The Journal of Experimental Biology 205, 3039–3054 (2002)
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JEB4218



# Mechanisms of ion transport in *Potamotrygon*, a stenohaline freshwater elasmobranch native to the ion-poor blackwaters of the Rio Negro

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*Accepted 3 July 2002*

## Summary

**Stingrays of the family Potamotrygonidae are the only stenohaline freshwater elasmobranchs. *Potomotrygon* sp. collected from the ion-poor blackwaters ($[Na^+]$, $[Cl^-]$ and $[Ca^{2+}]$=10–30 μmol $l^{-1}$, pH 6.1) of the Rio Negro, Amazonas, Brazil, were ammoniotelic (91% ammonia-N, 9% urea-N excretion) and exhibited blood chemistry ($Na^+$, $Cl^-$, urea, ammonia and glucose levels and osmolality) typical of freshwater teleosts. Unidirectional $Na^+$ and $Cl^-$ influx rates, measured with radiotracers, displayed saturation kinetics. The relationships for $Cl^-$ and $Na^+$ had similar $K_m$ values (300–500 μmol $l^{-1}$), but $J_{max}$ values for $Cl^-$ (approximately 950 μmol $kg^{-1}$ $h^{-1}$) were almost twice those for $Na^+$ (approximately 500 μmol $kg^{-1}$ $h^{-1}$). $Cl^-$ efflux rates varied with external concentration, but $Na^+$ efflux rates did not. There were no differences in the kinetic variables ($K_m$, $J_{max}$) for influx between animals acclimated to their native ion-poor blackwater or to ion-rich hard water, but efflux rates for both $Na^+$ and $Cl^-$ were lower in the former, yielding much lower balance points (external $Na^+$ or $Cl^-$ levels at which influx and efflux were equal). $Na^+$, $Cl^-$ and $Ca^{2+}$ uptake were all strongly inhibited by acute exposure to pH 4.0, but efflux rates and $Ca^{2+}$ binding to the body surface did not change. $Na^+$ influx was inhibited by amiloride ($10^{-4}$ mol $l^{-1}$) and by two of its analogs, phenamil ($4\times10^{-5}$ mol $l^{-1}$) and HMA ($4\times10^{-5}$ mol $l^{-1}$), with the latter being slightly more potent, while $Cl^-$ fluxes were unaffected. $Cl^-$ fluxes were insensitive to DIDS ($2\times10^{-5}$ mol $l^{-1}$ or $10^{-4}$ mol $l^{-1}$) and SITS ($10^{-4}$ mol $l^{-1}$), but both influx and efflux rates were strongly inhibited by DPC ($10^{-4}$ mol $l^{-1}$) and thiocyanate ($10^{-4}$ mol $l^{-1}$). Ammonia excretion was unresponsive to large changes in water $Na^+$ concentration, but was elevated by 70% during acute exposure to pH 4.0 and transiently inhibited by approximately 50% by amiloride and its analogues. The strategy of adaptation to ion-poor blackwater appears similar to that of some Rio Negro teleosts (Cichlidae) in which low-affinity transport systems are relatively sensitive to inhibition by low pH but are complemented by low diffusive loss rates. Ionic transport systems in these freshwater elasmobranchs, although superficially similar to those in some freshwater teleosts, may bear more resemblance to their presumed evolutionary precursors in marine elasmobranchs.**

Key words: Potamotrygonidae, freshwater elasmobranch, ion transport kinetics, $Na^+$ flux, $Cl^-$ flux, $Ca^{2+}$ flux, ammonia excretion, low pH, blackwater.

## Introduction

Stingrays of the family Potamotrygonidae are widespread throughout river systems of South America that drain into the Atlantic Ocean. While some members of the family Dasyatidae may complete their entire life cycle in freshwater (Compagno and Roberts, 1982; Johnson and Snelson, 1996), the potamotrygonid stingrays are considered to be the only group of elasmobranchs to have speciated exclusively within freshwaters (Lovejoy, 1996). In contrast to all other freshwater-tolerant elasmobranchs, potamotrygonid rays have lost their ability to live in seawater and are restricted in nature to waters with a salinity of less than 3 ppt (Brooks et al., 1981). Unique features include an inability to retain urea to any great extent when challenged with higher salinity (Thorson et al., 1967; Junqueira et al., 1968; Thorson, 1970; Griffith et al., 1973; Gerst and Thorson, 1977; Bittner and Lang, 1980), the absence of salt excretion by the rectal gland, which is

degenerate (Thorson et al., 1978), and modifications of the electroreceptive ampullae of Lorenzini for freshwater operation (Raschi and Mackanos, 1989).

The exact route and timing of evolutionary invasion from the ocean remains controversial, either from the Pacific Ocean during the late Cretaceous 90–100 million years ago (Brooks et al., 1981; Brooks, 1995) or from the northern coast of South America (i.e. Caribbean Atlantic) during the early Miocene 15–23 million years ago (Lovejoy, 1996; Lovejoy et al., 1998). Regardless of which scenario proves correct, it is apparent that the Potamotrygonidae have experienced as long a period for evolutionary adaptation to freshwater as many teleosts endemic to the same areas (Lovejoy et al., 1998). It is therefore possible that they have evolved ionoregulatory strategies similar to those of freshwater teleosts and different from those of their euryhaline cousins, stingrays of the family Dasyatidae. Indeed, it is known that the Potamotrygonidae are ammoniotelic rather than ureotelic (Gerst and Thorson, 1977; Barcellos et al., 1997) and regulate their blood salts, osmolality and urea at levels similar to those of teleosts (Thorson et al., 1967; Griffith et al., 1973; Gerst and Thorson, 1977; Mangum et al., 1978; Bittner and Lang, 1980) and at levels lower than those of freshwater-adapted Dasyatidae (Thorson, 1967; Shuttleworth, 1988; Piermarini and Evans, 1998). However, little is known about the details of the mechanisms involved.

Some species of the Potamotrygonidae are endemic to the most extreme freshwater environment of the continent, the acidic, ion-poor blackwaters of the Rio Negro and its tributaries, so called because of their high content of dissolved organic carbon (DOC) comprising humic, fulvic and other organic acids derived from the breakdown of jungle vegetation. Recently, Gonzalez and colleagues have begun to characterize ionoregulatory mechanisms in various teleost species endemic to these blackwaters (Gonzalez, 1996; Gonzalez et al., 1997, 1998, 2002; Gonzalez and Preest, 1999; Gonzalez and Wilson, 2001). Two basic strategies have emerged in the Rio Negro teleosts. In one strategy, as seen in many members of the Family Characidae, the presence of a high-affinity (i.e. low $K_m$), high-capacity $Na^+$ transport system, which is relatively insensitive to inhibition by low pH, compensates for high rates of diffusive loss. In the other, as seen in some members of the Family Cichlidae, the transporter is sensitive to inhibition by low pH, and affinity is much lower (i.e. higher $K_m$), although capacity may still be high. However, the key to adaptation in these species is that diffusive loss rates are much lower. In both strategies, diffusive loss rates are relatively resistant to stimulation by low pH, but show variable patterns of change in response to alterations in external $Na^+$ concentration.

With this background in mind, the objectives of the present study were several-fold. First, using radiotracers to measure unidirectional flux rates, we described the basic concentration-dependent kinetics of both $Na^+$ and $Cl^-$ uptake and efflux in a *Potamotrygon* species collected from and tested in its native blackwater. This point is important because Gonzalez et al. (2002) concluded that transport mechanisms of Rio Negro teleosts bred in captivity and/or held in other water qualities for long periods overseas by aquarists may differ from those of native fish in natural blackwater. Second, we examined whether these kinetic relationships changed when the freshwater rays were acclimated to ion-rich hard water. Third, we evaluated the responses of $Na^+$, $Cl^-$ and $Ca^{2+}$ transport and ammonia excretion to acute short-term exposure to low pH (pH 4.0) in natural blackwater. The only previous work on ammonia excretion in Rio Negro teleosts indicated that it generally increases at low pH and does not appear to be linked to $Na^+$ uptake (Wilson, 1996; Wilson et al., 1999). Lastly, we used a range of pharmacological inhibitors to characterize the nature of the $Na^+$, $Cl^-$ and ammonia transport systems in stingrays acclimated to and tested in blackwater. To date, little research has been done on $Cl^-$ or $Ca^{2+}$ transport or on the pharmacological characterisation of any of the transporters in Rio Negro fish.

## Materials and methods

### *Experimental animals*

Stingrays (70–770 g; mean 270±40 g, $N$=21) were collected in November–December 1999 by beach seine or throw-net from sandy, shallow regions of the Rio Negro and its tributaries between Barcelos and the Anavilhanas Archipelago. The systematics of *Potamotrygon* are controversial. The rays were morphologically uniform but heterogeneous in patterning. They were identified by a collector as *Potamotrygon thorsoni* but by local experts as *Potamotrygon* aff. *hystrix*, recognizing that *hystrix* may include up to three species (including *thorsoni*) and/or subspecies that hybridize and show considerable regional variability in color patterns (Brooks et al., 1981). We therefore refer to the fish as *Potamotrygon* sp. Since freshwater rays have protected status in Brazil, animal numbers were limited, and all procedures were designed to be non-lethal. Experiments were planned such that after exposure to any pharmacological treatment (always by addition of drugs to the water), animals were allowed 48 h for recovery prior to further pharmacological tests. In all cases, control measurements were made prior to tests to ensure that baseline conditions had been re-established.

After capture, most (16) of the rays were transported to the Laboratory of Ecophysiology and Molecular Evolution [Instituto Nacional de Pesquisas da Amazonia (INPA)], Manaus, Brazil, where they were held for 10 days in a recirculating, filtered tank (300 l) in which the INPA wellwater (which is very soft) had been supplemented with NaCl and limestone chips, a common procedure used to reduce the stress of captivity. This provided us with an opportunity to examine the kinetics of both $Na^+$ and $Cl^-$ uptake and efflux (see below) in this species after acclimation to ion-rich hard water (composition in Table 1). After completion of tests at INPA, the fish were transported back to the Anavilhanas Archipelago on board the research vessel *Amanai II*. The research vessel was moored in an embayment close to the Scientific Base of the Anavilhanas Archipelago of the Instituto Brasiliero do

Meio Ambiente e dos Recursos Naturais Renovaveis. During this time, the 300 l holding tank was continually flushed with fresh blackwater (which is very soft) from the Rio Negro (composition in Table 1), i.e. the original water quality from which the fish had been collected. The rays were held in this water for at least 5 days prior to further tests, and all other experiments were performed in this water quality on board the research vessel. During the 14-day period of research at Anavilhanas, an additional five rays were collected locally, from the wild and added to the pool of experimental animals. The fish were offered food (pellets, tubifex worms) but did not appear to eat in captivity. Holding and experimental temperatures were 28–31°C throughout.

### *Chemicals*

The radioisotopes $^{22}$Na (as NaCl), $^{36}$Cl (as HCl) and $^{45}$Ca (as $CaCl_2$) were manufactured by New England Nuclear (Dupont) and supplied by REM (Sao Paulo). Dimethylsulphoxide (DMSO), amiloride hydrochloride, 5-(*N*,*N*)-hexamethylene amiloride (HMA), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt (DIDS), 4-acetomido-4′-isothiocyanatostilbene-2,2′-disulfonic acid sodium salt (SITS), sodium thiocyanate, lithium heparin and tricaine methane sulfonate (MS-222) were all obtained from Sigma. Diphenylamine-2-carboxylic acid (DPC: *N*-phenylanthranilic acid) and phenamil methane sulphonate were obtained from RBI Pharmaceuticals.

### *General methods*

In all experiments, rays were placed in individual polyethylene containers and allowed to settle for at least 12 h prior to tests. The containers were fitted with airstones and lids and flushed with water from the holding reservoir or directly from the river at approximately 200 ml min$^{-1}$. Container volume was 2.6 l, except for the largest rays (450–770 g), for which 6 l chambers were used. 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.

In most experiments, unidirectional $Na^+$ and $Cl^-$ flux rates were determined simultaneously by adding both $^{22}$Na (a dual 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 gamma and scintillation counting (see below) was employed to monitor 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, approximately 1000× ambient concentrations, at the end of an experiment) demonstrated that there was no significant adsorption of either radioisotope to the walls of the container or the surface of the fish. Unidirectional influx rates were low in these fish, so flux periods of 2 h (or longer) were routinely employed. 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 total concentrations of water $Na^+$ (by flame photometry) and $Cl^-$ (by colorimetric assay). Influx rates ($J_{in}^X$, by convention positive) were calculated from the mean external specific activity and the disappearance of counts from the external water (factored by time, volume and fish mass), net flux rates ($J_{net}^X$) were calculated from the change in total $Na^+$ or $Cl^-$ concentration in the water (similarly factored) and unidirectional efflux rates ($J_{out}^X$, by convention negative) were calculated by difference, as outlined in detail by Wood (1988). This approach makes no assumptions about steady state and allows comparisons of different treatments over time. Experiments were designed such that internal specific activity, calculated from the measured accumulation of counts in the fish (i.e. cumulative disappearance 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'. In these same experiments, net ammonia flux rates were determined simultaneously by the appearance of total ammonia (measured colorimetrically) in the external water. Net urea-N excretion was similarly measured in a few experiments.

### *Measurements of the kinetics of $Na^+$ and $Cl^-$ uptake and efflux*

Kinetic relationships were determined by starting at the lowest water $Na^+$ and $Cl^-$ concentrations and then moving upwards in approximately twofold steps until approximately 2000 μmol l$^{-1}$ was reached. For stingrays acclimated to ion-rich hard water (Table 1), this was achieved by replacing the acclimation water immediately prior to the first test period with INPA wellwater ([$Na^+$]≈20 μmol l$^{-1}$, [$Cl^-$]≈20 μmol l$^{-1}$, [$Ca^{2+}$]≈10 μmol l$^{-1}$) supplemented 24 h previously with sufficient $CaCO_3$ to raise the measured [$Ca^{2+}$] to 912 μmol l$^{-1}$, approximately equal to that in the acclimation water. For stingrays acclimated to ion-poor Rio Negro blackwater (Table 1), the starting point was Rio Negro water. Radioisotopes (18.5 kBq of $^{22}$Na, 46.3 kBq of $^{36}$Cl or proportionately more for the largest rays) were added, allowed to mix for 10 min, and then an initial 15 ml water sample was taken, followed by a final 15 ml water sample after 2 h. Thereafter, sufficient quantities of NaCl, $^{22}$Na and $^{36}$Cl were added (as a single stock solution) to approximately double the concentrations of all in the external bath. The process was repeated until six flux determinations had been made, each at progressively twofold higher external $Na^+$ and $Cl^-$ concentrations and radioactivity. This procedure ensured that

Table 1. *Composition of ion-rich hard water and ion-poor Rio Negro water (soft water) to which the stingrays were acclimated*

| | [$Na^+$] (μmol l$^{-1}$) | [$Cl^-$] (μmol l$^{-1}$) | [$Ca^{2+}$] (μmol l$^{-1}$) | [DOC]* (mg C l$^{-1}$) | pH |
|---|---|---|---|---|---|
| Hard water | 2012 | 2192 | 897 | 2.60 | 6.5 |
| Rio Negro water | 30 | 23 | 10 | 8.35 | 5.9-6.1 |

*Dissolved organic carbon.

external specific activity remained approximately constant throughout the kinetics experiment. To achieve comparable resolution at these 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, 7 h and finally 10 h. As both $Na^+$ and $Cl^-$ uptake clearly exhibited saturation kinetics, the $J_{in}^{Na}$ and $J_{in}^{Cl}$ data for each individual were fitted separately to a Michaelis–Menten model by Eadie–Hofstee regression (Michal, 1985) to yield individual estimates of $K_m$ (affinity constant) and $J_{max}$ (maximum transport rate). Grand means ± S.E.M. were then calculated for all fish in a treatment group.

### *Measurement of $Ca^{2+}$ flux rates*

We found that the methodology used for $Na^+$ and $Cl^-$ flux determinations would not work for measuring unidirectional $Ca^{2+}$ fluxes, because $Ca^{2+}$ influx rates were lower and there was a significant adsorption of $^{45}Ca$ to the body surface of the stingrays (in all experiments) and to the walls of the container (in some experiments). Therefore, a longer-term measurement with correction for the latter was developed. The method assumed steady-state conditions and therefore allowed only a single measurement of $J_{in}^{Ca}$ under a single treatment condition. However, the method also provided an estimate of the amount of $Ca^{2+}$ bound to the surface of the fish. At the start of the experiment, 148 kBq of $^{45}Ca$ was added to the 2.0 l of water in each container, allowed to mix for 10 min, and 15 ml water samples (for measurement of $^{45}Ca$ radioactivity and total $Ca^{2+}$ concentration) were then taken at 0 h, 3 h, 6 h and 9 h. The slope of the regression line of the natural logarithm of total water radioactivity against time yielded the rate constant ($k$) for turnover, and the product of $k$ and the mean external total $Ca^{2+}$ pool, factored by mass, yielded an uncorrected estimate of $J_{in}^{Ca}$ (see Kirschner, 1970).

After the 9 h sample, the ray was gently transferred to new container containing 1.0 l of fresh Rio Negro water. A water sample was taken, and then 10 ml of 1 mol $l^{-1}$ $Ca(NO_3)_2$ was added to raise the ambient $Ca^{2+}$ concentration to 10 mmol $l^{-1}$ (approximately 1000× ambient concentration) for 'cold displacement'. A second water sample was taken, and the increase in $^{45}Ca$ radioactivity, factored by volume, mean specific activity of the original water and mass, was taken as the amount bound to the surface of the fish ($B$). Similarly, the water remaining in the original container (approximately 2.0 l) was spiked with 20 ml of 1 mol $l^{-1}$ $Ca(NO_3)_2$, and any increase in $^{45}Ca$ radioactivity that occurred, factored by volume and mean specific activity of the original water, was taken as the amount bound ($C$) to the container's surface. Corrected $J_{in}^{Ca}$ was then calculated as:

$$\text{corrected } J_{in}^{Ca} = \frac{(\text{uncorrected } J_{in}^{Ca} \times m \times 9) - (B \times m) - C}{m \times 9},$$

where $m$ is mass and 9 refers to 9 h sample. $J_{net}^{Ca}$ and $J_{out}^{Ca}$ were then calculated as for $Na^+$ and $Cl^-$ fluxes. The approach therefore assumes that $^{45}Ca$ simply equilibrates with pre-existing 'cold' $Ca^{2+}$ on surface sites and that these sites do not contribute to net fluxes.

### *Responses to low pH*

The responses to low pH in Rio Negro water were assessed by measuring unidirectional and net $Na^+$ and $Cl^-$ flux rates and net ammonia flux rates during a 2 h control period, followed by a 2 h low-pH period and a 2 h recovery period, using each fish as its own control. Water samples (15 ml) were taken at the beginning and end of each period. At 10 min prior to the start of the control period, 37 kBq of $^{22}Na$ and 92.5 kBq of $^{36}Cl$ 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 the pH to approximately 4.0 with 0.5 mol $l^{-1}$ $HNO_3$ and then raise it back to approximately 6.1 with 0.5 mol $l^{-1}$ KOH for recovery. In practice, because pH tended to rise during the low-pH period as a result of ammonia production by the fish, pH was monitored in each container every 30 min and was held between 3.8 and 4.2 by addition of more $HNO_3$. During the control and recovery periods, pH was held between 5.9 and 6.3.

To assess the effects of low pH on unidirectional $Ca^{2+}$ fluxes, two separate experiments were performed using the $Ca^{2+}$ methodology outlined above. In the first 9 h experiment (control), pH was held at approximately 6.1. In the second experiment, water pH was lowered to approximately 4.0 approximately 30 min before the start of the experiment and held at this level for the ensuing 9 h by monitoring at 30 min intervals.

### *Responses to pharmacological treatments*

We tested the effects of various pharmacological agents on the unidirectional and net flux rates of $Na^+$ and/or $Cl^-$ and the net flux rates of ammonia in Rio Negro water. The basic protocol was a 2 h control period followed by three successive 2 h experimental periods in the presence of the drug, using each fish as its own control. Water samples (15 ml) were taken at the beginning and end of each period. At 10 min prior to the start of the control period, 37 kBq of $^{22}Na$ and 92.5 kBq of $^{36}Cl$ were added to the external water (or proportionately more if the volume was greater than 2.0 l). In tests with amiloride ($10^{-4}$ mol $l^{-1}$), DIDS ($2\times10^{-5}$ mol $l^{-1}$), phenamil ($4\times10^{-5}$ mol $l^{-1}$) and HMA ($4\times10^{-5}$ mol $l^{-1}$), both $Na^+$ and $Cl^-$ exchanges were evaluated. In tests with DIDS ($10^{-4}$ mol $l^{-1}$), SITS ($10^{-4}$ mol $l^{-1}$), DPC ($10^{-4}$ mol $l^{-1}$) and thiocyanate ($10^{-4}$ mol $l^{-1}$), only $Cl^-$ exchanges were evaluated. In all cases, drugs were initially dissolved in DMSO, yielding a final concentration of 0.1% DMSO in the experimental water. The same concentration of DMSO was therefore used during the control periods.

### *Blood sampling*

At the end of the 14-day period on the *Amanai II*, stingrays acclimated to Rio Negro water were lightly anaesthetized in MS-222. Approximately 1 ml of blood was withdrawn non-terminally from either the caudal arch or the heart using a 23-gauge needle and syringe heparinized with 50 µl of 5000 i.u. $ml^{-1}$ lithium heparin. Haematocrit and plasma protein (by refractometry) were measured immediately after

centrifugation of blood in microcapillary tubes at 500 ***g*** for 10 min. The remainder of the plasma was separated by centrifugation at 10 000 ***g***, portioned, and frozen in liquid nitrogen for later analysis of $Na^+$, $Cl^-$, glucose, urea and total ammonia concentrations and osmolality.

### *Analytical methods*

$^{22}Na$ radioactivity was determined by manually counting 2 ml water samples in a Picker Cliniscaler gamma counter fitted with a 10 cm NaI crystal with a well. $^{22}Na$ plus $^{36}Cl$ radioactivity was determined by mixing 3.0 ml water samples with 7.5 ml of Ecolite fluor and manually counting on a Triathler portable scintillation counter. $^{36}Cl$ radioactivity was obtained by subtraction after correcting for differences in efficiency of $^{22}Na$ counting between the two instruments, determined by counting the same standards on both (Wood, 1988). $^{45}Ca$ radioactivity was determined directly by scintillation counting. Water and plasma total $Na^+$ and water total $Ca^{2+}$ concentrations were determined by flame photometry (CELM flame photometer) or atomic absorption spectrophotometry (Perkin Elmer 1100B), water DOC by a Rosemount total carbon analyzer, and water pH 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 concentrations by the salicylate hypochlorite method of Verdouw et al. (1978). In both assays, standards were made up with the same concentrations of DMSO and the appropriate drug, as in the experimental medium. In addition, we ran blanks to correct for the absorbance due to the natural color of blackwater, and confirmed that the slopes of the assays were not altered by this color. Water and plasma urea concentrations were determined by the diacetyl monoxime method of Rahmatullah and Boyde (1980). Plasma $Cl^-$ concentration was determined by coulometric titration (Radiometer CMT10), plasma osmolality by vapour pressure osmometry (Wescor 5100C), plasma protein concentration by refractometry (American Optical TS meter; Alexander and Ingram, 1980), plasma total ammonia concentration by the L-glutamate dehydrogenase method (Mondzac et al., 1965; Sigma kit 171-UV) and plasma glucose concentration by the hexokinase method (Bergmeyer, 1985; Sigma kit 17).

### *Statistical analyses*

All data are reported as means ± 1 S.E.M. (*N*). Relationships were assessed by analysis of variance (ANOVA), and individual means were compared using Student's paired or unpaired two-tailed *t*-tests, as appropriate, with the Bonferroni correction for multiple comparisons (Nemenyi et al., 1977). A significance level of $P \leq 0.05$ was used throughout.

## Results

### *Blood chemistry and nitrogen metabolism*

Concentrations for most measured plasma components (ions, glucose, urea and total ammonia and osmolality; Table 2) in these freshwater elasmobranchs held in Rio Negro blackwater were typical of those for freshwater teleosts, although plasma protein concentration was notably low. Nitrogen metabolism was very characteristic of the teleost pattern, with plasma urea concentration being very low relative to other elasmobranchs but approximately fourfold higher than plasma total ammonia concentration (eightfold higher on a per unit N basis). Urea-N excretion comprised only approximately 9% (58±10 μmol $kg^{-1}$ $h^{-1}$) and ammonia-N excretion approximately 91% (607±29 μmol $kg^{-1}$ $h^{-1}$; $N$=6) of the total N excretion.

Table 2. *Blood chemistry (plasma concentrations and haematocrit) of stingrays acclimated to ion-poor Rio Negro water (soft water)*

| | |
|---|---|
| [$Na^+$] (mmol $l^{-1}$) | 178.2±4.8 (11) |
| [$Cl^-$] (mmol $l^{-1}$) | 146.2±2.1 (11) |
| Osmolality (mosmol $kg^{-1}$) | 319.6±8.5 (11) |
| [Glucose] (mmol $l^{-1}$) | 2.36±0.28 (11) |
| [Urea] (μmol $l^{-1}$) | 1221±185 (11) |
| [Total ammonia] (μmol $l^{-1}$) | 306±61 (7) |
| [Plasma protein] (g 100 $ml^{-1}$) | 1.69±0.09 (10) |
| Haematocrit (%) | 15.5±1.4 (10) |

Values are means ± 1 S.E.M. (*N*).

### *Kinetics of $Na^+$ and $Cl^-$ uptake and efflux*

Both $J_{in}^{Na}$ and $J_{in}^{Cl}$ exhibited clear saturation kinetics as external $Na^+$ and $Cl^-$ concentrations were increased (Fig. 1). There were no differences in the kinetic variables ($K_m$, $J_{max}$) for these relationships between animals acclimated to their native ion-poor blackwater of the Rio Negro or to ion-rich hard water (Table 3), so single lines have been fitted to the data of Fig. 1. The relationships for $Cl^-$ were significantly different from those for $Na^+$, with 1.7- to twofold higher $J_{max}$ values but virtually identical $K_m$ values (Table 3). As an additional check that the relationships for Rio Negro water had not changed as a result of the animals' holding and manipulation in the

Table 3. *Kinetic variables for $Na^+$ and $Cl^-$ influx in stingrays acclimated to either ion-poor Rio Negro water (soft water) or ion-rich hard water*

| | Ion-poor Rio Negro water ($N$=7) | Ion-rich hard water ($N$=6) |
|---|---|---|
| $K_m^{Na}$ (μmol $l^{-1}$) | 468±100 | 331±86 |
| $J_{max}^{Na}$ (μmol $kg^{-1}$ $h^{-1}$) | 443±67 | 523±75 |
| $K_m^{Cl}$ (μmol $l^{-1}$) | 472±55 | 385±58 |
| $J_{max}^{Cl}$ (μmol $kg^{-1}$ $h^{-1}$) | 773±93† | 982±148† |

Values are means ± 1 S.E.M.

†Significantly different ($P \leq 0.05$) from corresponding value for $Na^+$.

There were no significant differences ($P$>0.05) in corresponding variables between the two acclimation treatments.

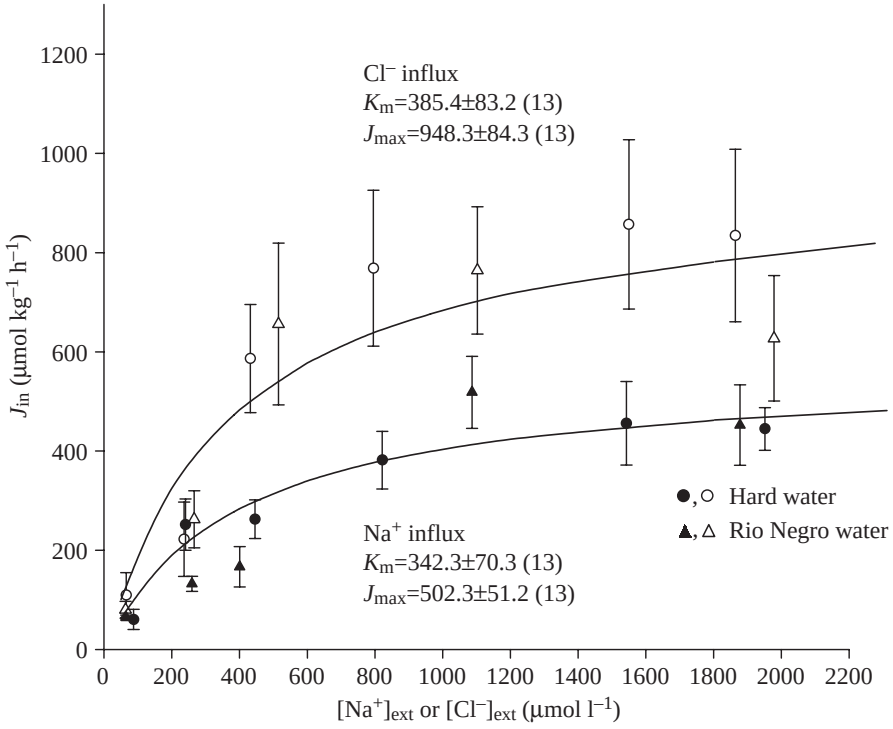


Fig. 1. Kinetic relationships for $Na^+$ (filled symbols) and $Cl^-$ (open symbols) influx rates in stingrays acclimated to either ion-poor Rio Negro water (soft water, triangles, $N$=7) or ion-rich hard water (circles, $N$=6). Values are means ± 1 S.E.M. There were no significant effects of acclimation water on the relationships. The lines shown represent the Michaelis–Menten equations derived from the mean values of $K_m$ (in μmol $l^{-1}$) and $J_{max}$ (in μmol $kg^{-1}$ $h^{-1}$) for all fish in both treatments ($N$=13) as shown.

laboratory, uptake kinetics of $Na^+$ and $Cl^-$ were also measured in four stingrays freshly collected from the Rio Negro. These yielded values of $K_m$=563±86 μmol $l^{-1}$ and $J_{max}$=400±73 μmol $kg^{-1}$ $h^{-1}$ for $Na^+$ and $K_m$=569±98 μmol $l^{-1}$ and $J_{max}$=487±160 μmol $kg^{-1}$ $h^{-1}$ for $Cl^-$, not significantly different from the corresponding values (Table 1) for fish acclimated to Rio Negro water in the laboratory. The slightly lower $J_{max}$ values in the freshly collected animals probably reflected their 2.5-fold greater mean body mass, and we therefore elected not to pool these data in the kinetic relationships.

To evaluate whether there was any effect of acclimation water chemistry on ammonia excretion or any direct coupling of ammonia excretion to $J_{in}^{Na}$, the net ammonia flux was measured at the lowest and highest external $Na^+$ concentrations used in establishing the kinetic relationships (Table 4). There were no differences in ammonia excretion associated with acclimation water chemistry, and no differences in ammonia excretion between the lowest and highest water $Na^+$ levels, despite approximately sevenfold differences in $J_{in}^{Na}$ at the two concentrations. The four freshly collected Rio Negro fish exhibited the same patterns (data not shown).

$J_{out}^{Na}$ did not vary significantly with external $Na^+$ concentration in either acclimation condition and was generally lower than $J_{in}^{Na}$, so that $J_{net}^{Na}$ was positive over most of the range tested (Fig. 2). In the lower range of external $Na^+$ levels, $J_{out}^{Na}$ was lower in the stingrays acclimated to Rio Negro water, with a significant difference at around 400 μmol $l^{-1}$ (Fig. 2). While the absolute differences appear small, the net effect was to shift the balance point (marked by arrows in Fig. 2), where $J_{in}^{Na}=J_{out}^{Na}$ (and hence $J_{net}^{Na}$ is zero), from 220 μmol $l^{-1}$ in ion-rich hard water to 75 μmol $l^{-1}$ in ion-poor Rio Negro water.

$J_{out}^{Cl}$ values were generally greater than $J_{out}^{Na}$ values at comparable external ion concentrations. In contrast to $J_{out}^{Na}$, $J_{out}^{Cl}$ varied significantly with external $Cl^-$ concentration under both acclimation conditions, in a manner similar to the influx relationship (Fig. 3). Over most of the range of external $Cl^-$ levels, there were no differences between the two treatments, but the relationships diverged around 300 μmol $l^{-1}$, with a significantly lower value of $J_{out}^{Cl}$ in the fish acclimated to Rio Negro water. As a result, the $J_{in}^{Cl}=J_{out}^{Cl}$ balance point

Table 4. *Ammonia excretion rates ($J_{Amm}$) and associated rates of $Na^+$ influx ($J_{in}^{Na}$) in stingrays acclimated to either ion-poor Rio Negro water (soft water) or ion-rich hard water*

| | Lowest $[Na^+]_{ext}$ | | Highest $[Na^+]_{ext}$ | |
|---|---|---|---|---|
| | $J_{in}^{Na}$ (μmol $kg^{-1}$ $h^{-1}$) | $J_{Amm}$ (μmol $kg^{-1}$ $h^{-1}$) | $J_{in}^{Na}$ (μmol $kg^{-1}$ $h^{-1}$) | $J_{Amm}$ (μmol $kg^{-1}$ $h^{-1}$) |
| Rio Negro water ($N$=7) | 65±12 | 507±80 | 409±66* | 452±81 |
| Hard water ($N$=6) | 61±27 | 635±44 | 444±43* | 579±58 |

Values were determined at the lowest and highest external $Na^+$ concentrations used in establishing the kinetic relationships of Fig. 1.
Values are means ± 1 S.E.M.
*Significantly different ($P \leq 0.05$) from the corresponding variable at the lowest $[Na^+]_{ext}$.
There were no significant differences ($P>0.05$) in corresponding variables between the two acclimation treatments.

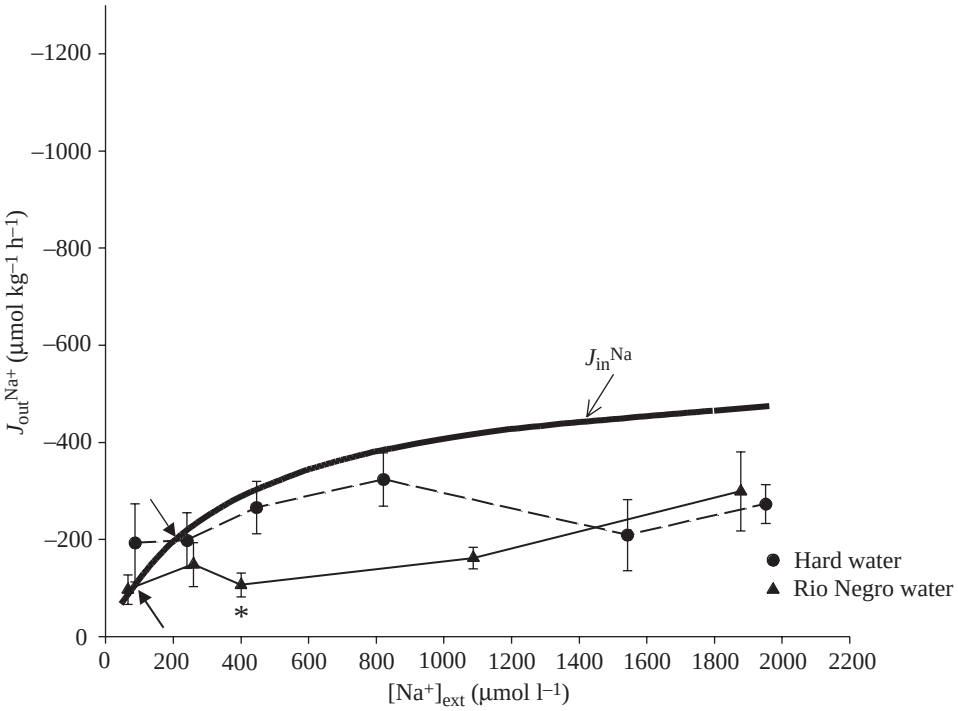


Fig. 2. The relationships between $Na^+$ efflux ($J_{out}^{Na}$) and external $Na^+$ concentration ($[Na^+]_{ext}$) in stingrays acclimated to either ion-poor Rio Negro water (soft water, triangles and solid line, $N$=7) or ion-rich hard water (circles and dashed line, $N$=6). Values are means ± 1 S.E.M. By ANOVA, the relationships (considering only the four lowest $Na^+$ concentrations) are significantly different, with a specific significant difference (*$P$≤0.05) around 400 μmol l$^{-1}$. The thicker line shows the uptake kinetic relationship for $J_{in}^{Na}$ from Fig. 1, and the arrows mark the intersection points of balance where $J_{in}^{Na}$=$J_{out}^{Na}$. Axes are drawn to the same scale as Fig. 3 to facilitate comparison.

(marked by arrows in Fig. 3) shifted from 460 μmol l$^{-1}$ in ion-rich hard water to 65 μmol l$^{-1}$ in ion-poor Rio Negro water.

All other experiments were performed with stingrays held in Rio Negro water. Under the conditions of our experiments, blackwater $Na^+$ and $Cl^-$ concentrations were generally around 20–40 μmol l$^{-1}$ (Table 1; i.e. slightly below the balance points), so the animals were in slight negative balance for both $Na^+$ and $Cl^-$ during control treatments (Figs 4–7). $J_{in}^{Na}$ and $J_{in}^{Cl}$ were low, approximately 30–80 μmol kg$^{-1}$ h$^{-1}$ (generally higher for $J_{in}^{Cl}$ than for $J_{in}^{Na}$), while efflux rates were approximately twice these values. DMSO (0.1%) had no effect on any of the flux rates measured.

### *Responses to low pH*

Acute exposure to pH 4.0 induced substantial reductions (approximately 80%) in both $J_{in}^{Na}$ and $J_{in}^{Cl}$ (Fig. 4A). However, there was no increase in net ion loss rates because efflux rates were slightly reduced (Fig. 4A). At the same time, net ammonia excretion was increased by approximately 70% (Fig. 4B). When the water pH was returned to 6.1 after 2 h of exposure to low pH, the recovery of $J_{in}^{Na}$ was only partial, and $J_{out}^{Na}$ was now significantly lower (i.e. $Na^+$ turnover was reduced). In contrast, $Cl^-$ fluxes returned to

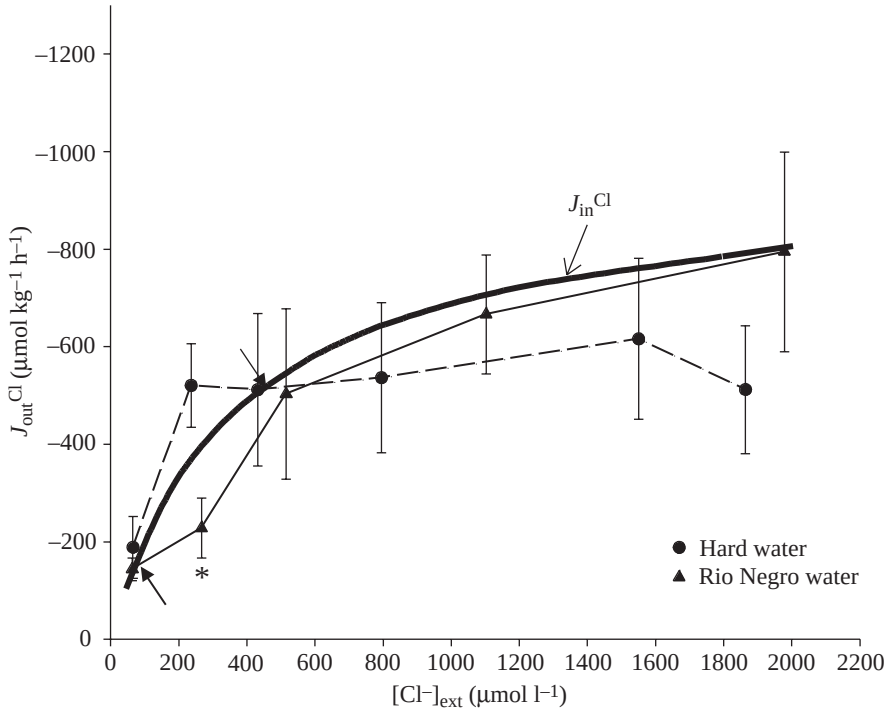


Fig. 3. The relationships between $Cl^-$ efflux ($J_{out}^{Cl}$) and external $Cl^-$ concentration ($[Cl^-]_{ext}$) in stingrays acclimated to either ion-poor Rio Negro water (soft water, triangles and solid line, $N$=7) or ion-rich hard water (circles and dashed line, $N$=6). Values are means ± 1 S.E.M. There was a specific significant difference between the two relationships (*$P$≤0.05) only around 300 μmol l$^{-1}$. The thicker line shows the uptake kinetic relationship for $J_{in}^{Cl}$ from Fig. 1, and the arrows mark the intersection points of balance where $J_{in}^{Cl}$=$J_{out}^{Cl}$.

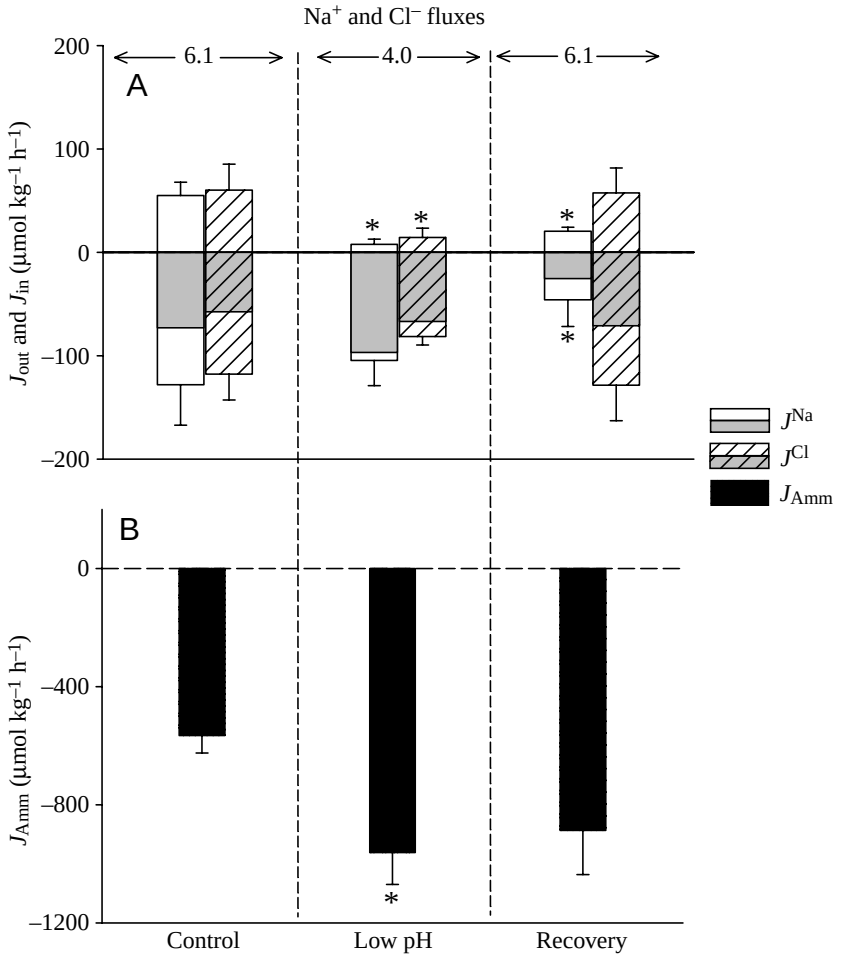


Fig. 4. The influence, in stingrays acclimated to ion-poor Rio Negro water, of acute exposure to pH 4.0 for 2 h, followed by recovery at control pH 6.1 for 2 h, on (A) unidirectional ($J_{in}$, $J_{out}$) and net flux rates of $Na^+$ (open bars) and $Cl^-$ (hatched bars) and (B) net ammonia flux rates ($J_{amm}$; filled bars). Values are means ± 1 S.E.M. ($N$=5). Standard errors have been omitted from net $Na^+$ and $Cl^-$ net flux rates (shaded bars) for clarity as they sometimes overlap those of the unidirectional flux rates. Positive (upward) values represent uptake into the fish; negative (downward) bars represent losses from the fish. Asterisks indicate means significantly different (*$P$≤0.05) from the corresponding control mean.

normal levels, and ammonia flux was no longer significantly elevated.

At pH 6.1, $J_{in}^{Ca}$ was only approximately 19 μmol kg$^{-1}$ h$^{-1}$ (Table 5) under control conditions, lower than $J_{in}^{Na}$ or $J_{in}^{Cl}$. $J_{out}^{Ca}$ was approximately 3.5 times higher than $J_{in}^{Ca}$, so $J_{net}^{Ca}$ was negative. Exposure to pH 4.0 completely abolished $J_{in}^{Ca}$, while $J_{out}^{Ca}$ and $J_{net}^{Ca}$ did not change significantly. Interestingly, the amount of $Ca^{2+}$ bound to the surface of the fish (6–7 μmol kg$^{-1}$) was not significantly altered by exposure to low pH (Table 5).

*Responses to pharmacological treatments*

Exposure to the cationic transport inhibitor amiloride ($10^{-4}$ mol l$^{-1}$) caused a 70% inhibition of $J_{in}^{Na}$, an effect that was stable throughout all three 2 h periods of the experimental treatment (Fig. 5A). There was no significant effect on $J_{out}^{Na}$ or $J_{net}^{Na}$, or on the unidirectional or net flux rates of $Cl^-$. The non-significant tendency for a large stimulation of $J_{in}^{Cl}$ and $J_{out}^{Cl}$ probably reflected the fact that the drug was presented as the hydrochloride salt, which therefore raised the water $Cl^-$ concentration by 100 μmol l$^{-1}$ to around 130 μmol l$^{-1}$. In this range, both $J_{in}^{Cl}$ (Fig. 1) and $J_{out}^{Cl}$ (Fig. 3) are very sensitive to small changes in external water $Cl^-$ concentration, and the increases were approximately equal to those expected from the kinetic relationships.

Exposure to the anionic transport inhibitor DIDS ($2\times10^{-5}$ mol l$^{-1}$) had no effect on $J_{in}^{Cl}$, $J_{out}^{Cl}$ or $J_{net}^{Cl}$ (Fig. 5B). Unidirectional and net flux rates of $Na^+$ were also unaffected.

To pursue the amiloride effect, two analogues (HMA, phenamil) of reputedly greater specificity were tested at a lower concentration ($4\times10^{-5}$ mol l$^{-1}$). Responses to the two drugs were very similar, although HMA appeared to be slightly more potent. Exposure to HMA ($4\times10^{-5}$ mol l$^{-1}$) induced a 90% inhibition of $J_{in}^{Na}$, which persisted throughout all three 2 h periods of the treatment (Fig. 6A). Phenamil ($4\times10^{-5}$ mol l$^{-1}$) caused a 70% inhibition of $J_{in}^{Na}$, which was significant during the first two experimental periods only (Fig. 6B). During the first and third periods of drug exposure, $J_{in}^{Na}$ was significantly lower in the presence of HMA than in the presence of phenamil. Both drugs caused significant increases in $J_{out}^{Na}$ and more negative values of $J_{net}^{Na}$ throughout the exposures, whereas there were no significant effects on unidirectional or net flux rates of $Cl^-$, apart from a decrease in $J_{in}^{Cl}$ at 2–4 h in the presence of HMA.

To pursue the surprising lack of response of $Cl^-$ transport to DIDS, both this drug and a related stilbene derivative (SITS)

Table 5. *The influence of exposure to low pH on unidirectional and net $Ca^{2+}$ flux rates and surface-bound [$Ca^{2+}$] in stingrays acclimated to ion-poor Rio Negro water (softwater)*

| | Control pH 6.1 ($N$=6) | Low pH 4.0 ($N$=7) |
|---|---|---|
| $J_{in}^{Ca}$ (μmol kg$^{-1}$ h$^{-1}$) | 19.0±1.2 | 0.5±1.6* |
| $J_{out}^{Ca}$ (μmol kg$^{-1}$ h$^{-1}$) | –68.0±29.2 | –89.9±21.7 |
| $J_{net}^{Ca}$ (μmol kg$^{-1}$ h$^{-1}$) | –49.0±28.0 | –89.4±20.2 |
| Surface-bound [$Ca^{2+}$] (μmol kg$^{-1}$) | 6.0±1.9 | 7.3±1.1 |

Values are means ± 1 S.E.M.
*Significantly different ($P$≤0.05) from corresponding value at control pH 6.1.

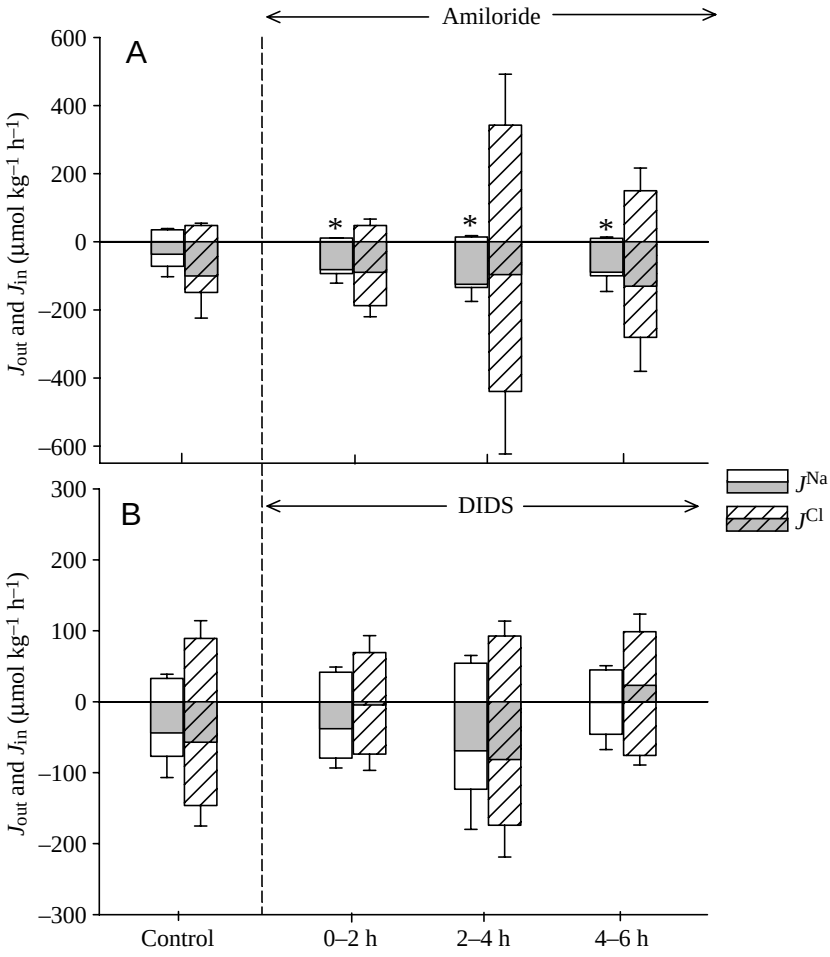


Fig. 5. The influence, in stingrays acclimated to ion-poor Rio Negro water, of (A) amiloride ($10^{-4}$ mol l$^{-1}$) and (B) DIDS ($2\times10^{-5}$ mol l$^{-1}$) on unidirectional ($J_{in}$, $J_{out}$) and net flux rates of $Na^+$ (open bars) and $Cl^-$ (hatched bars) during three successive 2 h periods. Values are means ± 1 S.E.M. ($N$=5). Other details as in the legend of Fig. 4.

were tested at a higher concentration ($10^{-4}$ mol l$^{-1}$). In addition, two other anionic transport blockers (DPC, thiocyanate) were evaluated at this same concentration. To increase the sensitivity for $Cl^-$ uptake measurement, only $^{36}Cl$ was used in these experiments, so that $^{36}Cl$ counts could be obtained by direct scintillation counting, avoiding the need for differential gamma and scintillation detection to eliminate $^{22}Na$ counts. Furthermore, DIDS, SITS and thiocyanate were all presented as $Na^+$ salts, thereby raising water $Na^+$ concentration significantly, which would have complicated interpretation of $Na^+$ flux rates.

Even at $10^{-4}$ mol l$^{-1}$, both DIDS (Fig. 7A) and SITS (Fig. 7B) were without significant effect on $J_{in}^{Cl}$, $J_{out}^{Cl}$ or $J_{net}^{Cl}$. However, both DPC (Fig. 7C) and thiocyanate (Fig. 7D) strongly inhibited $J_{in}^{Cl}$, reducing it to almost zero by the third 2 h period. $J_{out}^{Cl}$ was also strongly reduced during exposure to DPC and thiocyanate (significant in the second and third periods), so that $Cl^-$ turnover was greatly reduced, and therefore $J_{net}^{Cl}$ became only slightly more negative.

Exposure to amiloride (at $10^{-4}$ mol l$^{-1}$) and its analogues (HMA and phenamil at $4\times10^{-5}$ mol l$^{-1}$) exerted similar effects on net ammonia excretion, inhibiting it by approximately 50% during the first 2 h of treatment, with recovery in subsequent periods. This response was significant for amiloride (Fig. 8A) and HMA (Fig. 8B), but just below the level of significance ($P\leq0.10$) for phenamil (Fig. 8C). None of the anionic transport blockers tested had any effect on net ammonia excretion (data not shown).

## Discussion

### *Strategies of adaptation*

Here, we confirm earlier reports of typical 'teleostean' blood chemistry (low $Na^+$, $Cl^-$ and urea levels, Table 2; Thorson et al., 1967; Griffith et al., 1973; Gerst and Thorson, 1977; Mangum et al., 1978; Bittner and Lang, 1980) in potamotrygonid stingrays, very different from that of dasyatid stingrays (Piermarini and Evans, 1998) and other euryhaline elasmobranchs in freshwater (Shuttleworth, 1988), which have much higher levels of these plasma constituents. More importantly, we provide the first data on ion transport kinetics in the only stenohaline freshwater elasmobranch. To our knowledge, the only previously published information on ion transport in the family Potamotrygonidae appeared in a review article by Pang et al. (1977). These authors stated that, on the basis of preliminary experiments, 'the influx rate of $Na^+$ was rather low', the stingrays 'are capable of picking up $Na^+$ from surrounding water against a concentration gradient' and that 'the uptake system is saturable'. However, no data were reported. The present results demonstrate that this preliminary report was correct and extend the same conclusions about low rates of uptake occurring against large gradients with typical saturation kinetics to $Cl^-$ transport (Fig. 1). Indeed, this is the first time that $Cl^-$ transport has been characterized in any Amazonian blackwater fish.

At least superficially, the freshwater stingrays appear to be physiologically similar to some of the freshwater teleosts that are also endemic to the dilute blackwaters of the Amazon basin. These teleosts may or may not have shared the same route of invasion (Brooks, 1995; Lovejoy et al., 1998). Gonzalez et al. (2002) recently summarized kinetic variables and acid sensitivity for $Na^+$ transport in a number of teleost species collected from the Rio Negro. The data divided into two strategies for adaptation to acidic, ion-poor water: (i) high-capacity, high-affinity (i.e. low $K_m$) $Na^+$ transport systems relatively resistant to inhibition by low pH, but linked with high diffusive loss rates; and (ii) low-affinity (i.e. high $K_m$)

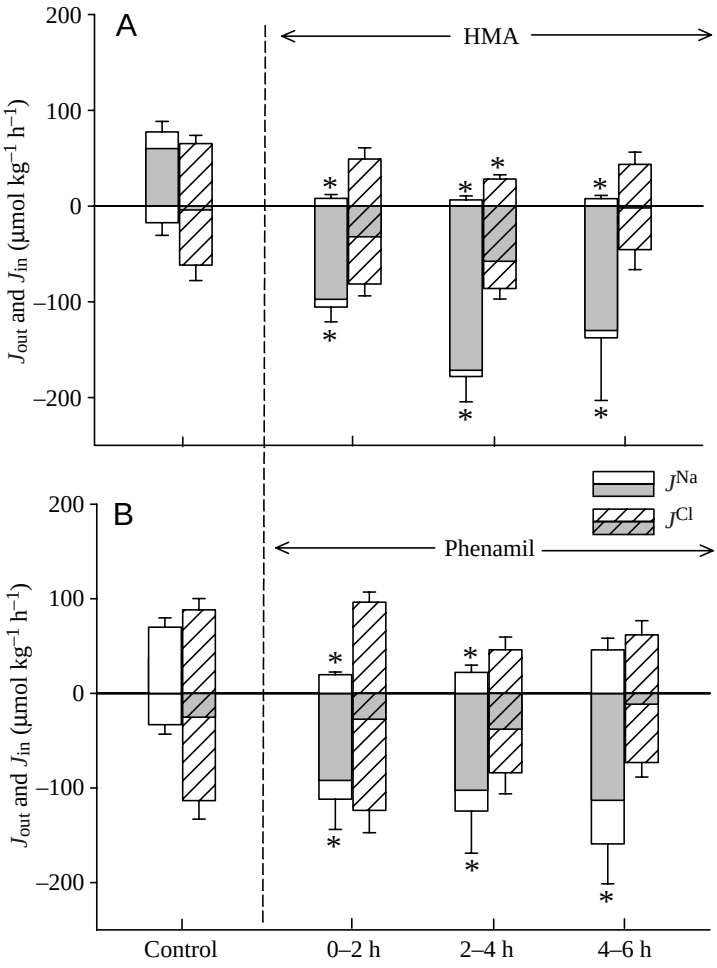


Fig. 6. The influence, in stingrays acclimated to ion-poor Rio Negro water, of (A) HMA ($4\times10^{-5}$ mol l$^{-1}$) and (B) phenamil ($4\times10^{-5}$ mol l$^{-1}$) on unidirectional ($J_{in}$, $J_{out}$) and net flux rates of $Na^+$ (open bars) and $Cl^-$ (hatched bars) during three successive 2 h periods. Values are means ± 1 S.E.M. ($N$=5). Other details as in the legend of Fig. 4.

transport systems relatively sensitive to inhibition by low pH, but complemented by low diffusive loss rates. The freshwater rays represent an extreme version of the second strategy, with $K_m$ values for $Na^+$ (and $Cl^-$) of approximately 400–500 μmol l$^{-1}$ (Fig. 1; Table 3), at least double that of any of the teleosts surveyed, $J_{max}$ values at the lower end of the range for the teleosts surveyed, normal influx and efflux rates of less than 200 μmol kg$^{-1}$ h$^{-1}$ and an 80% inhibition of influx but no increase in efflux or net loss rate upon challenge with pH 4.0 (Fig. 4). As pointed out by Gonzalez et al. (2002), this strategy, typical of Amazonian cichlids, is designed to limit net ion loss and thereby minimize physiological disturbance so as to 'wait out' exposures to especially acidic and dilute challenges.

Most of the teleosts surveyed by Gonzalez et al. (2002) exhibited $J_{in}^{Na}$=$J_{out}^{Na}$ balance points below 43 μmol l$^{-1}$, whereas the $Na^+$ and $Cl^-$ balance points in the stingrays were higher, 65–75 μmol l$^{-1}$ (Figs 2, 3), approximately twice the normal levels in the blackwater in which they were living (Table 1). Thus, the rays were in negative balance in their native water, but they were not feeding in our studies. We suspect the explanation is that these animals normally obtain significant supplementary ions from the diet, as documented for some teleosts (e.g. Smith et al., 1989). The importance of food-derived salts in successful adaptation to low environmental pH has recently been highlighted for salmonids (D'Cruz and Wood, 1998). A second explanation may be that the stingrays normally live at the bottom of the water column, just above the benthos; ion concentrations in the water column may be slightly higher here through remobilization from the sediments.

A surprising result was the absence of any shift in the $Na^+$ or $Cl^-$ influx kinetic curves between acclimation to ion-rich hard water and ion-poor Rio Negro water (Fig. 1; Table 3). The 'normal' pattern, documented in many freshwater teleosts and crustaceans (e.g. Shaw, 1959; Maetz, 1974; McDonald and Rogano, 1986; Perry and Laurent, 1989; Potts, 1994), is for a higher affinity (lower $K_m$) and/or greater $J_{max}$ upon acclimation to ion-poor water. The explanation may be that modification of ion uptake systems originally designed for function in seawater (see below) has been pushed to a maximum during the evolutionary invasion of freshwater, and there is no capacity in these elasmobranchs for further modification to differentiate between normal freshwater and very dilute freshwater. In this regard, examination of teleost families with a similar distribution to the Potamotrygonidae, and which today have both marine and blackwater representatives (e.g. Scianidae, Engraulidae, Clupeidae, Belonidae, Soleidae; Gery, 1969; Chao, 1978; Fink and Fink, 1979), may be instructive.

On the other hand, upon acclimation to ion-poor blackwater, there were subtle but significant reductions in both $J_{out}^{Na}$ and $J_{out}^{Cl}$, which substantially shifted the balance points to much lower external $Na^+$ and $Cl^-$ concentrations (Figs 2, 3). Efflux rates may consist of two components in these fish (see below), but it appears likely that an attenuation of the simple diffusive component *via* epithelial tightening was responsible for the overall reductions. This is a classic response to softwater acclimation (e.g. McDonald and Rogano, 1986). Possibly, DOC may also play a role. Since dissolved organic matter is now known to bind to fish gills, especially at low pH (Campbell et al., 1997), Gonzalez et al. (1998, 2002) have speculated that the high DOC content of ion-poor blackwaters (Table 1) may also be important in limiting gill permeability.

### *Mechanisms of $Ca^{2+}$ transport and binding*

To our knowledge, there are no previous measurements of unidirectional $Ca^{2+}$ fluxes (Table 5) in any elasmobranch, marine or freshwater, or in any Amazonian blackwater teleosts. In other freshwater teleosts, in which transport rates are similar, $Ca^{2+}$ uptake is active and is mediated by apical $Ca^{2+}$ channels and basolateral high-affinity $Ca^{2+}$-ATPase and/or

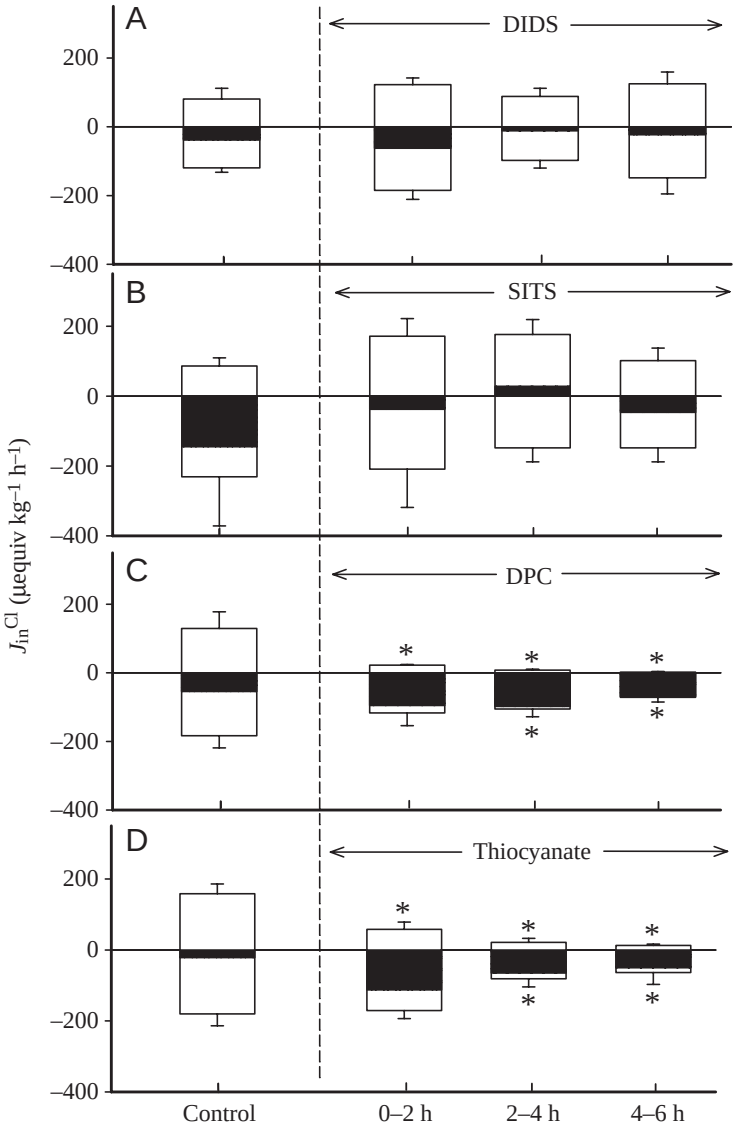


Fig. 7. The influence, in stingrays acclimated to ion-poor Rio Negro water, of (A) DIDS ($10^{-4}$ mol l$^{-1}$), (B) SITS ($10^{-4}$ mol l$^{-1}$), (C) DPC ($10^{-4}$ mol l$^{-1}$) and (D) thiocyanate ($10^{-4}$ mol l$^{-1}$) on unidirectional ($J_{in}$; open bars) and net (filled bars) flux rates of $Cl^-$ during three successive 2 h periods. Values are means ± 1 S.E.M. ($N$=5). Other details as in the legend to Fig. 4.

$Na^+/Ca^{2+}$ exchange mechanisms in gill ionocytes (Flik et al., 1995; Jurss and Bastrop, 1995; Perry, 1997). As for $Na^+$ and $Cl^-$ balance, these stingrays were in negative $Ca^{2+}$ balance when acclimated to their native blackwater (Table 5), again suggesting an important role for dietary uptake or ionic microclimates in benthic waters in normal homeostasis. In two northern hemisphere teleost species, Hobe et al. (1984) reported 65–75% reductions of $J_{in}^{Ca}$ upon acute exposure to pH 4.0. In stingrays, $J_{in}^{Ca}$ was abolished at pH 4.0 (Table 5), similar to the severe reduction of $J_{in}^{Na}$ and $J_{in}^{Cl}$ during acid exposure (Fig. 4). Interestingly, for all three ions, there was no stimulation of the efflux components. Gonzalez and co-workers (Gonzalez and Dunson, 1989; Gonzalez et al., 1997, 1998, 2002; Wood et al., 1998; Gonzalez and Preest, 1999; Wilson et al., 1999; Gonzalez and Wilson, 2001) have developed the idea that some blackwater teleosts are able to minimize or prevent low-pH-induced increases in diffusive permeability by having very high affinity $Ca^{2+}$ binding sites which stabilize external cell junctions. The present measurements showing that surface-bound $Ca^{2+}$ in stingrays are unaffected by exposure to pH 4.0 (Table 5) provide direct support for this hypothesis.

### *Mechanisms of $Na^+$ and $Cl^-$ transport*

In the following discussion, we assume that ion exchanges occur mainly at the gills, as in other fish. However, given the large surface area and soft texture of the well-perfused skin (which becomes pink with blood flow during hypoxia), we cannot eliminate a significant role for the skin in both ion uptake and efflux, and of course for the renal system in ion efflux (see Mangum et al., 1978). Another important caveat is that the present experiments were performed on whole animals, in which side effects of the pharmacological treatments or water chemistry manipulations may potentially confound interpretation. These might include changes in transepithelial potential (Kirschner, 1997) or alterations in the potency of drugs by trapping in mucus or by interactions with dissolved organic matter in the natural blackwater (Campbell et al., 1997).

The presence of traditional Michaelis–Menten saturation kinetics (Fig. 1) for both $J_{in}^{Na}$ and $J_{in}^{Cl}$ (Fig. 1) against large water-to-blood concentration gradients (Table 1) suggests that both occur by carrier-mediated active transport in these freshwater elasmobranchs. They were of different magnitude and responded entirely differently to various pharmacological treatments (Figs 5, 6), suggesting that they are independent, as in most freshwater teleosts (Kirschner, 1970, 1997; Goss et al., 1992; Potts, 1994; Jurss and Bastrop, 1995; Perry, 1997; Evans et al., 1998). $J_{out}^{Cl}$ appeared to exhibit a strong kinetic linkage with $J_{in}^{Cl}$ (Fig. 3), a conclusion reinforced by the inhibitory effects of several drugs on both components (Fig. 7C,D). This pattern is reminiscent of the ‘exchange diffusion’ phenomenon seen in many freshwater teleosts and crustaceans (e.g. Shaw, 1960; Kerstetter and Kirschner, 1972; de Renzis, 1975; Wood et al., 1984; Goss and Wood, 1990), in which the same transport mechanism may perform both $Cl^-/Cl^-$ self-exchange and vectorial transport (e.g. $Cl^-$/base exchange). The phenomenon can be equally well explained by an exchange protein or a selective channel mechanism linked to an electrogenic pump (Potts, 1994). Exchange diffusion has also been seen for $Na^+$ transport where $Na^+/Na^+$ and $Na^+$/acid exchange may co-exist (e.g. Shaw, 1959; Wood and Randall, 1973; Goss and Wood, 1990), but this was not apparent in the stingrays. $J_{out}^{Na}$ and $J_{in}^{Na}$ appeared to be kinetically independent (Fig. 2), and did

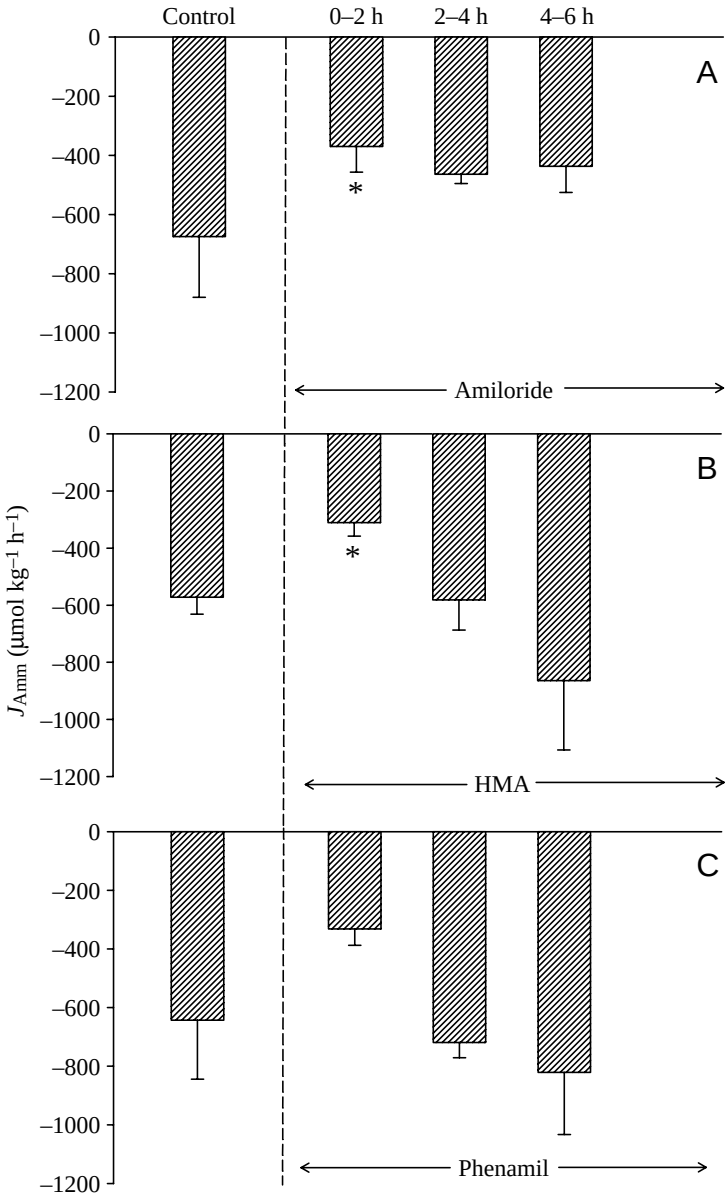


Fig. 8. The influence, in stingrays acclimated to ion-poor Rio Negro water, of (A) amiloride ($10^{-4}$ mol l$^{-1}$), (B) HMA ($4\times10^{-5}$ mol l$^{-1}$) and (C) phenamil ($4\times10^{-5}$ mol l$^{-1}$) on net ammonia flux rates ($J_{amm}$). Values are means ± 1 S.E.M. ($N$=5). Other details as in the legend of Fig. 4.

not co-vary when $J_{in}^{Na}$ was pharmacologically inhibited (Figs 5A, 6A,B). Interestingly, Gonzalez et al. (2002) reported kinetic linkage between $J_{out}^{Na}$ and $J_{in}^{Na}$ in approximately half the Rio Negro teleost species they surveyed ($Cl^-$ fluxes were not measured), but were unable to relate the presence or absence of this exchange diffusion phenomenon to either of the strategies outlined above.

According to theory (Shaw, 1959; Kirschner, 1970; Wood and Randall, 1973; de Renzis, 1975; Goss and Wood, 1990), the exchange diffusion components of efflux, when present, are always superimposed on the simple diffusive components, and the latter are predominant at low external $Na^+$ and $Cl^-$ concentrations. It was probably a reduction in this simple diffusive permeability, rather than a change in the exchange diffusion component, which explained the lower values of $J_{out}^{Na}$ and $J_{out}^{Cl}$, and the resulting reductions in $J_{in}$=$J_{out}$ balance points upon acclimation to Rio Negro blackwater (Figs 2, 3).

Amiloride ($10^{-4}$ mol l$^{-1}$) inhibited $J_{in}^{Na}$ by 70% in these freshwater stingrays (Fig. 5A). In this regard, the stingray is similar to most freshwater teleosts, in which a 60–95% blockade of $Na^+$ uptake induced by this concentration of amiloride has been widely reported (e.g. Kirschner et al., 1973; Perry and Randall, 1981; Perry et al., 1981; Wright and Wood, 1985; Wilson et al., 1994; Nelson et al., 1997; Clarke and Potts, 1998; Patrick and Wood, 1999). Interestingly, in two native Rio Negro characids that demonstrated the high-capacity, high-affinity, low-pH-resistant-strategy of $Na^+$ transport (i.e. opposite to that of the rays), amiloride ($10^{-4}$ mol l$^{-1}$) had little effect (13–26% inhibition of $J_{in}^{Na}$; Gonzalez et al., 1997; Gonzalez and Preest, 1999). However, amiloride at this concentration is a relatively non-selective drug, blocking both $Na^+$ channels and $Na^+/H^+$ exchangers (Benos, 1982). In an attempt to separate these two possibilities in the stingrays, we employed two drugs of reputedly greater specificity (HMA, phenamil) at lower concentration ($4\times10^{-5}$ mol l$^{-1}$). In higher vertebrate systems (cell lines), HMA is reported to be over 500 times more potent against $Na^+/H^+$ exchange and only 1/30$^{th}$ as potent against $Na^+$ channels as amiloride (Kleyman and Cragoe, 1988). In contrast, phenamil is reported to be 17 times more potent against $Na^+$ channels, and only 1% as potent against $Na^+/H^+$ exchange as amiloride (Kleyman and Cragoe, 1988). While HMA was somewhat more effective than phenamil (90% *versus* 70% inhibition, with greater persistence), our results were not clear-cut because both drugs strongly reduced $J_{in}^{Na}$ (Fig. 6A,B). Amiloride analogue specificities defined in mammalian cell lines may not necessarily apply in intact lower vertebrates. No definitive conclusion as to the importance of $Na^+/H^+$ exchange *versus* $Na^+$ channel/$H^+$-ATPase systems in mediating $Na^+$ uptake can be drawn.

Stilbene drugs such as DIDS and SITS are effective blockers of $J_{in}^{Cl}$ in some freshwater teleosts (Perry and Randall, 1981; Perry et al., 1981) although they are ineffective in others (Marshall et al., 1997). In the present study, these agents were completely ineffective against $Cl^-$ transport in freshwater stingrays, even at concentrations ($10^{-4}$ mol l$^{-1}$) above those ($2\times10^{-5}$ mol l$^{-1}$) usually considered to be specific for blockade of $Cl^-/HCO_3^-$ exchange (Figs 5B, 7A,B). We took precautions to shield these agents from light and to make them up immediately before use, and we also ran one test in water without DOC to ensure that the lack of effect was not due to immobilization of DIDS by dissolved organic molecules in blackwater. Nevertheless, from this result, we cannot eliminate a role for anion exchange in $Cl^-$ uptake, because many members of the anion exchange family are insensitive to stilbenes.

Thiocyanate is considered to be a more general blocker (competitive and non-competitive) of many anion exchange systems and has been shown to inhibit $Cl^-$ transport in several freshwater teleosts (Epstein et al., 1973; Kerstetter and Kirschner, 1974; de Renzis, 1975; Perry et al., 1984), even those in which stilbenes are ineffective (Marshall et al., 1997). In the stingrays, at a concentration ($10^{-4}$ mol $l^{-1}$) only slightly higher than that of $Cl^-$ in the water, thiocyanate almost abolished $J_{in}^{Cl}$ and also strongly reduced $J_{out}^{Cl}$ (Fig. 7D). These actions are very similar to those reported in the goldfish, which were interpreted as a blockade of both $Cl^-/HCO_3^-$ exchange and $Cl^-/Cl^-$ exchange diffusion in the very thorough study of de Renzis (1975). Since it is known that thiocyanate is a 'sticky' anion that will compete for and block $Cl^-$ channels, while DIDS and SITS are generally ineffective against $Cl^-$ channels, at least when applied externally (McCarty, 2000), this raised the possibility that a $Cl^-$ channel might be involved.

DPC ($10^{-4}$ mol $l^{-1}$) was employed because it is widely effective in blocking $Cl^-$ channels (Gogelein, 1988; Distefano et al., 1985; Chang and Loretz, 1993; McCarty, 2000), including those recently characterized in isolated gill cells of freshwater teleost fish (O'Donnell et al., 2001), although it has never been tested on intact freshwater teleosts. In the stingrays, DPC ($10^{-4}$ mol $l^{-1}$) essentially duplicated the action of thiocyanate, abolishing $J_{in}^{Cl}$ and greatly reducing $J_{out}^{Cl}$, indicating blockade of both vectorial transport and $Cl^-/Cl^-$ exchange diffusion. While this would suggest the involvement of a $Cl^-$ channel in both processes, the result must be treated with caution because it is unclear where this channel is located. DPC is lipophilic, so even though it was applied in the external medium, it may well have penetrated to the basolateral membrane of gill ionocytes, thereby blocking basolateral $Cl^-$ channels, which in turn would disrupt the whole transcellular $Cl^-$ transport pathway at the 'exit' step rather than the 'entry' step. A further caveat is that DPC at this concentration has also been reported to block $Cl^-/HCO_3^-$ exchange in at least one system (Reuss et al., 1987). Whatever the mechanism of $Cl^-$ uptake, given a negative intracellular potential, any reasonable estimate of intracellular $Cl^-$ concentration in the low millimolar range and an extracellular concentration in the low micromolar range, it would have to be 'energized' at the apical membrane, perhaps by a local elevation of intracellular $HCO_3^-$ concentration coupled to intracellular carbonic anhydrase and/or $H^+$-ATPase or $Cl^-/HCO_3^-$-ATPase systems.

### *Nitrogen metabolism and ammonia excretion*

Urea-N excretion was low (approximately 60 μmol $kg^{-1}$ $h^{-1}$) and comparable to rates previously reported in other freshwater Potamotrygonidae, while ammonia-N excretion was high (500–600 μmol $kg^{-1}$ $h^{-1}$) but in the mid-range of previously reported values (200–1000 μmol $kg^{-1}$ $h^{-1}$), confirming the ammoniotelic nature of these fish (Goldstein and Forster, 1971; Gerst and Thorson, 1977; Barcellos et al., 1997). The high rate of ammonia-N production may have resulted from metabolism of endogenous protein in these non-feeding animals. Since net ammonia flux was approximately eight- to tenfold higher than normal $J_{in}^{Na}$ in animals held in Rio Negro blackwater, any contribution from $Na^+/NH_4^+$ exchange was presumably small, and this was in accord with the complete insensitivity of ammonia excretion to approximately sevenfold variations in $J_{in}^{Na}$ in the kinetic experiments (Table 4). Furthermore, the 70% increase in ammonia excretion at pH 4.0, when $J_{in}^{Na}$ was severely reduced, was consistent with a dominant role for simple $NH_3$ diffusion along $P_{NH_3}$ gradients (Wilson, 1996; Wilkie, 1997). It was therefore surprising that amiloride and its analogues (HMA, phenamil) all caused a transitory 50% depression in ammonia excretion (Fig. 8). Indeed, this was larger than the marginal decreases in ammonia efflux (0–30%) normally seen in freshwater teleosts in response to amiloride blockade of $J_{in}^{Na}$ (Kirschner et al., 1973; Wright and Wood, 1985; Wilson et al., 1994; Nelson et al., 1997; Clarke and Potts, 1998; Patrick and Wood, 1999). While originally seen as evidence for $Na^+/NH_4^+$ exchange, the latter observations have more recently been interpreted as an inhibition of 'diffusion trapping' of $NH_3$ in gill boundary layer water associated with blockade of $H^+$ extrusion *via* $Na^+/H^+$ and/or $Na^+$ channel/$H^+$-ATPase systems (Wilson, 1996; Wilkie, 1997). Perhaps this diffusion trapping is particularly important in the poorly buffered blackwater, especially if $H^+$ and $NH_3$ excretion sites are 'downstream' from alkalizing sites (e.g. $HCO_3^-$ excretion) on the gills. A related explanation is that blockade of $H^+$ excretion mechanisms by amiloride and its analogs may also reduce internal pH, thereby trapping more ammonia internally as $NH_4^+$ and transiently reducing the internal $P_{NH_3}$ and, therefore, the $P_{NH_3}$ gradient for outward $NH_3$ diffusion.

### *Evolutionary perspective and future directions*

Based on the limited knowledge of branchial $Na^+$ and $Cl^-$ transport in marine elasmobranchs (for a review, see Shuttleworth, 1988), it is possible to put the present results in an evolutionary perspective. In marine elasmobranchs, the rectal gland normally performs the bulk salt excretion, and the gills usually achieve a very small net uptake of $Na^+$ and $Cl^-$. The gills exhibit unidirectional ion flux rates that are only a small fraction of those in marine teleosts and are quantitatively more similar to those of freshwater teleosts. Unidirectional $Cl^-$ fluxes are generally greater than unidirectional $Na^+$ fluxes (Bentley et al., 1976). Although the fine details of both fluxes are unknown, the system appears to be designed for pH regulation, normally achieving a small net uptake of $Na^+$ in exchange for 'acid', as in freshwater teleosts (Payan and Maetz, 1974; Evans et al., 1979). These characteristics are all similar to those we have identified in these freshwater stingrays and suggest that gill transport mechanisms already present in marine elasmobranchs may have been modified for operation in dilute external solution during the evolutionary invasion of freshwater by the Potamotrygonidae.

In the euryhaline cousins of the Potamotrygonidae, the Dasyatidae, Piermarini and Evans (2000, 2001) have recently identified two types of ionocyte in the gills on the basis of immunocytochemistry, one of which stains for $Na^+/K^+$-ATPase and the other for $H^+$-ATPase. After acclimation to freshwater,

the expression of both enzymes increased, as measured by western blotting, and the $H^+$ATPase appeared to become localized in the basolateral membrane. Based solely on these data, for no flux measurements or pharmacological characterizations were carried out, Piermarini and Evans (2001) have proposed a model (see their Fig. 7) whereby basolateral $Na^+/K^+$-ATPase energizes an apical $Na^+/H^+$ exchanger in one cell type, while basolateral $H^+$-ATPase energizes an apical $Cl^-/HCO_3^-$ exchanger in the other. This scheme is certainly unusual compared to most current models for the gills of freshwater teleosts (Goss et al., 1992; Potts, 1994; Jurss and Bastrop, 1995; Perry, 1997; Kirschner, 1997; Evans et al., 1998), but is coherent with our present data on potamotrygonid stingrays. Nevertheless, it must be emphasized that the present pharmacological results cannot conclusively differentiate between $Na^+/H^+$ exchange and a $Na^+$ channel/$H^+$-ATPase system on the apical membrane, or between a $Cl^-$ channel and $Cl^-/HCO_3^-$ exchange on this membrane. Clearly, the next step is to apply all techniques simultaneously (flux measurements, pharmacology, immunocytochemistry and molecular characterization of transporters) to these two different families of freshwater stingrays, one euryhaline, the other stenohaline.

We thank the captain and crew of the *Amanai II* for their hospitality and expertise, Maria Lucia Goes de Araujo (Projeto Potamotrygon-ACEPOAM) and the fishermen of INPA for the collection of stingrays, the Director of the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renovaveis for allowing us to use the Scientific Base of the Anavilhanas Archipelago, Dr M. J. O'Donnell for helpful advice, Dr R. Playle for DOC analyses and Gamble Technology (Mississauga, Ontario, Canada) for loan of the Triathler scintillation counter. This work was supported by an NSERC (Canada) research grant to C.M.W., a Royal Society (UK) research grant (RSG 21085) to R.W.W., an NSF (USA) international dissertation enhancement grant to M.L.P., a University Professorship award from the University of San Diego to R.J.G. and a research grant from the Brazilian Research Council to A.LV. C.M.W. is supported by the Canada Research Chair Program and A.L.V. was the recipient of a research fellowship from the National Research Council of Brazil.

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