered by experiments in which addition of Ca^{2+} to test water causes a reduction or even an elimination of elevated rates of ion losses at low pH (McDonald et al. 1980; McDonald and Wood 1981; McDonald 1983). Extending this concept, it has been proposed that the basis of tolerance to low pH is an increased branchial affinity for Ca^{2+}, which resists displacement at low pH (Hunn 1985; Gonzalez and Dunson 1987, 1989; McDonald et al. 1991). Indeed, several studies have indicated a very high branchial affinity for Ca^{2+} in fish that are tolerant of low pH (McWilliams 1982; Freda and McDonald 1988; Gonzalez and Dunson 1989), including a study of an Amazonian species (Gonzalez et al. 1997).

While our results support the concept that control of ion efflux is key for survival, they also indicate that for the Rio Negro species tested here, in contrast to previous findings, Ca^{2+} may not be involved to any great extent in regulation of branchial permeability at low pH. In our tests, raising the Ca^{2+} concentration 10-fold had little or no effect on rates of ion loss in the three species tested. This insensitivity of ion losses to water Ca^{2+} concentration suggests the possibility of a novel mechanism for the control of paracellular tight junction permeability, one that does not involve Ca^{2+}.

Of course, given the way our experiments were performed, several alternative explanations cannot be ruled out. For instance, it is possible that an actual reduction in diffusive efflux in water with increased Ca^{2+} concentration was masked by a simultaneous drop in active uptake. Since we measured only net Na^+ and Cl^- fluxes and not unidirectional movement (i.e., radioisotope fluxes; legal restrictions prevented the use of radioisotopes in this project) of Na^+ and Cl^- across the gills, we cannot determine the response of the unidirectional fluxes to added Ca^{2+}. However, given the overall elevated rates of ion loss, such masking seems unlikely.

Another possibility is that the Rio Negro fish possess such a high branchial affinity for Ca^{2+} that tight junction binding sites are saturated even in 10 μmol L^{-1} water. This seems reasonable, since these fish inhabit waters with typical Ca^{2+} concentrations that are 10 μmol L^{-1} or less. If this is the case, then raising water Ca^{2+} concentration would have no additional effect on branchial permeability or ion balance. A similar conclusion was reached with the acid-tolerant banded sunfish and yellow perch. For the sunfish, raising water Ca^{2+} concentration from 0 to 50 μmol L^{-1} at pH 3.25 causes Na^+ losses to drop by more than 50%, but additional Ca^{2+} produces no further reductions (Gonzalez and Dunson 1989). Na^+ losses of yellow perch (*Perca flavescens*) were found to be insensitive to water Ca^{2+} concentration at a variety of pH's, although the
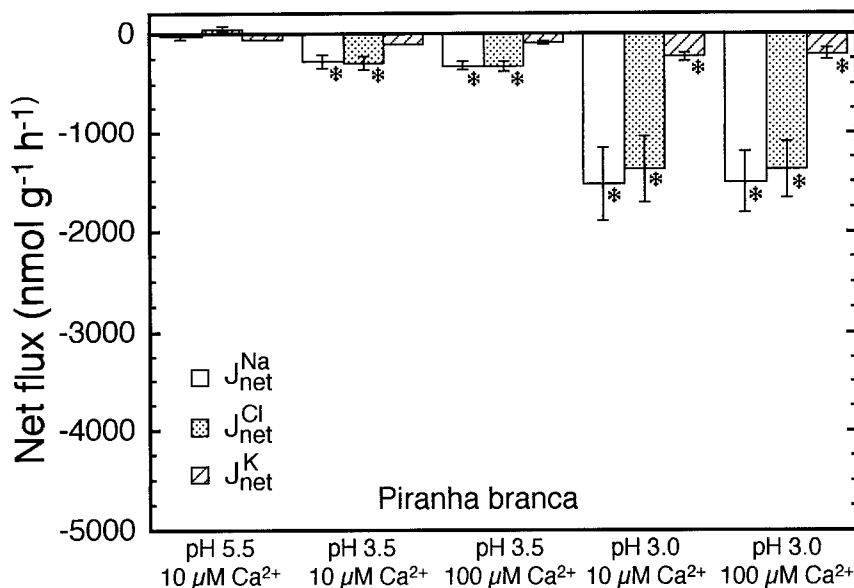
Figure 4. Effects of water pH and Ca^{2+} concentration on net Na^+ flux (J_{net}^{Na}), net Cl^- flux (J_{net}^{Cl}), and net K^+ flux (J_{net}^{K}) of piranha branca (*Serrasalmus* cf. *holandi*) from the Rio Negro. Fluxes were measured during the first hour of exposure to each low pH. Values are means $\pm$ SE. The *y*-axis is drawn with the same scale as Figure 1 to facilitate comparisons. Asterisks indicate significant differences from corresponding fluxes at pH 5.5.

lowest concentration tested, 50 μmol L^{-1}, may have been too high to detect any effect (Freda and McDonald 1988).

One final, interesting alternative is that the organic compounds that give the Rio Negro its tea color (and its name) somehow play a role in eliminating the effects of water Ca^{2+} concentration. Furch (1984) found an average total carbon concentration in the Rio Negro of 10.5 mg L^{-1}. Given this large quantity of organic compounds in the river, it is possible that they could interact directly with the branchial tight junctions and influence permeability. Dissolved organic compounds have been shown to bind to North American amphibian egg membranes and prevent hatching (Karns 1983; Freda et al. 1989). It also seems possible that the organic compounds could effectively bind to Ca^{2+} in the water, rendering it unavailable to the fish (Freda et al. 1989), and promote the development of a new mechanism to limit branchial permeability. Further studies are needed to identify the possible involvement of dissolved organic substances in ion regulation of Rio Negro fish.

While our results raise questions concerning the role of water Ca^{2+} in regulating branchial ion permeability in Rio Negro fish, they also prompt questions about other aspects of models of ion regulation that have been developed in recent years from studies of North American and European species. For example, Lin and Randall (1995) argue, on the basis of their examination of proton pumps in trout, that Na^+ uptake

is impossible below about pH 5.0. In their model, active extrusion of H^+ produces a transmembrane potential that drives Na^+ uptake through channels in the apical membrane. As pH falls, H^+ excretion is progressively inhibited, and Na^+ uptake falls. At about pH 5.0, H^+ excretion is completely inhibited and Na^+ uptake ceases. However, if Rio Negro fish can survive extended periods (perhaps their whole life) at very low pH's (≤ 4.5), it seems likely that they can actively transport salts at these pH's. A recent study provides some support for this notion. The blackskirt tetra experiences partial inhibition of Na^+ uptake at pH 4.0, but at pH 4.5, when loss rates are stimulated, they exhibit a rapid stimulation of Na^+ uptake (Gonzalez et al. 1997). Interestingly, support also comes from studies of some North American and European fish. Yellow perch and brown trout (*Salmo trutta*), for instance, are able to take up salts at pH 4.0 (McWilliams 1982; Freda and McDonald 1988), which is well below the proposed theoretical limit. It seems we must begin to reevaluate our model to make it consistent with these results.

Finally, the exceptional acid tolerance of all five species tested here suggests that this tolerance is a general characteristic of fishes of the Rio Negro (Dunson et al. 1977). Given that it is estimated that over 1,000 species from many different families inhabit the Rio Negro, there may be a variety of different patterns of ion regulation in these fish. Further studies of these species are likely to yield novel mechanisms of ion regulation.
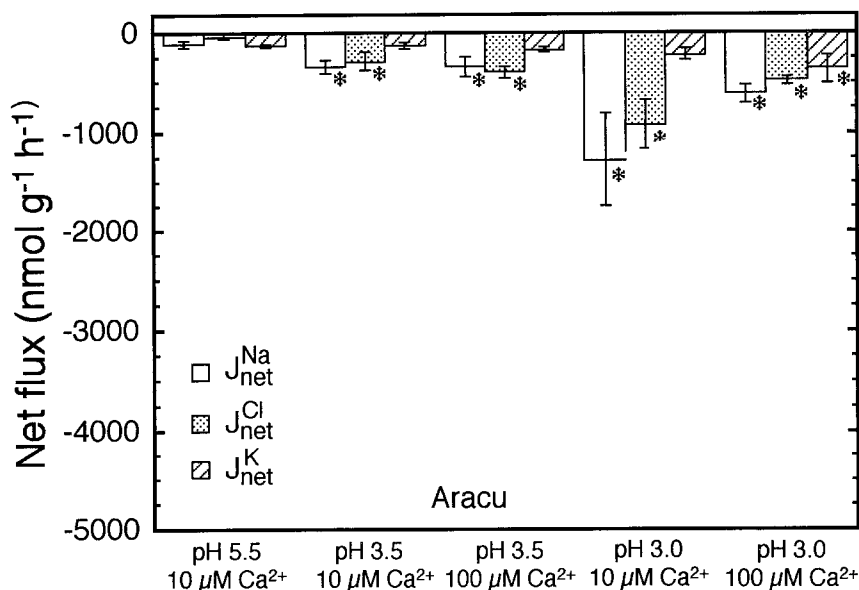
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Figure 5. Effects of water pH and Ca^{2+} concentration on net Na^+ flux (J_{net}^{Na}), net Cl^- flux (J_{net}^{Cl}), and net K^+ flux (J_{net}^{K}) of aracu (*Leporinus fasciatus*) from the Rio Negro. Fluxes were measured during the first hour of exposure to each low pH. Values are means ± SE. The *y*-axis is drawn with the same scale as Figure 1 to facilitate comparisons. Asterisks indicate significant differences from corresponding fluxes at pH 5.5.
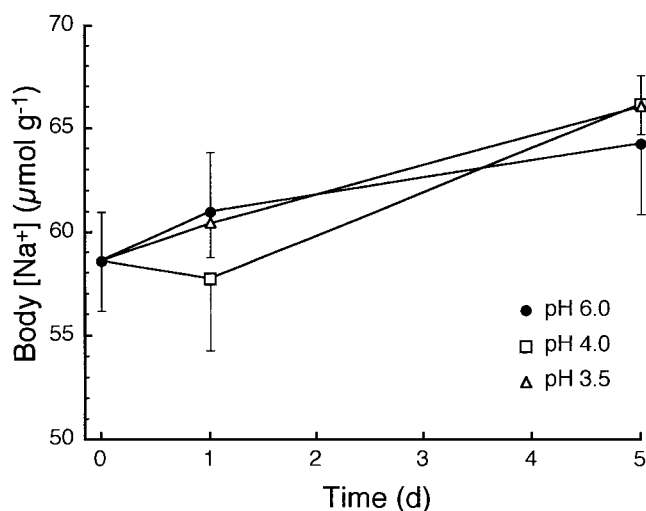
Figure 6. Body Na^+ concentration of cardinal tetras (*Paracheirodon axelrodi*) during 5 d of exposure to pH 6.0, 4.0, or 3.5. Values are means ± SE.

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