

Protection by Natural Blackwater against Disturbances in Ion Fluxes Caused by Low pH Exposure in Freshwater Stingrays Endemic to the Rio Negro

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ABSTRACT

Stenohaline freshwater stingrays (*Potamotrygon* spp.) are endemic to the very dilute (Na^+ , Cl^- , $\text{Ca}^{2+} \leq 30 \mu\text{mol L}^{-1}$), often acidic blackwaters of the Rio Negro despite gill Na^+ and Cl^- transport characteristics that appear unfavorable (high K_m , low J_{max}). We evaluated the possible protective role of blackwater itself, which is rich in dissolved organic carbon (DOC), as well as the importance of Ca^{2+} in allowing this tolerance of dilute, acidic conditions. Responses of stingrays in natural blackwater (DOC = 8.4 mg L^{-1}) were compared with those in a natural reference water with similar ion levels but low DOC (0.6 mg L^{-1}). Comparing these two water types, we found that differences in Na^+ and Cl^- unidirectional fluxes (J_{in}^X , J_{out}^X ; measured with radiotracers) and net fluxes (J_{net}^X), influx and outflux ki-

netic relationships, and net ammonia excretion (J_{Amm}) were generally small at pH 6.3, though the balance points where $J_{\text{in}} = J_{\text{out}}$ shifted from $>300 \mu\text{mol L}^{-1}$ in reference water (low DOC) to about $100 \mu\text{mol L}^{-1}$ in blackwater (high DOC). In reference water, both $J_{\text{in}}^{\text{Na}}$ and $J_{\text{in}}^{\text{Cl}}$ were inhibited $>90\%$, both $J_{\text{out}}^{\text{Na}}$ and $J_{\text{out}}^{\text{Cl}}$ more than doubled, and J_{Amm} did not change at pH 4.0. In blackwater, the inhibition of influxes was attenuated, the increases in outflux did not occur, and J_{Amm} increased by 60% at pH 4.0. Addition of $100 \mu\text{mol L}^{-1} \text{Ca}^{2+}$ to reference water prevented the increases in $J_{\text{out}}^{\text{Na}}$ and $J_{\text{out}}^{\text{Cl}}$ and allowed J_{Amm} to increase at pH 4.0, which demonstrates that the gills are sensitive to Ca^{2+} . However, addition of Ca^{2+} to blackwater had no effect on the responses to pH 4.0. Addition of commercial humic acid to reference water did not duplicate the effects of natural Rio Negro blackwater at the same DOC level; instead, it greatly exacerbated the increases in $J_{\text{out}}^{\text{Na}}$ and $J_{\text{out}}^{\text{Cl}}$ at low pH and prevented any protective influence of added Ca^{2+} . Thus, blackwater DOC appears to be very different from commercial humic acid. Biogeochemical modeling indicated that blackwater DOC prevents Ca^{2+} binding, but not H^+ binding, to the gills and that the protective effects of blackwater cannot be attributed to its higher buffer capacity or its elevated Al or Fe levels. Natural DOC may act directly at the gills at low pH to exert a protective effect and, when doing so, may override any protective action of Ca^{2+} that might otherwise occur.

Introduction

The ion-poor acidic blackwaters of the Rio Negro and its tributaries, characterized as “slightly contaminated distilled water” by Sioli (1968, p. 271), support a surprising diversity of fish life that is thought to represent about 8% of the world’s ichthyofauna (reviewed by Val and Almeida-Val 1995). These blackwaters are so called because of their dark tea color, which is caused by a high content of dissolved organic carbon (DOC) comprising humic, fulvic, and other organic acids derived from the breakdown of jungle vegetation (Leenheer 1980; Sioli 1984; Ertel et al. 1986; Kuchler et al. 1994). In addition to the many teleost families with representatives endemic to blackwaters (Gery 1969; Fink and Fink 1979; Val and Almeida-Val 1995), one family of elasmobranchs, the Potamotrygonidae, has successfully colonized this extreme habitat. These freshwater sting-

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rays are unique in being the only truly stenohaline freshwater elasmobranchs. Although elasmobranchs have a marine origin, the potamotrygonid rays are thought to have speciated exclusively within freshwaters and have lost their ability to live in seawater (Brooks 1995; Lovejoy 1996; Lovejoy et al. 1998). Their rectal gland is nonfunctional, they have very little urea in their body fluids, and they cannot elevate internal urea levels to any great extent when challenged with elevated external osmolality (Thorson et al. 1967, 1978; Junqueira et al. 1968; Thorson 1970; Griffith et al. 1973; Gerst and Thorson 1977; Bittner and Lang 1980). Therefore, they appear to be completely adapted to ion-poor media; indeed, in nature, they are restricted to waters with salinity less than 3 ppt (Brooks et al. 1981).

Recently, we examined the mechanisms and concentration dependence (“Michaelis-Menten kinetics”) of Na^+ and Cl^- uptake in these animals (Wood et al. 2002). Rather surprisingly, the affinity of the branchial transport systems for both of these ions was very low, indicated by high K_m values around 300–500 $\mu\text{mol L}^{-1}$, which are approximately 15-fold higher than the natural concentrations of Na^+ and Cl^- in the Rio Negro water from which they were collected. In comparison with 12 teleost species collected from the same waters and surveyed by Gonzalez et al. (2002), the K_m values of the stingrays were at least twofold higher and as much as 30-fold higher, while the J_{max} values were at the lower end of the teleost range. Furthermore, influx kinetics of the stingrays exhibited no differences between animals acclimated to ion-rich hard water and those acclimated to ion-poor Rio Negro water, and influx rates were inhibited about 80% by acute exposure to pH 4.0. Rather than influx rates, it appeared to be efflux rates that were particularly well adapted to life in blackwater. The rates were low overall and were lower in animals acclimated to blackwater than they were in those acclimated to ion-rich hard water. Most important, when the blackwater pH was lowered to 4.0, unidirectional efflux rates did not increase, and the net loss rate did not change substantially (Wood et al. 2002). These influx and efflux characteristics of the stingrays seem to represent an extreme version of one of two strategies identified by Gonzalez et al. (2002) for adaptation to blackwater conditions, most notably by members of the family Cichlidae. As pointed out by Gonzalez et al. (2002), this strategy appears designed to limit net ion loss and thereby minimize physiological disturbance so as to “wait out” exposures to especially acidic and dilute challenges.

Gonzalez et al. (1998, 2002) speculated that the high DOC content of blackwaters (Table 1) may be protective against dilute and acidic conditions, especially in limiting gill permeability. In support of this idea, they reported that the effects of low pH exposure in two teleost species were less severe in natural Rio Negro water than they were in an artificial medium made from distilled water with the same concentrations of Na^+ , Cl^- , and Ca^{2+} but no DOC. Because dissolved organic matter is now known to bind to fish gills at low pH (Campbell et al. 1997), the DOC might be acting in a similar manner to the

Table 1: Composition of reference water (INPA well water) and blackwater (from the Rio Negro)

Parameter	Reference Water	Blackwater
Na^- ($\mu\text{mol L}^{-1}$)	19	30
Cl^- ($\mu\text{mol L}^{-1}$)	21	23
Ca^{2+} ($\mu\text{mol L}^{-1}$)	11	10
Mg^{2+} ($\mu\text{mol L}^{-1}$)	2	6
K^+ ($\mu\text{mol L}^{-1}$)	16	18
DOC (mg C L^{-1})	.6	8.4
pH	6.3–6.5	5.9–6.1

Note. DOC = dissolved organic carbon.

well-known action of elevated water Ca^{2+} in stabilizing tight junctions, thereby limiting paracellular losses at low pH, as first suggested by Kullberg et al. (1993). Gonzalez et al. (1998) also reported that large increases in water Ca^{2+} concentration, which are normally very protective against the stimulatory effect of low pH on ion leakage (McDonald 1983; Wood 2001), had no protective effect in three acid-tolerant teleost species exposed to low pH in their native Rio Negro water.

In this study on potamotrygonid stingrays of the Rio Negro, we first examined the effects of blackwater on Na^+ and Cl^- influx and efflux kinetics at circumneutral pH. Our comparative medium (reference water) was another natural water with similar levels of major ions but low in DOC because synthetic media made from distilled water and salts may lack essential micronutrients. We then critically evaluated the possible protective role of natural blackwater on the responses of unidirectional Na^+ and Cl^- influx and efflux rates to low pH exposure as well as responses in ammonia excretion. We also evaluated the actions of added Ca^{2+} in reference water and of a commercially available DOC (Aldrich humic acid) added to reference water at the same concentration as natural blackwater DOC at both circumneutral and low pH. Ca^{2+} was also tested in natural blackwater and in the presence of commercially available DOC. The metal contents and titration characteristics of the waters were considered as possible modifying factors. The results reveal important actions and interactions of the various water qualities at low pH, effects that are not seen at circumneutral pH.

Material and Methods

Experimental Animals

The stingrays (70–770 g; mean = 270 ± 40 [SEM] g; $N = 21$) used in this study were collected in November and December 1999 from the Rio Negro and its tributaries between Barcelos and the Anavilhanas Archipelago. The systematics of the genus *Potamotrygon* are unsettled. The fish were uniform in morphology but variable in color pattern. They were diagnosed by local experts to represent a single species, either *Potamotrygon thorsoni* or *Potamotrygon* aff. *hystrix*. The latter classi-

fication recognizes that *hystrix* may include up to three species (including *thorsoni*) and/or subspecies that hybridize and show considerable regional variability in patterning (Brooks et al. 1981). We therefore refer to the fish as *Potamotrygon* sp.

Most (16) of the rays were furnished by a collector (Projeto Potamotrygon, Association of Ornamental Fish Breeders and Exporters of Amazonas [ACEPOAM], Barcelos) and shipped to the Laboratory of Ecophysiology and Molecular Evolution (Instituto Nacional de Pesquisas da Amazonia [INPA], Manaus) where they were held for 10 d in a recirculating, filtered 300-L tank in which the INPA well water (which is very soft) had been supplemented with NaCl and limestone chips, which substantially raised the levels of major ions ($\text{Na}^+ = 2.0$, $\text{Cl}^- = 2.2$, $\text{Ca}^{2+} = 0.9 \text{ mmol L}^{-1}$) This is a common procedure used to reduce the stress of captivity. Six of the fish were then transferred to a 100-L tank continually flushed with the soft INPA well water, which is low in ions and DOC and is hereafter referred to as reference water (composition as in Table 2). This well water had been vigorously aerated to achieve full equilibration with atmospheric O_2 and CO_2 tensions. After 5 d of holding in reference water, the kinetics of both Na^+ and Cl^- uptake and efflux were determined.

After completion of tests at INPA, the 16 fish were transported back to the Anavilhanas Archipelago of the Rio Negro on board the research vessel *Amanai II*, where all further experiments were performed. The research vessel was moored at the Scientific Base of the Anavilhanas Archipelago of the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis. An additional five rays were caught locally and added to the pool of experimental animals. Fresh blackwater from the river (i.e., the original water quality from which the fish had been collected) was continually flushed through the 300-L holding tank during this time. Blackwater was similar in composition to reference water with respect to the major ions Na^+ , Cl^- , Ca^{2+} , and K^+ but was substantially higher in DOC (composition in Table 1). Note that throughout this study, all

measurements of water ionic concentrations are total and therefore do not distinguish free ionic concentrations from DOC-bound ions. The rays were held in this water for at least 5 d before further tests, and all other experiments were performed on board the *Amanai II* during a 14-d period of research at Anavilhanas. Throughout, the rays were held in blackwater. For comparison tests, reference water from the INPA well was stored on board the *Amanai II* in plastic carboys normally used for drinking water storage.

Because freshwater rays have protected status in Brazil, animal numbers were limited, and all procedures were designed to be nonlethal. Experiments were planned such that low pH exposures were limited to moderate acidity (pH 4.0) and lasted only 2 h. The animals were allowed a 48-h recovery before further tests. In all cases, control measurements were made before testing to ensure that baseline conditions had been re-established. Holding and experimental temperatures were 28°–31°C throughout. The fish were offered food (pellets, tubifex worms) but did not appear to eat in captivity.

General Methods

At least 12 h before all flux rate measurements, rays were placed in individual polyethylene containers fitted with airstones and lids. Container volume was 2.6 L, except for the largest rays (450–770 g), for which 6-L chambers were used. Containers were flushed at about 200 mL min^{-1} with blackwater pumped directly from the river in most trials or with reference water (INPA well water) in the first kinetics experiment. At the start of all flux experiments, the inflowing water was stopped, and the volume was set to a known volume, generally 2.0 L for fish up to 450 g and 3.0 or 4.0 L as appropriate for the largest rays.

The radioisotopes ^{22}Na (as NaCl) and ^{36}Cl (as HCl), manufactured by New England Nuclear (Dupont), were supplied by REM (Sao Paulo). Unidirectional Na^+ and Cl^- flux rates were determined simultaneously by adding both ^{22}Na (a dual

Table 2: Measured mean water calcium concentrations during the three experimental periods of the paired trials

Treatment	Control	Low pH	Recovery
Blackwater	22.7 ± 1.9	31.7 ± 3.3	51.5 ± 6.4
Blackwater + Ca	140.0 ± 7.4	158.0 ± 10.6	159.3 ± 10.7
Reference water	18.7 ± 3.6	25.4 ± .7	45.2 ± 3.4
Reference water + Ca	116.0 ± 3.0	140.9 ± 1.4	145.3 ± 4.5
Reference water + HA	16.6 ± 2.9	22.4 ± 1.9	40.2 ± 5.1
Reference water + HA + Ca	107.4 ± 2.6	118.7 ± 3.8	131.6 ± 6.2

Note. In all three paired trials, water calcium was left at background levels or nominally raised by 100 $\mu\text{mol L}^{-1}$. The increases in calcium concentration were significant ($P \leq 0.05$) and close to the nominal target of 100 $\mu\text{mol L}^{-1}$. However, there were no significant differences among the three trials at comparable periods, although in all trials, calcium concentrations increased significantly between control and recovery periods due to calcium loss by the fish. Means ± 1 SEM ($N = 5-6$). HA = Aldrich humic acid.

gamma and beta emitter) and ^{36}Cl (a beta emitter only, neutralized with KOH) to the external water in a 1 : 2.5 ratio (Wood 1988). Differential scintillation and gamma counting were used to separate the counts of the two radioisotopes. Unidirectional influx was followed by monitoring the disappearance of ^{22}Na and ^{36}Cl counts into the fish over time. Initial tests using “cold displacement” (a rinse with 20 mmol L⁻¹ NaCl, ~1,000 times ambient concentrations, at the end of an experiment) demonstrated that there was no significant loss of either radioisotope to the surface of the fish or to the walls of the container. Water samples (generally 15 mL) were taken at the beginning and end of each flux period for the measurement of ^{22}Na and ^{36}Cl radioactivity and the total concentrations of water Na⁺, Cl⁻, total ammonia, and, in some trials, Ca²⁺. Unidirectional Na⁺ and Cl⁻ influx rates (J_{in}^{X} ; by convention positive) were calculated from the disappearance of counts from the external water (factored by time, volume, and fish mass) and the mean external specific activity. Net flux rates ($J_{\text{net}}^{\text{X}}$) were calculated from the change in total Na⁺ or Cl⁻ concentration in the water (similarly factored), and unidirectional efflux rates ($J_{\text{out}}^{\text{X}}$; by convention negative) were calculated by difference, as outlined in detail by Wood (1988). Internal specific activity, calculated from the cumulative disappearance of counts from the water and an estimated internal Na⁺ or Cl⁻ pool of 40 mmol kg⁻¹, was never more than 5% of measured external specific activity at the end of an experiment, so there was no need for “backflux correction.” Net ammonia flux rates were calculated from the appearance of total ammonia in the external water (factored by time, volume, and fish mass).

Measurements of the Concentration Kinetics of Na⁺ and Cl⁻ Influx and Efflux

Kinetic relationships were determined by starting at the lowest water Na⁺ and Cl⁻ concentrations (either reference water from the INPA well or blackwater; Table 1) and then moving progressively upward in steps, each representing an approximate twofold increase in concentration. At the first concentration, radioisotopes (0.25 μCi ^{22}Na , 0.625 μCi ^{36}Cl per liter; 1 μCi = 37 kBq) were added to the chambers and allowed to mix for 10 min, and then an initial 15-mL water sample was taken, followed by a final 15-mL water sample at 2 h. Thereafter, sufficient NaCl plus ^{22}Na and ^{36}Cl were added (as a single stock solution) to approximately double the concentrations of all three in the external bath, which thereby maintained the same specific activities. The process was repeated sequentially to generate five or six points on the kinetic curves, each with measurements of J_{in}^{X} , $J_{\text{out}}^{\text{X}}$, and $J_{\text{net}}^{\text{X}}$. To achieve comparable resolution at progressively higher external concentrations, the length of the flux period was gradually increased from 2 h to 2.5 h, 3.25 h, 4.5 h, and finally, 7–10 h. The $J_{\text{in}}^{\text{Na}}$ and $J_{\text{in}}^{\text{Cl}}$ data for each individual fish were fitted separately to a Michaelis-Menten model by Eadie-Hofstee regression (Michal 1985) to yield in-

dividual estimates of the K_m (affinity constant) and J_{max} (maximum transport rate) constants. Grand means (\pm SEM) were then calculated for all fish in a treatment group.

Responses to Low pH: Influence of Water Type, Ca²⁺, and Humic Acid

In these experiments, a common control pH of ~6.3 was used because the natural pH of blackwater was 5.9–6.1, while the natural pH of reference water was 6.4–6.6 (Table 1). The basic experimental protocol was a 2-h initial period at control pH, followed by a 2-h period at pH 4.0, followed by a 2-h recovery period at pH 6.3. Each fish was used as its own control. In all cases, the fish were exposed to the treatment water throughout the experiment. Responses were assessed by measurements of unidirectional and net Na⁺ and Cl⁻ flux rates and net ammonia flux rates. At 10 min before the start of the control period, 0.5 μCi ^{22}Na and 1.25 μCi ^{36}Cl per liter were added to the external water (or proportionately more if the volume was greater than 2.0 L). A 20-min interval between each period was used to lower pH to ~4.0 with 0.5 M HNO₃ and then raise it back to ~6.3 for recovery with 0.5 M KOH. In practice, pH tended to rise, especially during the low pH period, because of ammonia production by the fish. Therefore, pH was monitored in each container every 30 min and was held between 3.8 and 4.2 by addition of more HNO₃ as necessary. During the control and recovery periods, pH was held between 6.1 and 6.5. Water samples (15 mL) were taken at the beginning and end of each period.

These experiments were run as paired comparisons, with each treatment in the pair applied to five or six animals on the same day. The first experiment compared responses to pH 4.0 exposure in blackwater versus reference water. The second experiment compared responses to pH 4.0 exposure in reference water versus reference water plus 100 $\mu\text{mol L}^{-1}$ of added Ca²⁺. The third experiment compared responses to pH 4.0 exposure in blackwater versus blackwater plus 100 $\mu\text{mol L}^{-1}$ of added Ca²⁺. The fourth experiment compared responses to pH 4.0 exposure in reference water with added Aldrich humic acid versus reference water with added Aldrich humic acid plus 100 $\mu\text{mol L}^{-1}$ of added Ca²⁺.

In the second, third, and fourth experiments, the water Ca²⁺ concentration was raised by the nominal value of 100 $\mu\text{mol L}^{-1}$ by adding the required volume of a 1 M Ca(NO₃)₂ stock solution to the appropriate water in the animal's chamber 20 min before the start of the experiment. As the rays are known to lose Ca²⁺ to the dilute water, especially at low pH (Wood et al. 2002), water samples were taken at the beginning and end of each 2-h period to determine the actual mean Ca²⁺ concentrations achieved. These were comparable in the three series and demonstrated that the actual elevations in Ca²⁺ concentrations were close to the nominal target (Table 2).

In the fourth experiment, our goal was to add sufficient

Aldrich humic acid to reference water to raise its DOC to a level equivalent to that of blackwater. However, as we had no means of directly measuring DOC during this field expedition, we estimated its concentration spectrophotometrically in the following manner. A series of 12 standards ranging from 0 to 24 mg L⁻¹ of added Aldrich humic acid (total humic acid, not total C) were made up in reference water. Aldrich humic acid is approximately 50% C by weight. These humic acid standards were used to construct calibration curves of absorbance versus humic acid content at both 330 and 400 nm, using a 1-cm cuvette (Thurman 1985). When Rio Negro water was read against this standard curve, its total content was equivalent to 12.7 mg L⁻¹ humic acid at 400 nm and 15.5 mg L⁻¹ humic acid at 330 nm. On this basis, 15 mg L⁻¹ of Aldrich humic acid was added to the reference water in the tests of the fourth experiment. In practice, this approach worked well because samples brought back to North America and later analyzed for true DOC with a Rosemount analyzer yielded a measured true DOC content of 0.6 mg L⁻¹ for reference water, 8.4 mg L⁻¹ for blackwater (Table 1), and 8.5 mg L⁻¹ for reference water with added Aldrich humic acid.

Analytical Methods

²²Na plus ³⁶Cl radioactivity was determined by mixing 3.0-mL water samples with 7.5 mL Ecolite fluor and counting on a Triathler portable scintillation counter. ²²Na radioactivity was determined by counting 2.0-mL water samples in a Picker Cliniscaler gamma counter fitted with a 10-cm NaI crystal in a well. ³⁶Cl radioactivity was obtained by subtraction after correcting for differences in volume and in efficiency of ²²Na counting between the two instruments. The latter was determined by counting the same standards on both (Wood 1988). Water total Na⁺, K⁺, Ca²⁺, and Mg²⁺ concentrations were determined by flame photometry (CELM flame photometer) or atomic absorption spectrophotometry (Perkin Elmer 1100B), and water pH was determined using an Orion model 266 portable meter and electrode. Water total Cl⁻ concentrations were determined by the colorimetric assay of Zall et al. (1956), and water total ammonia was determined by the salicylate hypochlorite method of Verdouw et al. (1978). Water samples were returned to North America for DOC analyses by a Rosemount total carbon analyzer and for determination of buffer curves. Water buffer curves were determined using a Radiometer GK2401C combination electrode connected to a PHM 84 meter. We continually bubbled 10-mL samples of blackwater, reference water, and reference water plus Aldrich humic acid with CO₂-free air and initially alkalinized them to pH 9.4 by the addition of KOH. The samples were then titrated with standardized 0.02 N HCl (Sigma) to pH 3.4 in steps of approximately 0.2 pH units using a Gilmont microburette and waiting 3 min between each acid addition. Titrations were performed in triplicate on the same electrode, and the results were averaged. In addition, a single

250-mL sample of blackwater and a single 250-mL sample of reference water were acidified with 0.1% trace metal grade HNO₃ and analyzed at McMaster University for a range of cationic metals (Al, Fe, Cu, Mn, Pb, Zn, Ni, Ag, and Cd) by graphite furnace atomic absorption spectrophotometry (Varian AA-1275 with GTA furnace atomizer) against Fisher certified multielement standards (see Table 3).

Statistical Analyses

All data are reported as means ± 1 SEM (*N*). Relationships were assessed by ANOVA, and individual means were compared using Student's paired or unpaired two-tailed *t*-test, as appropriate, with the Bonferroni correction for multiple comparisons (Nemenyi et al. 1977). The reference water data of the second experiment were tested against those of the first experiment at every time, and similarly, the blackwater data of the third experiment were tested against those of the first experiment. In both cases, there were no significant differences, so the respective data sets were pooled for the purpose of reference water versus blackwater comparison. A significance level of *P* ≤ 0.05 was used throughout.

Results

Concentration Kinetics of Na⁺ and Cl⁻ Uptake and Efflux in Blackwater and Reference Water

By ANOVA, the concentration kinetics of influx (at circum-neutral pH) did not differ significantly between reference water and blackwater for J_{in}^{Na} (Fig. 1A) but did for J_{in}^{Cl} (Fig. 1B); J_{max}^{Cl} was significantly greater in blackwater by 70%. There were no differences in K_m values for either ion. J_{out}^{Cl} (Fig. 2B), but not J_{out}^{Na} (Fig. 2A), increased significantly with external concentration in both waters. Between reference water and blackwater, there were no significant differences in outflux relationships for either ion. Nevertheless, because of small differences, the "balance points" at which influx and outflux relationships intersected (i.e., where $J_{in} = J_{out}$) shifted substantially from >300

Table 3: Metal concentrations ($\mu\text{g L}^{-1}$) of reference water (INPA well water) and blackwater (from the Rio Negro)

Metal	Reference Water	Blackwater
Al	6	77
Fe	<1	51
Cu	7	<1
Mn	2	8
Pb	1	1
Zn	7	1

Note. Ni, Ag, and Cd were below detectable levels (<1 $\mu\text{g L}^{-1}$) in both water types.

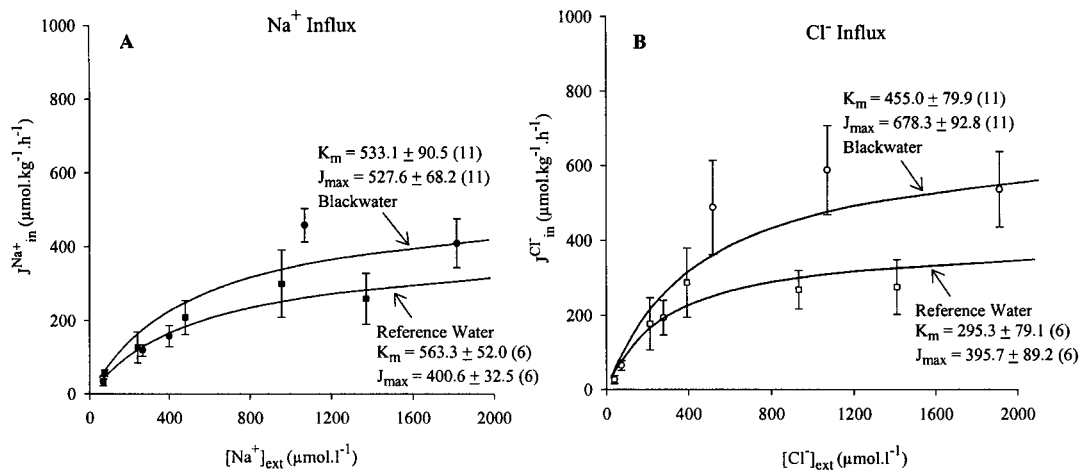


Figure 1. Kinetic relationships for (A) Na^+ and (B) Cl^- influx rates in stingrays acclimated to either reference water (squares; $N = 6$) or blackwater (circles; $N = 11$). Means ± 1 SEM. By ANOVA, there was a significant effect ($P \leq 0.05$) of water type on the Cl^- relationship but not on the Na^+ relationship. The mean values of K_m and J_{\max} , determined from individual animals and then averaged, are shown, illustrating the significantly higher J_{\max} for Cl^- influx in blackwater-acclimated fish. There were no other significant differences.

$\mu\text{mol L}^{-1}$ in reference water to about $100 \mu\text{mol L}^{-1}$ in blackwater for both Na^+ and Cl^- .

Responses to Low pH in Blackwater versus Reference Water

At control pH 6.3, there were no significant differences in unidirectional or net fluxes of either Na^+ or Cl^- (Fig. 3A) or in net ammonia excretion (J_{Amm} ; Fig. 3B) between rays held in reference water or blackwater. However, when rays were exposed to pH 4.0, their responses showed significant differences between the two water qualities. In reference water, $J_{\text{in}}^{\text{Na}}$ and $J_{\text{in}}^{\text{Cl}}$ were both inhibited by $>90\%$ at pH 4.0, while $J_{\text{out}}^{\text{Na}}$ and $J_{\text{out}}^{\text{Cl}}$ were both stimulated about 2.5-fold, which resulted in highly negative values of $J_{\text{net}}^{\text{Na}}$ and $J_{\text{net}}^{\text{Cl}}$ (Fig. 3A). All these changes were significant relative to the control period. However, when rays were exposed to pH 4.0 in blackwater, the inhibitions of $J_{\text{in}}^{\text{Na}}$ and $J_{\text{in}}^{\text{Cl}}$ were attenuated, and increases in $J_{\text{out}}^{\text{Na}}$ and $J_{\text{out}}^{\text{Cl}}$ were prevented. Therefore, $J_{\text{net}}^{\text{Na}}$ and $J_{\text{net}}^{\text{Cl}}$ became only slightly more negative. On return to pH 6.3, $J_{\text{in}}^{\text{Na}}$ and $J_{\text{in}}^{\text{Cl}}$ fully recovered in both water qualities. However, the stimulation of $J_{\text{out}}^{\text{Na}}$ and $J_{\text{out}}^{\text{Cl}}$ persisted in the rays exposed in reference water but not in those exposed in blackwater.

Rays exposed to pH 4.0 in reference water showed no changes in J_{Amm} (Fig. 3B). However, the same exposure in blackwater caused a significant 60% increase in J_{Amm} , which was corrected during the recovery period. The differences in the J_{Amm} response between the two water qualities were significant. Thus, blackwater completely protected both Na^+ and Cl^- efflux components, slightly protected the influx components, and promoted increased J_{Amm} during low pH exposure as well as the recovery of Na^+ and Cl^- balance in the postexposure period.

Influence of Ca^{2+} on the Responses to Low pH in Reference Water versus Blackwater

In the second experiment, the addition of $100 \mu\text{mol L}^{-1}$ Ca^{2+} to reference water had no effect on unidirectional Na^+ and Cl^- fluxes (Fig. 4A) or net ammonia flux (Fig. 4B) at control pH 6.3. However, the presence of elevated Ca^{2+} in reference water completely prevented the increases in both $J_{\text{out}}^{\text{Na}}$ and $J_{\text{out}}^{\text{Cl}}$ and thereby prevented the increases in $J_{\text{net}}^{\text{Na}}$ and $J_{\text{net}}^{\text{Cl}}$ during exposure to pH 4.0 and the subsequent recovery period (Fig. 4A). The decreases in $J_{\text{in}}^{\text{Na}}$ and $J_{\text{in}}^{\text{Cl}}$ during acid exposure and their restoration on return to control pH were not significantly altered. High Ca^{2+} also facilitated a significant increase in ammonia excretion at low pH, an effect not seen in reference water alone (Fig. 4B). Measured water Ca^{2+} concentrations for these exposures are given in Table 2.

In the third experiment, the addition of the same level of Ca^{2+} to blackwater was completely without effect on the responses to low pH exposure (Fig. 5A, 5B). However, note here that the presence of blackwater alone (in contrast to reference water) already prevented any increases in $J_{\text{out}}^{\text{Na}}$ and $J_{\text{out}}^{\text{Cl}}$ at pH 4.0 (Fig. 5A) while facilitating an increase in J_{Amm} (Fig. 5B). Indeed, in blackwater, there were no significant effects of increased Ca^{2+} on any of the parameters monitored.

Influence of Humic Acid on the Responses to Low pH in Reference Water

The fourth experiment evaluated whether the addition of commercially available DOC (Aldrich humic acid) to reference water to match the DOC level of blackwater would offer the same protective effects on the responses to low pH exposure. The

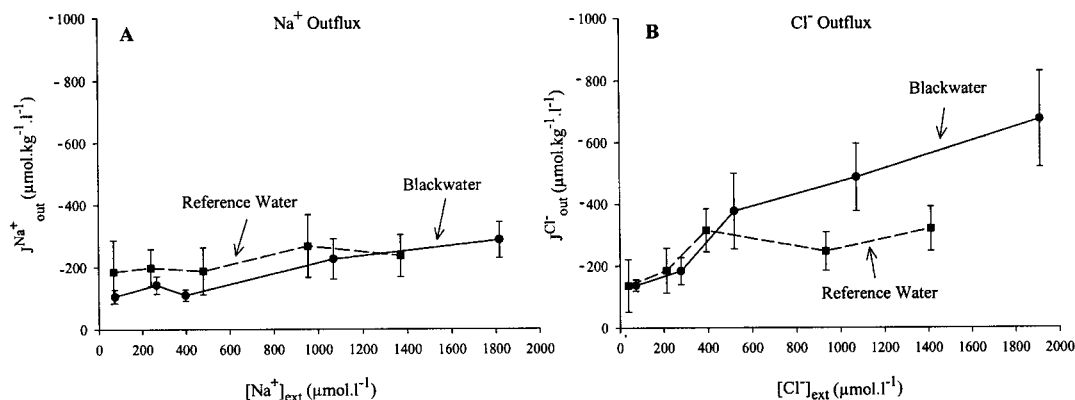


Figure 2. Relationships between (A) Na⁺ outflux and external Na⁺ concentration and (B) Cl⁻ outflux and external Cl⁻ concentration in stingrays acclimated to either reference water (squares; $N = 6$) or blackwater (circles; $N = 11$). Means ± 1 SEM. By ANOVA, there were no significant effects of water type on the relationships, though the trend for increasing Cl⁻ outflux with increasing external Cl⁻ concentration was significant ($P \leq 0.05$).

comparison trial evaluated whether added humic acid, like natural blackwater, would prevent any influence of added Ca²⁺. Humic acid alone exerted no effects at control pH 6.3 (compared with either blackwater or reference water at the same pH), but surprisingly, it greatly exacerbated the increases in both J_{out}^{Na} and J_{out}^{Cl} and therefore J_{net}^{Na} and J_{net}^{Cl} at low pH (Fig. 6A). Na⁺ and Cl⁻ effluxes increased to more than eightfold higher than the pH 6.3 control levels and approximately threefold higher than levels seen during acid exposure in reference water alone (cf. Figs. 3A, 4A). However, the addition of 100 μmol L⁻¹ Ca²⁺ had no protective effect whatsoever in the presence of humic acid (Fig. 6A). Indeed, both J_{out}^{Na} and J_{out}^{Cl} at low pH were about sixfold greater in comparison with the responses seen at low pH when Ca²⁺ alone was added to reference water (cf. Fig. 4A). The pattern of recovery was not altered. However, the presence of added humic acid did facilitate an increase in J_{Amm} at pH 4.0 that was similar to the response in blackwater alone and in both reference water and blackwater when additional Ca²⁺ was present (cf. Figs. 3B, 4B, 5B) but absent in reference water alone (cf. Figs. 4B, 5B). These effects of humic acid were seen only at low pH regardless of the presence or absence of added Ca²⁺.

Discussion

Overview

These results confirm the rather unusual low affinity, low capacity Na⁺ and Cl⁻ uptake kinetics (Fig. 1), and low efflux rates (Fig. 2) previously reported for these stenohaline elasmobranchs (Wood et al. 2002). However, they do not support the suggestion that the lower Na⁺ and Cl⁻ efflux rates seen in blackwater relative to ion-rich hard water were due to some component of the blackwater itself because the same low efflux rates were seen in reference water (Fig. 2) that was equally low

in ions but virtually lacking in DOC (Table 1). Very likely, the low efflux rates were simply a function of the ion-poor condition of the water, which is known to increase epithelial “tightness” (e.g., McDonald and Rogano 1986). The tendency for J_{out}^{Cl} , but not J_{out}^{Na} , to increase with increasing external ion concentration (Fig. 2) likely reflected an exchange diffusion component to Cl⁻ turnover (e.g., De Renzis 1975; Wood et al. 2002).

At circumneutral pH 6.3, the influence of blackwater relative to reference water on Na⁺ and Cl⁻ influx kinetics was not dramatic (Fig. 1A, 1B). Because the K_m values for both relationships were much greater than normal environmental concentrations in blackwater (Table 1), there was relatively minor influence on actual J_{in}^{Na} and J_{in}^{Cl} values of rays living in these two media; this was confirmed by measurements of J_{in}^{Na} and J_{in}^{Cl} during the control periods of Figures 3, 4, and 5. Furthermore, J_{out}^{Na} and J_{out}^{Cl} relationships did not differ significantly between blackwater and reference water (Fig. 2). Nevertheless, the balance points for both Na⁺ and Cl⁻ (i.e., the concentrations at which influx and outflux values were theoretically equal) shifted from >300 μmol L⁻¹ in reference water to about 100 μmol L⁻¹ in blackwater, an important adaptation. This finding is in agreement with our earlier conclusion (Wood et al. 2002) and suggests a normal role for dietary ion uptake because the balance points are still above ambient concentrations (Table 1).

Clearly, blackwater has important protective actions against the negative ionoregulatory effects of low pH exposure in these freshwater elasmobranchs (Fig. 3). There was a modest protection of Na⁺ and Cl⁻ influxes so that these were not so strongly inhibited by acidity. A similar observation was recorded by Gonzalez et al. (2002), who compared the responses of the Rio Negro teleost *Geophagus* sp. to pH 3.75 exposure in blackwater versus ion-supplemented distilled water. More important, there was a marked protection of Na⁺ and Cl⁻ effluxes so that

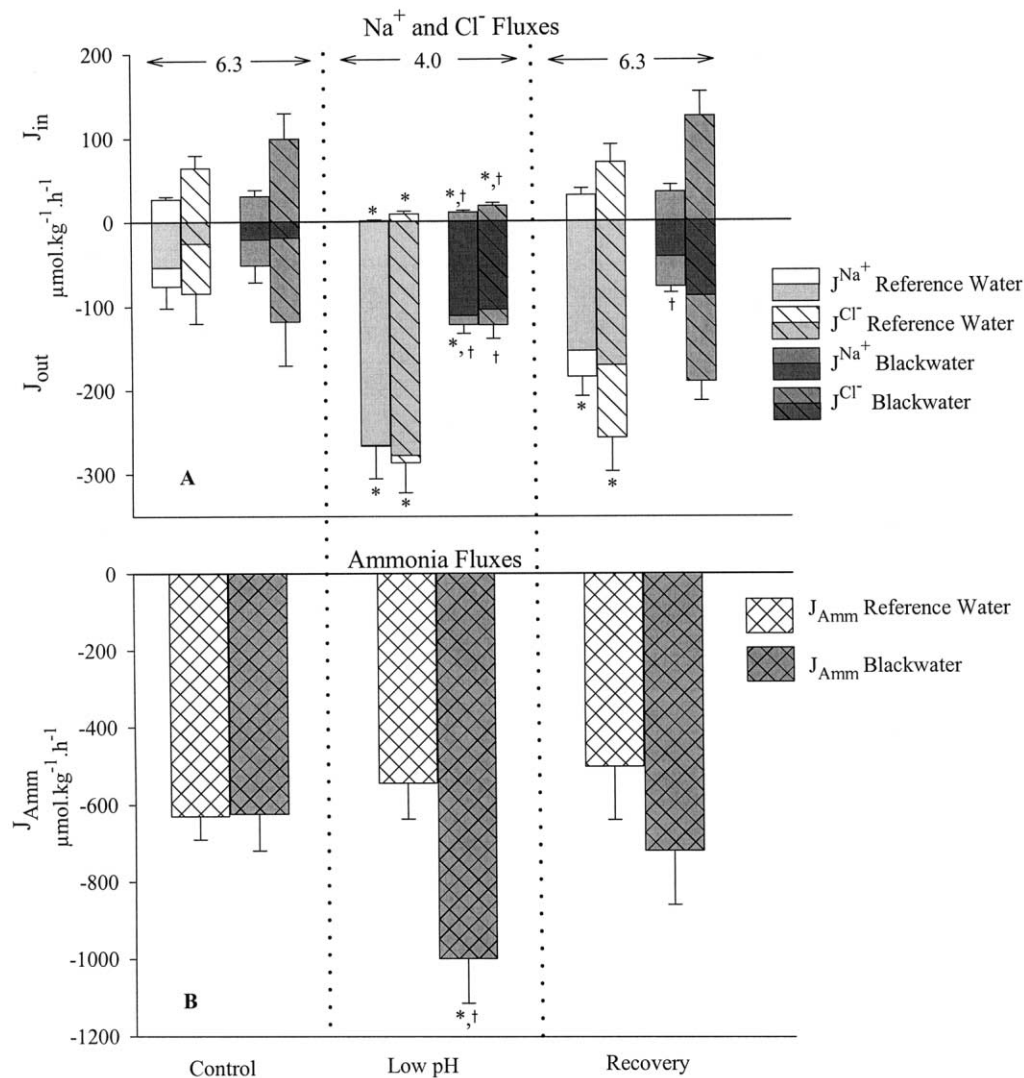


Figure 3. The influence, in stingrays tested in either reference water or blackwater, of acute exposure to pH 4.0 for 2 h, followed by recovery at control pH 6.3 for 2 h, on (A) unidirectional and net flux rates of Na⁺ (open bars) and Cl⁻ (hatched bars) and (B) net ammonia flux rates (cross-hatched bars). Means \pm 1 SEM ($N = 11-12$). SEMs have been omitted from net Na⁺ and Cl⁻ net flux rates (darker shaded bars) for clarity because they sometimes overlap those of the unidirectional flux rates. Positive (upward) bars represent uptake into the fish; negative (downward) bars represent losses from the fish. Asterisks indicate means significantly different ($P \leq 0.05$) from the corresponding control mean; daggers indicate means significantly different ($P \leq 0.05$) from the corresponding mean in reference water during the same period.

increases in both J_{out}^{Na} and J_{out}^{Cl} at low pH and during recovery were greatly attenuated in *Potamotrygon* sp. (Fig. 3A). Again, a similar phenomenon was reported for J_{out}^{Na} in *Geophagus* sp. exposed to pH 3.75 and in another Rio Negro teleost, *Pimelodes* sp., during recovery from pH 3.75 (Gonzalez et al. 2002). Therefore, net ion losses during and after low pH exposure are greatly attenuated by blackwater in both teleosts and elasmobranchs. Blackwater also facilitated an increase in J_{Amm} at low pH in *Potamotrygon* sp. (Figs. 3B, 5B). Clearly, there is something about natural blackwater that is beneficial to both stingrays and teleosts in tolerating the bouts of low pH that are

common in this dilute environment (Val and Almeida-Val 1995).

The addition of Ca²⁺ to reference water mimicked these protective actions of natural blackwater on ionic efflux, but not on the influx components, and also stimulated J_{Amm} at low pH (Fig. 4). In contrast, Ca²⁺ addition to blackwater had no effect on the (already attenuated) responses to environmental acidity (Fig. 5), and the addition of commercial humic acid to reference water did not duplicate the actions of either blackwater or Ca²⁺ supplementation on the responses to environmental acidity. Added humic acid actually exacerbated Na⁺ and Cl⁻ losses

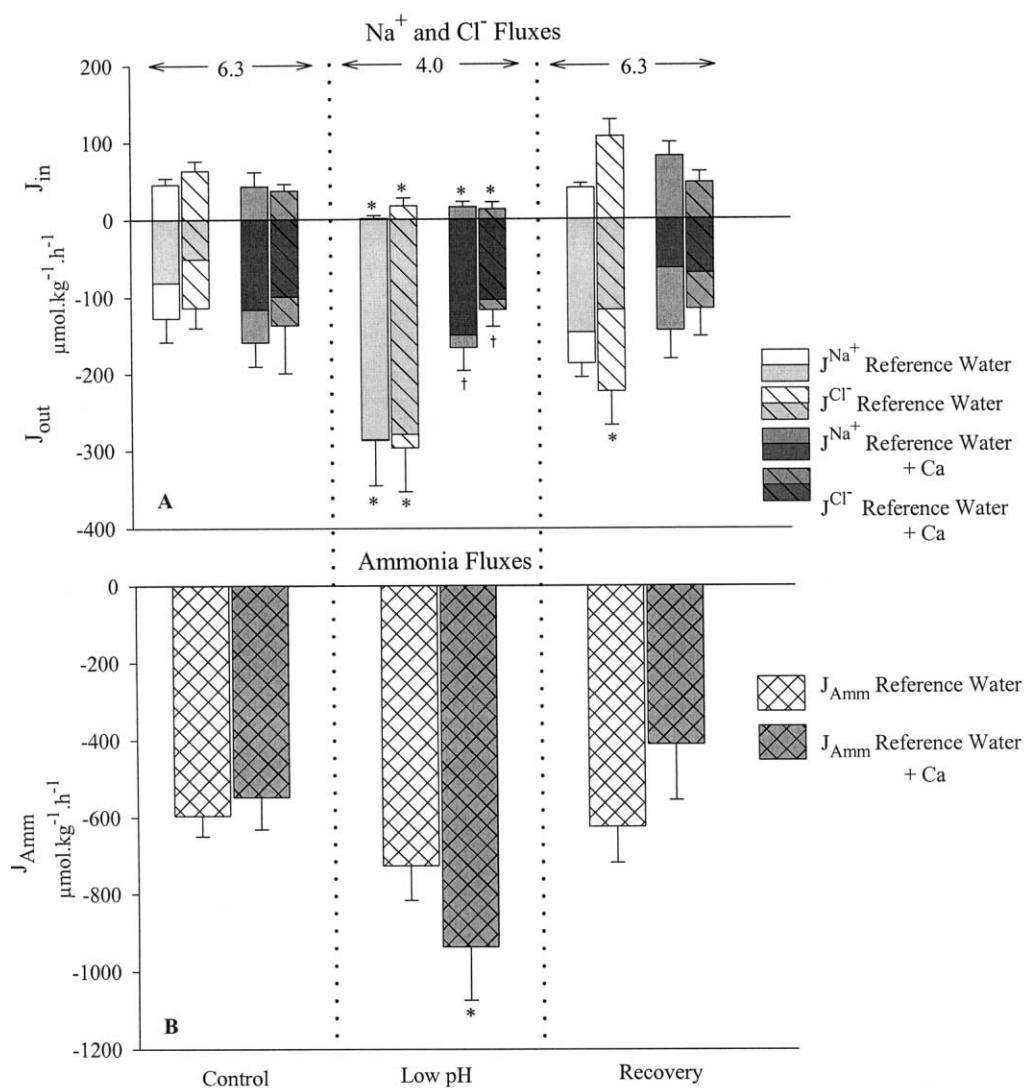


Figure 4. The influence, in stingrays tested in either reference water or reference water supplemented with nominally $100 \mu\text{mol L}^{-1} \text{Ca}^{2+}$, of acute exposure to pH 4.0 for 2 h, followed by recovery at control pH 6.3 for 2 h, on (A) unidirectional and net flux rates of Na^+ (open bars) and Cl^- (hatched bars) and (B) net ammonia flux rates (cross-hatched bars). The mean measured Ca^{2+} concentrations in each period are tabulated in Table 2. Means ± 1 SEM ($N = 5-6$). SEMs have been omitted from net Na^+ and Cl^- net flux rates (darker shaded bars) for clarity because they sometimes overlap those of the unidirectional flux rates. Positive (upward) bars represent uptake into the fish; negative (downward) bars represent losses from the fish. Asterisks indicate means significantly different ($P \leq 0.05$) from the corresponding control mean; daggers indicate means significantly different ($P \leq 0.05$) from the corresponding mean in reference water during the same period due to the presence of added Ca^{2+} .

during low pH exposure and eliminated the protective action of Ca^{2+} supplementation (Fig. 6).

Possible Mechanisms of Low pH, Ca^{2+} , DOC, and Blackwater Effects on Ion Fluxes

The inhibitory effect of low environmental pH on active branchial ion uptake is well known in teleost fish (for reviews, see McDonald 1983; Reid 1995; Wood 2001). The inhibition of

J_{in}^{Na} was originally explained as H^+ competition with Na^+ on an apical Na^+/H^+ or $\text{Na}^+/\text{NH}_4^+$ exchange mechanism (e.g., Maetz and Garcia-Romeu 1964; Wright and Wood 1985) but more recently has been attributed to an H^+ gradient-induced slowing of an apical electrogenic H^+ ATPase that energizes an apical Na^+ channel in gill ionocytes (e.g., Lin and Randall 1993). The mechanism of J_{in}^{Cl} inhibition remains unknown and is usually attributed to some sort of indirect effect such as coupling to the inhibition of J_{in}^{Na} or depletion of intracellular HCO_3^- in

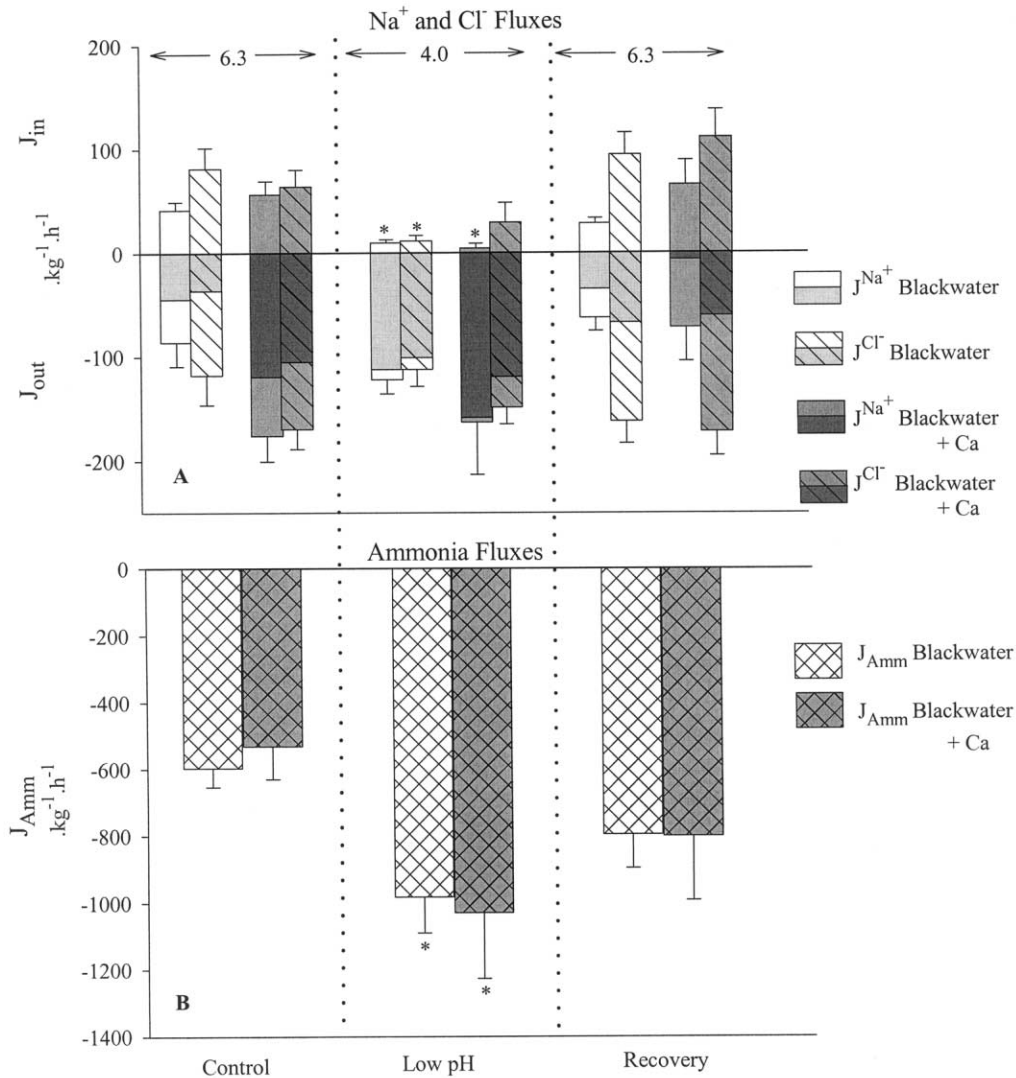


Figure 5. The influence, in stingrays tested in either blackwater or blackwater supplemented with nominally $100 \mu\text{mol L}^{-1} \text{Ca}^{2+}$, of acute exposure to pH 4.0 for 2 h, followed by recovery at control pH 6.3 for 2 h, on (A) unidirectional and net flux rates of Na^+ (open bars) and Cl^- (hatched bars) and (B) net ammonia flux rates (cross-hatched bars). The mean measured Ca^{2+} concentrations in each period are tabulated in Table 2. Means ± 1 SEM ($N = 5-6$). SEMs have been omitted from net Na^+ and Cl^- net flux rates (darker shaded bars) for clarity because they sometimes overlap those of the unidirectional flux rates. Positive (upward) bars represent uptake into the fish; negative (downward) bars represent losses from the fish. Asterisks indicate means significantly different ($P \leq 0.05$) from the corresponding control mean. There were no significant differences from the corresponding mean in blackwater during the same period due to the presence of added Ca^{2+} .

gill ionocytes, which thereby starves the apical $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism (e.g., Wood 2001).

Recent pharmacological studies on ion transport in *Potamotrygon* sp. (Wood et al. 2002) and recent molecular and immunocytochemical studies on a euryhaline stingray, *Dasyatis sabina* (Piermarini and Evans 2000, 2001), both suggest that ion transport mechanisms may be different from those of teleosts in these freshwater elasmobranchs. Indeed, the currently available data are coherent with a model (Piermarini and Evans

2001; Wood et al. 2002) whereby basolateral Na^+ , K^+ ATPase energizes an apical Na^+/H^+ exchanger in one type of ionocyte, while basolateral H^+ ATPase energizes an apical $\text{Cl}^-/\text{HCO}_3^-$ exchanger or Cl^- channel in another, the two mechanisms being entirely independent. By this model, inhibition of J_{in}^{Na} is easily explained by simple H^+ competition at the apical Na^+/H^+ exchanger, but the inhibition of J_{in}^{Cl} is again difficult to explain. Regardless, if natural DOC can bind to the apical membranes of the ionocytes at low pH, as indicated by the work of Camp-

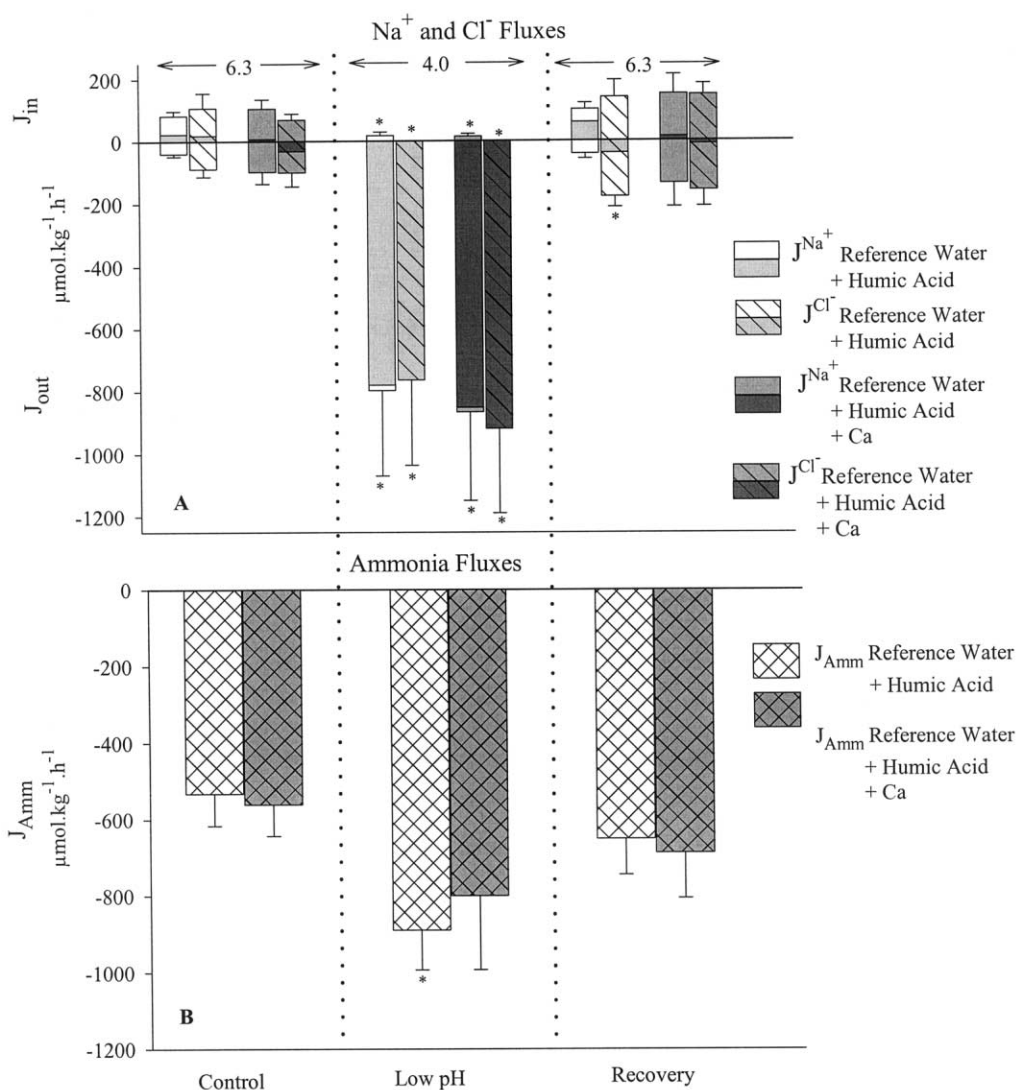


Figure 6. The influence, in stingrays tested in either reference water plus humic acid or reference water plus humic acid supplemented with nominally $100 \mu\text{mol L}^{-1} \text{Ca}^{2+}$ at control pH 6.3, of acute exposure to pH 4.0 for 2 h, followed by recovery at control pH 6.3 for 2 h, on (A) unidirectional and net flux rates of Na^+ (open bars) and Cl^- (hatched bars) and (B) net ammonia flux rates (cross-hatched bars). The mean measured Ca^{2+} concentrations in each period are tabulated in Table 2. The humic acid concentration was set to equal the DOC concentration of blackwater. Means ± 1 SEM ($N = 5-6$). SEMs have been omitted from net Na^+ and Cl^- net flux rates (darker shaded bars) for clarity because they sometimes overlap those of the unidirectional flux rates. Positive (upward) bars represent uptake into the fish; negative (downward) bars represent losses from the fish. Asterisks indicate means significantly different ($P \leq 0.05$) from the corresponding control mean. There were no significant differences from the corresponding mean in reference water plus humic acid during the same period due to the presence of added Ca^{2+} . However, note the much higher efflux and net flux rates during pH 4.0 exposure due to the presence of humic acid, relative to the trials of Figures 3, 4, and 5.

bell et al. (1997), then there are several possible mechanisms by which blackwater might protect against the inhibition of $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ by environmental acidity. For example, these organic molecules could protect against or displace H^+ ions, they could help concentrate Na^+ and Cl^- ions (by complexation) locally at the apical membrane of the gill, or they could help to deliver the ions to the uptake sites, roles often attributed to mucus

(e.g., Handy 1989). It is interesting that commercial humic acid did not have this beneficial effect, but it may have very different binding characteristics than does natural blackwater DOC.

In freshwater teleosts, increases in Na^+ and Cl^- efflux at low environmental pH are usually attributed to the leaching of Ca^{2+} from paracellular "leakage" channels on the gills (e.g., McWilliams 1983; Marshall 1985; Reid 1995), and the protective

action of elevated environmental Ca^{2+} levels is usually attributed to a reversal of that effect (e.g., McDonald 1983; Wood 2001). This would explain the protective effect of high Ca^{2+} seen for stingrays in reference water (Fig. 4). However, in stingrays acclimated to blackwater, the same elevation in water Ca^{2+} had no effect, but effluxes were already protected by some aspect of the blackwater itself (Fig. 5). Gonzalez et al. (1998) similarly reported that the same increase in water Ca^{2+} concentration had no protective effect in three acid-tolerant teleost species exposed to low pH in their native Rio Negro blackwater. Gonzalez et al. (1998) hypothesized that either gill sites were insensitive to Ca^{2+} or they were already fully saturated with Ca^{2+} such that Ca^{2+} elevation would have no effect. In the stingrays, the observed protective effects of Ca^{2+} in reference water (Fig. 4) eliminate the first explanation. Furthermore, using a radiolabeled calcium displacement technique, Wood et al. (2002) demonstrated that exposure to pH 4.0 in blackwater did not cause any displacement of surface-bound Ca^{2+} from the whole stingray. These results are consistent with the second explanation of Gonzalez et al. (1998) and indicate that Ca^{2+} is bound with very high affinity in blackwater-acclimated fish. Furthermore, it is possible that in blackwater, added Ca^{2+} is less available to the gills anyway after being immediately complexed by the dissolved organic molecules. However, there is a third possibility: perhaps in blackwater, the paracellular leakage sites are already fully protected by DOC, which may bind to the branchial epithelium at low pH (Kullberg et al. 1993; Campbell et al. 1997), or by some other aspect of blackwater composition that renders Ca^{2+} binding irrelevant under these conditions.

The tests with commercial humic acid added to reference water were designed to address this third possibility. The results were surprising and revealed a marked exacerbation of diffusive losses at low pH rather than a protective action of DOC and a complete elimination of the protective effect of Ca^{2+} (Fig. 6). Clearly, commercial humic acid does not duplicate the effects of natural blackwater, but it does prevent any protective action of added Ca^{2+} . The most likely explanation for this is that commercial humic acid has such a high affinity and/or capacity for cations that it strips Ca^{2+} from the gills at low pH and completely immobilizes the added Ca^{2+} . Commercial humic acid may have very different binding characteristics than does natural blackwater DOC. Indeed, the water titration data (Fig. 7) discussed subsequently reveal a greater buffer capacity in natural blackwater than in reference water plus commercial humic acid. The DOC of the Rio Negro and its tributaries has been studied intensively (Leenheer 1980; Ertel et al. 1986; Kuchler et al. 1994) and is much more complex than Aldrich humic acid, which is extracted from peat. Only a little more than 50% of the total blackwater DOC is composed of "humic substances," and of these, fulvic acids, operationally defined by acid solubility at $\text{pH} < 2.0$, predominate over true humic acids, defined by acid insolubility at $\text{pH} < 2.0$ (Thurman 1985). Al-

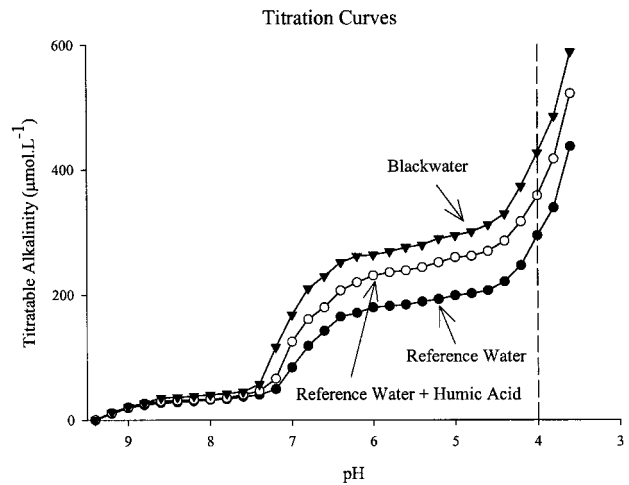


Figure 7. Titration curves of blackwater, reference water, and reference water plus commercial humic acid at the same DOC concentration as blackwater. The data are means of triplicate determinations, which were virtually identical. The reference point of pH 4.0 for calculation of pH changes at the gills is marked (dashed line).

drich humic acid itself is treated with strong chelating agents to remove metals during the preparative process, so it might be expected to more effectively bind Ca^{2+} and strip it from the gills. Furthermore, Aldrich humic acid is more aromatic in structure than are most natural organic matters, a property correlated with stronger binding of biologically relevant metals (Richards et al. 2001; M. Schwartz and R. C. Playle, unpublished results). A future challenge will be to isolate blackwater DOC without changing its chemistry for physiological tests.

Responses of Ammonia Excretion

Large increases in J_{Amm} at low pH occurred only in blackwater and not in reference water (Figs. 3, 5) but were facilitated by the addition of either Ca^{2+} (Fig. 4) or commercial humic acid (Fig. 6) to reference water. This increase may be an adaptive response because enhanced NH_3 efflux will raise the pH of the gill boundary water, especially in poorly buffered soft water (Playle and Wood 1989). Water buffer curves (Fig. 7) were determined for the principal reason of assessing how important this effect might be and whether it would vary among the different water qualities. The overall buffer capacity of blackwater was greater than that of reference water, and the addition of Aldrich humic acid to the same DOC level could account for only about half of the difference. The results are quantitatively similar to those reported by Leenheer (1980) for Amazonian blackwater samples. However, these differences occurred in the circumneutral pH range (pH 6.0–7.5); at around pH 4.0, the buffer curves for the three waters were very similar. If we assume a standard fish ventilation volume of 20 L kg^{-1}

h^{-1} (Wood 2001), complete mixing, and that all ammonia excretion occurred as NH_3 , the unchanged J_{Amm} of about $600 \mu\text{mol kg}^{-1} \text{h}^{-1}$ during acid exposure in reference water (Figs. 3B, 4B) would have raised the pH at the gills from 4.0 to 4.11, whereas the elevated J_{Amm} of about $1,000 \mu\text{mol kg}^{-1} \text{h}^{-1}$ during acid exposure in blackwater (Figs. 3B, 5B) as well as in reference water plus humic acid (Fig. 6B) would have raised the pH at the gills from 4.0 to 4.21. This reduction in H^+ ion concentration due to elevated J_{Amm} is about 20% and would probably be greater in the gill boundary layer in the more likely scenario that mixing is not complete. However, these experiments investigated only acute responses to low pH. A chronic elevation of J_{Amm} as a protective response to low pH would obviously require either an enhanced metabolic rate or a change in metabolism to a greater reliance on protein oxidation. There are currently no data on the chronic responses to low pH in any of these Amazonian fishes.

Recently, we reported that J_{Amm} at circumneutral pH in blackwater was strongly inhibited by amiloride and amiloride analogs (Wood et al. 2002), and we interpreted the observation as an inhibition of "diffusion trapping" of NH_3 in the gill boundary layer due to blockade of Na^+ -coupled H^+ extrusion (Wilson 1996a; Wilkie 1997). Clearly, diffusion trapping should be enhanced at low environmental pH, so why this occurred only in blackwater but not in reference water is unclear. Because blackwater partially protects $J_{\text{in}}^{\text{Na}}$, perhaps it also partially protects H^+ extrusion and thereby enhances diffusion trapping and ammonia excretion. However, the addition of either calcium or commercial humic acid did not protect sodium influx yet still promoted the elevation of J_{Amm} at low pH. In several temperate teleosts exposed to alkaline water where diffusion trapping should be severely inhibited, the addition of Ca^{2+} is known to facilitate J_{Amm} (Yesaki and Iwama 1992; Iwama et al. 1997) and also to enhance H^+ -ATPase activity in the gills (Lin and Randall 1993).

The Potential Effects of Naturally Occurring Metals

In natural waters, some of the toxic effects of low pH on ionoregulation may be due to metals such as Al and Cu, which are naturally present and benign at circumneutral pH but highly toxic at low pH due to pH-dependent shifts in speciation (McDonald et al. 1989; Brown and Waring 1996; Wilson 1996b). The cationic forms of these metals may inhibit influxes via actions on Na^+ channels and $\text{Na}^+ \text{K}^+$ ATPase (e.g., Staurnes et al. 1984; Lauren and McDonald 1987) and may stimulate effluxes both via displacement of Ca^{2+} from paracellular junctions and by inflammatory and physical damage mechanisms (Brown and Waring 1996; Wilson 1996b; Wood 2001). The situation is further complicated by the fact that Al^{3+} predominates around pH 4.0 and may actually mimic the protective effects of Ca^{2+} (Schofield and Trojnar 1980; Baker and Schofield 1982). Furthermore, DOC-rich waters draining from forest soils

are often rich in metals, and the organic acid molecules are capable of binding the metals, which would potentially reduce bioavailability and toxicity to fish gills (e.g., Wicklund-Glynn et al. 1992; Playle et al. 1993a, 1993b; Roy and Campbell 1997; Marr et al. 1999). For this reason, we analyzed the reference water and the blackwater for a range of metals (Table 3).

In the natural reference water, only Cu was slightly elevated to levels of potential concern for fish in very soft water (McDonald et al. 1989; Canadian Council of Ministers of the Environment 1999; Santore et al. 2001). However, in the virtual absence of DOC (Table 1), Cu speciation does not change appreciably between pH 6.3 and pH 4.0, so it would not have been a cause of the responses to low pH. In blackwater, the levels of most metals (Table 3) were in general accord with the data of Furch (1984) for Rio Negro water, though they were actually somewhat lower in absolute concentration. Fe and Al were markedly elevated in natural blackwater, the latter to a level known to exert ionoregulatory toxicity in teleosts if not immobilized by DOC (Wood and McDonald 1987; Brown and Waring 1996; Wilson 1996b; Canadian Council of Ministers of the Environment 1999; Gensemer and Playle 1999). Natural DOC will already be partly saturated with metals, but strong chelating agents are used to remove metals during the preparation of Aldrich humic acid. Clearly, we are dealing with a multifactorial situation, and some simple biogeochemical modeling may illuminate the results (e.g., Playle et al. 1993a, 1993b; Playle 1998; Gensemer and Playle 1999; Santore et al. 2001).

Biogeochemical Modeling

The interactions of H^+ , Ca^{2+} , and aluminum at the gills and with DOC were modeled using the MINEQL⁺ chemical equilibrium program (version 4.0; Schecher and McAvoy 1992) according to Gensemer and Playle (1999), with the addition of Ca^{2+} -DOC binding (Meinelt et al. 2001). In brief, key assumptions were that total gill binding sites are present at $1 \mu\text{M}$, that aluminum (as Al^{3+}) binds to these sites with a conditional equilibrium binding constant ($\log K_{\text{Al-gill}}$) of 6.5, and that comparable values for Ca^{2+} ($\log K_{\text{Ca-gill}}$) and H^+ ($\log K_{\text{H-gill}}$) are both 4.0. Complexation of Al^{3+} , H^+ , and Ca^{2+} by DOC was modeled using $1.0\text{-}\mu\text{mol}$ binding sites per 0.3 mg C L^{-1} DOC (i.e., $28 \mu\text{M}$ at 8.5 mg C L^{-1} ; Table 1), with $\log K_{\text{Al-DOC}} = 8.4$, $\log K_{\text{Al-H-DOC}} = 13.1$, $\log K_{\text{H-DOC}} = 4.0$, $\log K_{\text{Ca-DOC}} = 5.0$, and $\log K_{\text{Fe-DOC}} = 9.4$ (for details, see Playle et al. 1993a; Gensemer and Playle 1999; Meinelt et al. 2001). The water chemistry given in Tables 1 and 3 was used, and simulations were run open to the atmosphere with ionic strength interactions enabled and gibbsite formation allowed. Note that this modeling could not take into account possible direct binding of DOC to gill sites because no constants are yet available. Also note that the metal-DOC conditional equilibrium binding constants were not derived from Aldrich humic acid or blackwater DOC but rather

a variety of organic matter sources, so the modeling results must be considered tentative.

The principal conclusions of this exercise were as follows. First, the complexing capacity for H^+ of natural DOC in blackwater was not sufficient to appreciably alter the binding of H^+ to gill sites at pH 4.0, which amounted to about 47% saturation of available gill sites in both reference water and blackwater versus about 0.5% at pH 6.3 in both water qualities. Second, adding $100 \mu\text{mol L}^{-1} \text{Ca}^{2+}$ to reference water did increase gill Ca^{2+} binding at pH 4.0 from 9% to 37% and reduced H^+ binding from 46% to 31%. Third, despite its biologically protective effect, the presence of natural DOC in blackwater actually reduced gill Ca^{2+} binding slightly at pH 4.0 in the simulations, from 9% (in reference water alone) to 5% (in blackwater alone) in the absence of added Ca^{2+} and from 37% to 33% in the presence of added Ca^{2+} . Again, gill H^+ binding was not appreciably altered relative to the situation in reference water with added Ca^{2+} . Finally, the DOC in natural blackwater completely prevented Al binding to the gills (i.e., 0% vs. 71% saturation of gill sites if natural blackwater DOC was not present). Similarly, Fe, present only in blackwater, was completely bound up by DOC under all conditions and therefore would not interact at the gills.

Overall, these modeling results support the view that natural DOC may act directly at the gills to exert a protective effect at low pH and, when doing so, may override any protective action of Ca^{2+} that might otherwise occur. The protective action of natural blackwater cannot be attributed to Al or Fe binding to the gills (and thereby mimicking calcium) at low pH, and indeed, the complete binding of Al to natural blackwater DOC may be very important in preventing aluminum-induced toxicity in the Rio Negro.

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