THE JOURNAL OF EXPERIMENTAL ZOOLOGY 279:208–219 (1997)

Characterization of Ion and Acid-Base Transport in the Fresh Water Adapted Mummichog (*Fundulus heteroclitus*)

MARJORIE L. PATRICK,[1*] PETER PÄRT,[2] WILLIAM S. MARSHALL,[3] AND CHRIS M. WOOD[1]

[1]*Department of Biology, McMaster University, Hamilton, Ontario, Canada L8S 4K1*

[2]*Department of Ecotoxicology, University of Uppsala, S-752-36 Uppsala, Sweden*

[3]*Department of Biology, St. Francis Xavier University, Antigonish, Nova Scotia, Canada B2G 1C0*

ABSTRACT We examined whether ionoregulatory mechanisms of fresh water *Fundulus heteroclitus* in vivo are similar to those of typical freshwater species (e.g., rainbow trout, goldfish, and catfish). Under control conditions ($[NaCl]_{ext}$ ~1 mmol/l), the mummichog exhibits very large Na^+ influx and efflux rates but virtually no Cl^- influx and a small Cl^- efflux component. External NaCl levels were varied to reveal a saturable, low affinity (K_m = 1,723 ± 223 μmol/l), high capacity (J_{max} = 2,258 ± 288 nEq/g/h) Na^+ uptake system that was independent of both Na^+ efflux and ammonia excretion. A measurable Cl^- influx did not occur until NaCl levels surpassed 2 mmol/l and did not saturate within the freshwater range, suggesting a completely different uptake mechanism. Cl^- efflux was also independent of Cl^- influx. A systemic acidosis (intraperitoneal HCl injection) was induced in order to investigate the connection between ionoregulation and acid-base balance. The acidosis did not affect influx rates but induced an elevated Cl^- efflux and an attenuated Na^+ efflux. This resulted in an excess of net Cl^- loss over Na^+ loss which effected a net acid excretion by strong ion difference theory. These results concur with the measured acid-base fluxes which indicate that over 50% of the acid load was excreted within 4 h by differential efflux modulation. Therefore an ion/acid-base link does exist in the mummichog but differs in nature from that of other freshwater fish. Indeed, virtually all of these findings differ from the current model for most other teleosts, indicating that alternate models of ionoregulation in fresh water exist. *J. Exp. Zool. 279:208–219, 1997.*

The predominant habitats for the mummichog (*Fundulus heteroclitus*), a euryhaline teleost, are the estuaries and salt marshes along the eastern seaboard of North America from the Gulf of the St. Lawrence to northeastern Florida (Scott and Crossman, '73). They tolerate the rapid salinity changes that occur with the tidal cycle and have been documented to survive salinities from 120‰ (approximately fourfold seawater) down to 0‰ (fresh water) (Griffith, '72). Indeed, several populations of landlocked, freshwater mummichogs have been documented (Klawe, '57; Denoncourt et al., '78; Samaritan and Schmidt, '82). From this information, it appears that the mummichog is a truly euryhaline teleost that can readily withstand a wide range of salinities. In order to do so, it must possess specialized ionoregulatory abilities.

Most of the research on the ionoregulatory abilities of *F. heteroclitus* has focused on the seawater fish. The basic mechanisms have been well characterized by in vivo work and even more so by in vitro studies using opercular epithelium preparations. This body of *Fundulus* research has made an important contribution to our understanding of branchial ion transport in marine teleosts (reviewed by Karnaky, '80, '86; Evans et al., '82; Zadunaisky, '84; Pequeux et al., '88; Wood and Marshall, '94). In contrast, only a few studies have been performed on fresh water mummichogs (Potts and Evans, '66, '67; Epstein et al., '69; Pickford et al., '70; Hannah and Pickford, '81; Hossler et al., '85; Gonzalez et al., '89), and little is known of the exact mechanisms involved. The opercular epithelium preparation of fresh water

*Correspondence to: Marjorie L. Patrick, Dept. of Ecology and Evolutionary Biology, University of California, Irvine, CA 92697-2525. E-mail: mlpatric@uci.edu

Received 12 December 1996; revision accepted 23 May 1997.

F. heteroclitus has similarly received little attention (see Wood and Marshall, '94).

Our present understanding of the ionoregulatory mechanisms in the gills of freshwater teleosts comes largely from work with species such as goldfish, catfish, and salmonids. Details of these processes have been presented in several recent reviews (McDonald et al., '89; Wood, '91; Goss et al., '92; Kirschner, '96; Potts, '94) and are described briefly below.

In fresh water, fish are hyperosmotic to their environment and suffer a continuous diffusive loss of Na^+ and Cl^-. Both ions are actively extracted from the external medium at the gill epithelium by saturable ion exchange mechanisms to maintain internal levels. The uptake of Na^+ and Cl^- is associated with acid-base regulation; Cl^- is exchanged for HCO_3^-, but the exact mechanism of Na^+ uptake remains unclear. One model proposes an electroneutral exchange of Na^+ with a proton (H^+) or ammonium ion (NH_4^+). Another suggests a Na^+ channel/H^+ pump configuration. In the latter, active H^+ extrusion establishes an inward, electrochemical gradient for Na^+. Recently, a secondary ion/acid-base link involving Na^+ and Cl^- efflux components has been identified. Na^+ and Cl^- effluxes can be differentially adjusted resulting in a charge discrepancy which can establish a net H^+ flux (Hyde and Perry, '87; McDonald et al., '89; Goss and Wood, '90a,b; Goss et al., '92). The nature of ammonia excretion and the link to Na^+ uptake remains unsettled, although recent studies support the traditional view that NH_3 moves passively across the gill epithelium. Upon exiting the gill, protons extruded by either Na^+ transport mechanism bind to ammonia, thereby maintaining the diffusive gradient (e.g., Wilson et al., '94).

In view of the importance of *F. heteroclitus* as a seawater model system, both in vivo and in vitro, our eventual goal is to develop this euryhaline species as a comparable model system in fresh water (Wood and Marshall, '94). As a first step, the objective of the present study was to evaluate whether the fresh water mummichog ionoregulates in vivo by mechanisms similar to those outlined above for other freshwater species or whether it departs from our current freshwater model. In particular, we have characterized the external concentration dependence of Na^+ and Cl^- uptake and loss and therefore the affinities, maximum capacities, and possible influx/efflux linkages of the transport systems as well as the possible linkage of ammonia excretion to Na^+ uptake. Further, intraperitoneal injections of NaCl and HCl were administered, the latter to induce systemic acidosis, to investigate how the freshwater mummichog copes with ionic and acid-base disturbances and whether a coupling of acid-base flux to Na^+ and Cl^- regulation is apparent.

MATERIALS AND METHODS

Holding conditions

Mummichogs of both sexes, weighing 2.4–10.0 g, were collected from a brackish estuary located near Antigonish, Nova Scotia, and were held at St. Francis Xavier University, Antigonish, or were air-shipped to McMaster University, Hamilton, Ontario. At both locations, the fish were held indoors in 500 l fiberglass tanks containing 10% seawater at ambient temperature (18–25°C). The water was aerated and charcoal-filtered. Fish were fed a 1:2 mixture of Tetramin/Tetramarin daily.

Ten to 14 days prior to experiment, 10–12 fish were placed in a tank containing 60 l of aerated and filtered fresh water. Water was replaced at 3 l/h. The composition of this defined freshwater medium was (all in millimoles per liter) 1.0 Na^+, 1.0 Cl^-, 0.1 Ca^{2+}, 0.06 Mg^{2+}, 0.02 K^+, titration alkalinity to pH = 4.0, 0.28, pH = 6.8–7.2. Fish were fed daily until 4 days before the experiment. For the HCl and NaCl injection trials, mummichogs were held for 28 days under the same acclimation conditions.

The night prior to experiments, fish were weighed and placed in individual darkened 250 ml Nalgene beakers with lids and allowed to settle overnight. Mummichogs used in uptake kinetic studies received exchange flow (200 ml/h) and aeration overnight, whereas the fish used in the injection study were held in a static 200 ml volume with vigorous aeration for no longer than 6 h prior to experimentation.

To ensure that our experimental protocols did not produce confounding stress, we monitored two sensitive indicators of stress in fish, ion balance and acid-base status. In both the kinetic and injection series, the chambers were periodically siphoned and refilled, a process which took less than 30 seconds. When this exchange procedure was tested on otherwise untreated mummichogs, there was no effect on Na^+, Cl^-, and acid-base fluxes. Further, in the injection study, each fish experienced one episode of handling which included a peritoneal cavity injection. We performed an additional test (data not presented) in which the same injection protocol was followed minus the injectate (i.e., handling and needle insertion only). There was no effect on Na^+, Cl^-, and acid-base

fluxes. Unlike trout, in which a few seconds of handling alone can more than double Na^+ loss (Gonzalez and McDonald, '92), mummichogs do not appear to be stressed by brief handling or air exposure. We are confident that our experimental methods minimized the potential for stress artifact.

Ion Uptake Kinetics

Th rates of Na^+ and Cl^- uptake were measured in six different NaCl concentrations ranging from 500–8,000 μmol/l to determine if transport was carrier-mediated and saturable (i.e., conforming to Michaelis-Menten first-order kinetics). Na^+ and Cl^- unidirectional effluxes and ammonia excretion were also measured to determine if external [NaCl] influenced these components. Sodium (N = 11) and chloride (N = 6) uptake kinetic experiments were run independently but were performed using identical protocols. Mummichogs in individual containers were exposed to each [NaCl] sequentially. Flux periods varied in time according to the [NaCl] exposure: 500 and 800 μmol/l, 1.0 h; 1,300 and 2,300 μmol/l, 1.5 h; 4,200 μmol/l, 2 h; 8,000 μmol/l, 3 h.

These kinetic experiments were performed in the defined freshwater medium (see above), made initially without the addition of NaCl. At the start of each new flux, water was siphoned from the container holding the fish, and 105 ml of defined medium was immediately added, together with an aliquot of 1.0 M NaCl stock (either 22 KBq/ml ^{22}Na or 37 KBq/ml ^{36}Cl), to set the desired concentration. The fish were allowed 10 min to settle. Water samples (20 ml) were taken at the beginning and end of each flux period and were frozen immediately for subsequent analysis of ammonia, Na^+, Cl^-, ^{22}Na, and ^{36}Cl. Once the flux period had ended, the water was siphoned out of each container and the cycle repeated with the next higher [NaCl].

Injection studies

The purpose of this study was to measure fluxes of sodium, chloride, and acid-base equivalents in response to a systemic acidosis induced by an isosmotic load of HCl injected intraperitoneally. NaCl injection of the same dose was performed as the control. To confirm that an acidosis was induced, in a separate series (see below) blood acid-base status was measured prior to and following injection of both HCl and NaCl.

After the overnight settling period for the animals, the chambers were flushed and 250 ml fresh water added. Ninety minutes later, the chambers were flushed again, and 105 ml fresh water was added. ^{22}Na and ^{36}Cl fluxes were measured in separate batches of fish. Isotope solution was added to each chamber (7.4 KBq/chamber for both ^{22}Na and ^{36}Cl) and allowed to mix for 10 min. Water samples (20 ml) were taken at 0, 1, and 2 h intervals to define the control, preinjection fluxes. Following the control period, the flux containers were flushed, and the fish were given an intraperitoneal injection (1,000 nEq/g) of either 140 mM HCl (N = 16) or 140 mM NaCl (N = 14). One at a time, the fish were given an intraperitoneal injection of either HCl or NaCl solution via a 50 μl gas-tight Hamilton syringe. The fish were rinsed, blotted dry, inspected for visual signs of leakage (obviously leaking fish were rejected), returned to their containers filled with fresh water, and allowed to recover for 15–20 min. This injection procedure took less than 1 min for each fish. Containers were flushed again, and 105 ml fresh water and isotope was added. Water samples were taken initially and hourly for 4 h, with the water changed after hour 2, and were subsequently analyzed for ammonia, Na^+, Cl^-, ^{22}Na, ^{36}Cl, and titration alkalinity.

Blood acid-base measurements

In fish of this small size (2–10 g), it was not possible to measure blood acid-base status by cannulation. Nevertheless, it was still possible to sample blood by terminal caudal puncture, tonometer it to a fixed P_{CO_2} to remove any "respiratory" disturbance (i.e., P_{CO_2} elevation) associated with sampling, and then determine the remaining "metabolic" disturbance by measuring pH and HCO_3^-. Blood samples were taken under control conditions (preinjection) and at 0.5 and 3.0 h after injection of HCl or NaCl. At the time of sampling, fish (N = 5–8 at each point) were killed by a blow to the head, and a blood sample (typically 50–100 μl) was quickly drawn into a heparinized gas-tight Hamilton 100 μl syringe by caudal puncture. Each sample was then equilibrated to the same P_{CO_2} of 2.43 torr, which was chosen as a typical in vivo P_{CO_2} for teleost fish. The tonometer was a sealed 1.5 ml bullet centrifuge tube fitted with inflow and outflow PE50 tubing to carry the gas in and out. This was attached to a vigorous shaker in a water bath at 22.5°C. The gas mixture (P_{CO_2} = 2.43 torr in air) was generated by a gas mixing pump and passed through a humidifying flask prior to reaching the tonometer. Tests showed that 20 min equilibration was adequate. The equilibrated sample was then injected into a Radiometer micro pH electrode (Radiometer E5021, Copenhagen, Denmark) thermostatted to

22.5°C and coupled to a Radiometer pHM 71 Mk2 meter. The sample was subsequently retrieved from the pH capillary electrode and centrifuged for 5 min in a sealed capillary tube; then the plasma was decanted (back into a Hamilton syringe) for measurement of total CO_2 using a Corning (Corning, NY) 965 total CO_2 analyzer.

Analytical methods and calculations

Ammonia concentrations (T_{Amm}) were determined by a micromodification of the salicylate-hypochlorite assay (Verdouw et al., '78). Titratable alkalinity (TA) was determined by titration of 10 ml water samples with standardized HCl to pH = 4.0 as described by McDonald and Wood ('81). The difference between initial and final TA measurements represents the total base flux which includes ammonia excreted as NH_3. The difference between total ammonia and TA excretion is the net acidic equivalent movement. All measurements were made using a Radiometer GK2041C pH electrode coupled to a PHM82 pH meter. Titration acid (0.005 N or 0.02 N in different experiments) was dispensed using a Gilmont (Barrington, IL) microburette. All water samples were measured within 12 h of sampling and aerated 10 min prior to titration and another 15 min after titration to pH 4.2 before the titration to pH = 4.0 was completed. This ensured complete removal of CO_2.

Na^+ and Cl^- concentrations of water samples were determined with an atomic absorption spectrophotometer (Varian AA-1275 or 375, Palo Alto, CA) and coulometric titration (Radiometer CMT-10), respectively. To assay radioactivity (^{22}Na and ^{36}Cl), we prepared duplicate 2 ml water samples with 10 ml cocktail scintillant (ACS; Amersham, Arlington Heights, IL) and counted them on a scintillation counter (LKB 1217 Rack Beta; Pharmacia-LKB, Uppsala, Sweden). Rate of uptake (J_{in}) of Na^+ and Cl^- (in nEq/grams/hour), as measured by disappearance of radioactivity from the water, was calculated from the equation

$$J_{in} = \frac{\text{volume}}{\text{weight}} \cdot \frac{1}{\text{time}} \cdot (cpm_1 - cpm_2) \cdot \frac{1}{\overline{SA}}$$

where cpm_1 and cpm_2 are the activities of the isotope (cpm/ml) at the start and end of the flux period respectively, and SA is the mean specific activity of the water. The net ion flux (J_{net}) was calculated by

$$J_{net} = \frac{\text{volume}}{\text{weight}} \cdot \frac{1}{\text{time}} \cdot ([ion]_1 - [ion]_2)$$

where $[ion]_1$ and $[ion]_2$ are the water concentrations at the start and end of flux period, respectively. The rate of efflux (J_{out}) was determined as the difference between J_{net} and J_{in}.

Net titratable alkalinity (J^{TA}_{net}) and ammonia excretion (J^{Amm}_{net}) rates were calculated using the above J_{net} equation but using the starting and ending TA and T_{Amm} concentrations accordingly.

The relationship between $[NaCl]_{ext}$ and Na^+ uptake was examined using Michaelis-Menten analysis for first-order one substrate kinetics. Values of J_{max} (the maximum uptake rate) and apparent K_m (the $[Na^+]$ at which uptake is 50% of J_{max}) for individual fish were calculated using Eadie-Hofstee regression analysis (Michal, '85). To generate the curve fitted to the data, we determined grand means (N = 11) for J_{max} and K_m and substituted them into the Michaelis-Menten equation:

$$J_{in} = \frac{J_{max} \cdot [Na^+]_{ext}}{K_m + [Na^+]_{ext}} .$$

Chloride uptake over the range of $[NaCl]_{ext}$ tested did not exhibit saturation kinetics and therefore could not be analyzed in this manner.

Statistical analysis

All values are presented as mean ± SEM. Comparisons between control and postinjection means at $P \leq 0.05$ were tested by Student's paired *t*-test (two-tailed) with the *t* values adjusted according to the Bonferroni table for multiple comparison (Nemeyi et al., '77). Comparisons between treatment groups at the same times employed Student's unpaired *t*-tests (two-tailed).

RESULTS

Na^+ and Cl^- uptake kinetics

The rate of Na^+ uptake exhibited a distinctive hyperbolic relationship over the range of $[NaCl]_{ext}$, indicating that transport became saturated (Fig. 1A). The J_{max} and K_m for Na^+ were determined to be 2,258 ± 288 nEq/g/h and 1,723 ± 223 μmol/l (N = 11), respectively. In contrast, Cl^- uptake was approximately linear with respect to external Cl^- concentration and did not appear to exhibit saturation. The first $[NaCl]_{ext}$ concentration at which J^{Cl}_{in} was significantly different from zero was 2,300 μmol/l, well above the K_m for Na^+ uptake. However, by 8,000 μmol/l $[NaCl]_{ext}$, J^{Cl}_{in} did approximate J^{Na}_{in} at the same concentration (Fig. 1B).

In contrast to uptake of Na^+ and Cl^-, effluxes

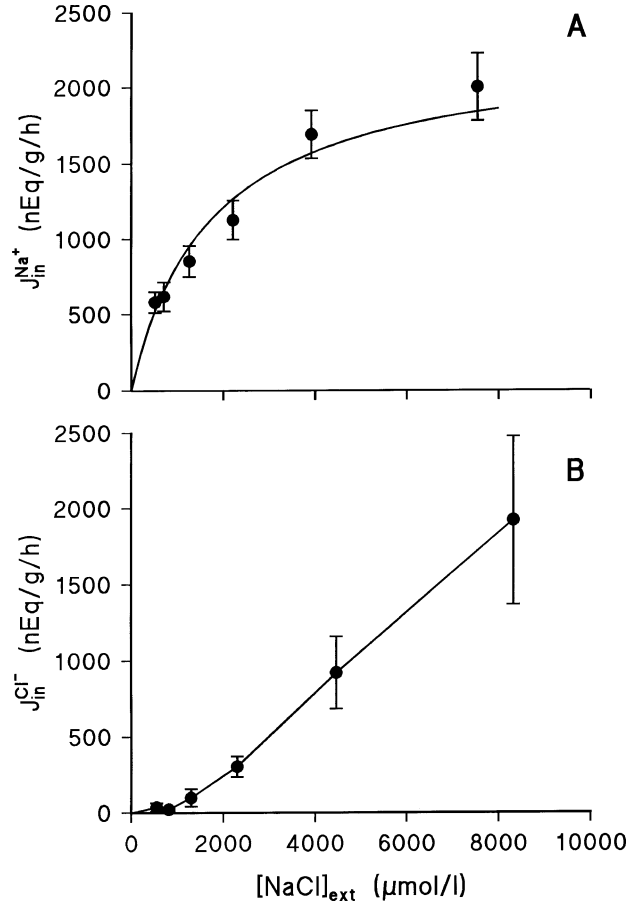

Fig. 1. The influence of external [NaCl] on uptake (J_{in}) of (**A**) Na^+ and (**B**) Cl^-. Values are mean ± 1 SEM. N = 11 for Na^+; N = 6 for Cl^-. The line fitted to the Na^+ influx data is a Michaelis-Menten equation with K_m = 1,723 μmol/l and J_{max} = 2,258 nEq/g/h (see text). The line is fitted by eye to the Cl^- influx data.

were unchanged throughout the range of concentrations tested and averaged approximately –750 and –250 nEq/g/h, respectively (Fig. 2A). This suggests that effluxes are not influenced by external NaCl levels and are not directly coupled to uptake. Throughout the NaCl concentration range tested, Na^+ fluxes were consistently larger than those for Cl^- (Figs. 1A, B, 2A).

Figures 1 and 2 indicate that different minimum concentrations are required to maintain Na^+ and Cl^- balance. The Na^+ concentration where influx equalled outflux was 980 μmol/l. For Cl^-, the balance point was 1,620 μmol/l. The defined freshwater medium used for holding the fish contained approximately 1,000 μmol/l NaCl. This concentration approximates the Na^+ balance point but is lower than the Cl^- balance point, suggesting that the fish were in Na^+ equilibrium but in negative Cl^- balance.

It appeared that net ammonia excretion was not coupled to Na^+ uptake since net ammonia excretion also remained unchanged throughout the tested range of external Na^+ concentrations (Fig. 2B).

Injection studies

Intraperitoneal HCl injection induced a significant systemic metabolic acidosis by 0.5 h (Fig. 3A, B). Both blood pH and HCO_3^- dropped relative to preinjection controls and relative to the NaCl injected fish sampled at 0.5 h. These differences from the NaCl-injected group (but not from the preinjection controls) persisted at 3 h. Interestingly, both pH_a (by 3 h) and plasma HCO_3^- (by 0.5 h) were significantly elevated in response to NaCl injection (Fig. 3A, B).

Prior to injection, the control flux rates of Na^+

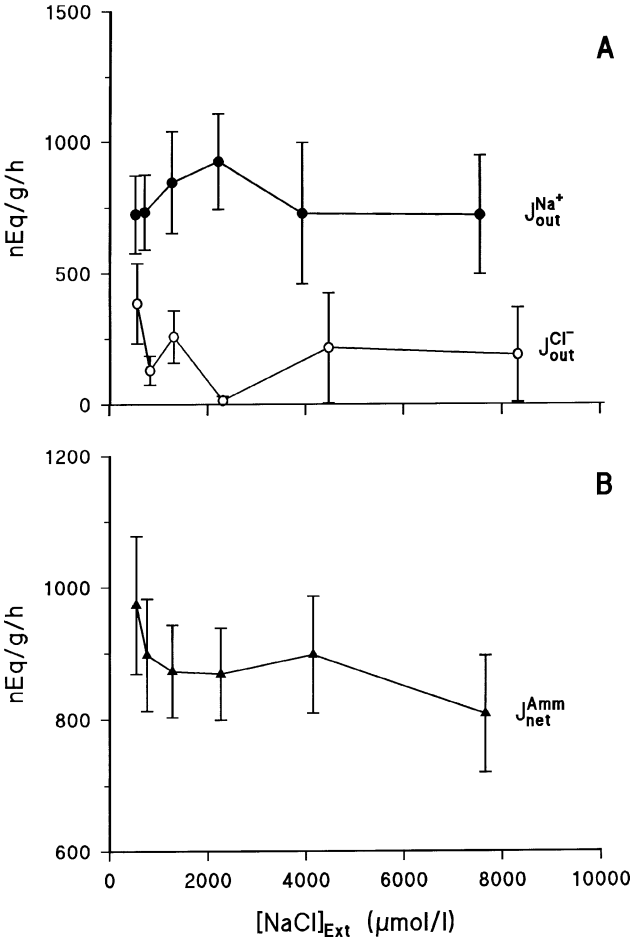

Fig. 2. The influence of external [NaCl] on whole-body (**A**) efflux (J_{out}) of Na^+ (thick line) and Cl^- (thin line) and (**B**) net ammonia excretion ($J^{Amm}{}_{net}$). Mean ± 1 SEM. N = 11 for Na^+; N = 6 for Cl^-; N = 16 for ammonia. There was no significant variation with external [NaCl] within any variable ($P \leq 0.05$).

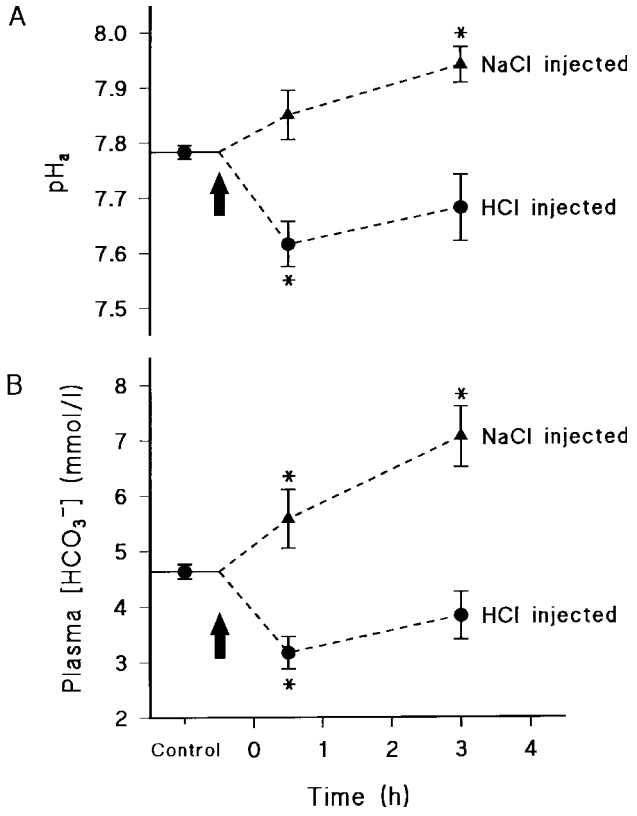

Fig. 3. Blood pH (**A**) and plasma $[HCO_3^-]$ (**B**) under control conditions and following isosmotic NaCl and HCl intraperitoneal injections (1,000 nEq/g). Mean ± 1 SEM. N = 6 for control; N = 5 for NaCl; N = 7–8 for HCl treatments. *Significant difference from the control values ($P \leq 0.05$).

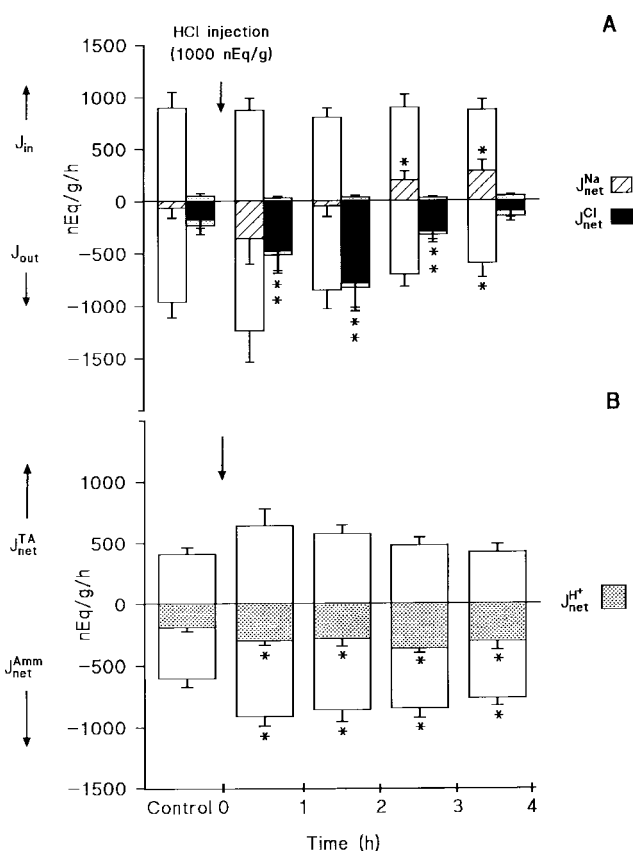

Fig. 4. The effect of an intraperitoneal injection of 1,000 nEq/g HCl on whole-body (**A**) influx (J_{in}), efflux (J_{out}), and net flux (J_{net}) of Na^+, Cl^-, and (**B**) net titratable alkalinity flux (J^{TA}_{net}), net acid movement (J^{H+}_{net}), and net ammonia excretion (J^{Amm}_{net}). Mean ± 1 SEM. N = 9 for Na^+ and the acid-base fluxes; N = 7 for Cl^-. *Significant difference from the control rates ($P \leq 0.05$).

and Cl^- were very different from one another, in accord with the kinetic analyses. At this acclimation $[NaCl]_{ext}$ of 1,000 μmol/l, there was a vigorous turnover of Na^+ with unidirectional influxes and effluxes of approximately 900 nEq/g/h and a resulting net flux which was not significantly different from zero (Figs. 4A, 5A). In contrast, unidirectional Cl^- influx was negligible. Cl^- efflux rates were much lower than Na^+ efflux rates; nevertheless, Cl^- net flux rates were negative (significantly below zero) (Figs. 4A, 5A).

Acid injection produced a gradual drop in Na^+ efflux which became significant in hour 4; since Na^+ uptake was unchanged, a net Na^+ gain resulted (Fig. 4A). This positive net Na^+ flux was significantly different from the control value in hours 3 and 4. In contrast, HCl injection stimulated Cl^- loss due to a twofold elevation of efflux. Elevated Cl^- efflux and negative net flux persisted for 3 h before returning to control levels in the final hour of the experiment.

An injection of NaCl equimolar to that of HCl produced a very different pattern of ionic flux responses. Na^+ and Cl^- effluxes were stimulated by 60% and 100%, respectively, resulting in negative net fluxes of both ions which were approximately equal to one another (Fig. 5A). Na^+ efflux remained significantly elevated for 2 h and Cl^- efflux for 1 h. Both Na^+ and Cl^- influx rates remained unchanged; thus, the response to the NaCl injection was the stimulation of net losses. Over 4 h, the entire NaCl load was eliminated.

Control acid-base fluxes in all of the treatments indicate that net H^+ excretion was close to zero, as net ammonia excretion (J^{Amm}_{net}) equalled net base excretion (J^{TA}_{net}) (Figs. 4B, 5B). From this we conclude that most of the titratable alkalinity measured was likely ammonia in its unprotonated from (NH_3). A significant increase in net H^+ excretion was initiated within the first hour after HCl injection and remained at the same elevated

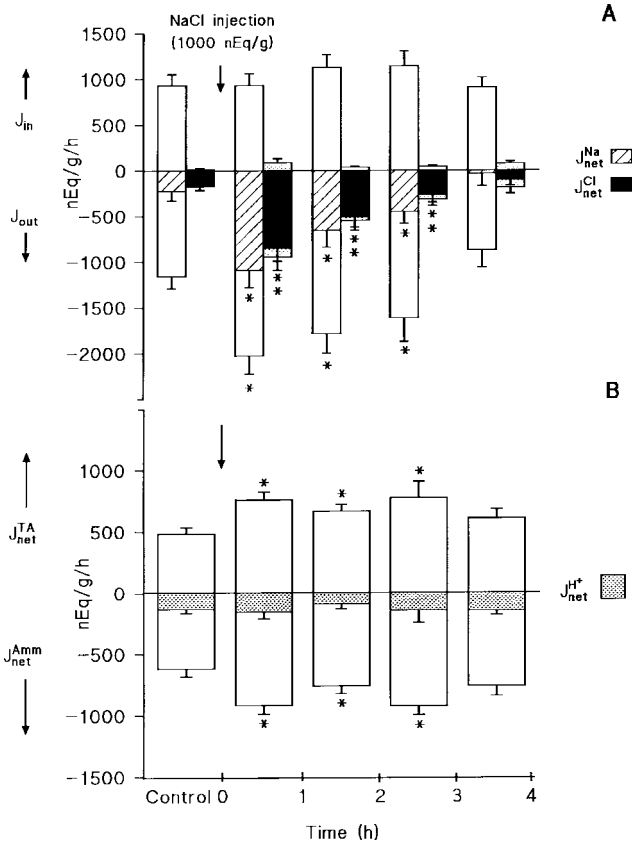

Fig. 5. The effect of an intraperitoneal injection of 1,000 nEq/g NaCl on whole-body (**A**) influx (J_{in}), efflux (J_{out}), and net flux (J_{net}) of Na^+, Cl^-, and (**B**) net titratable alkalinity flux ($J^{TA}{}_{net}$), net acid movement ($J^{H+}{}_{net}$), and net ammonia excretion ($J^{Amm}{}_{net}$). Mean ± 1 SEM. N = 8 for Na^+, Cl^-, and the acid-base fluxes. *Significant difference from the control rates ($P \leq 0.05$).

level over the 4 h period (Fig. 4B). A significant elevation of ammonia excretion followed the same pattern, whereas $J^{TA}{}_{net}$ did not change significantly. NaCl injection similarly caused increases in both ammonia and titratable base excretion, significant during the first 3 h, but did not stimulate acid excretion (Fig. 5B).

The present measurements also allowed evaluation of acid-base balance by means of strong ion difference (SID) theory, as described by Stewart ('83) and used successfully in several recent studies on fish (see Discussion). This provides a means of quantifying net acid-base balance independent from that of the titration approach. In brief, Na^+ and Cl^- are considered to be the major strong cations and anions, respectively, moving across the gills. An excess of Na^+ loss over Cl^- loss will thereby dictate a net base loss of acid uptake, whereas an excess of Cl^- loss over Na^+ loss will signify a net acid loss or base uptake (i.e., $J^{H+} = J^{Cl} - J^{Na}$, in terms of net fluxes).

Preinjection control Na^+ and Cl^- net fluxes in both treatments (Figs. 4A, 5A) were essentially equal, suggesting that the SID flux, or net acid movement, was zero. This is in agreement with the acid-base fluxes measured by titration (Figs. 4B, 5B). Although Na^+ and Cl^- net losses were both elevated during the first 3 h, there was negligible difference between the two throughout the 4 h following NaCl injection (Fig. 5A). This coincides with the unchanged $J^{H+}{}_{net}$ determined in Figure 5B. A different picture emerges from the HCl injection series, where a larger net Cl^- loss over Na^+ (Fig. 4A) throughout the postinjection period denotes a net acid loss. By the fourth hour, there was a net Na^+ gain, while net Cl^- flux resumed its preinjection level.

By calculating the total amount of H^+ excreted, relative to the preinjection control rate, over the 4 h, the mummichog was able to excrete almost half of the acid load (i.e., ~475 nEq/g) as measured by the titration approach. In the same manner, by the SID approach, the difference between net Cl^- and Na^+ fluxes above the control value over the 4 h (i.e., ~800 nEq/g) could easily account for all of the acid excreted.

DISCUSSION

Na^+ and Cl^- fluxes under control conditions

Under control conditions ([NaCl] = 1,000 μmol/l), Na^+ unidirectional fluxes in *F. heteroclitus* are substantial. The large Na^+ efflux component (~750 nEq/g/h) may reflect the inability to abolish the paracellular permeability to Na^+ which is a prominent feature of the standard ion transport model for seawater teleosts (Silva et al., '77; Zadunaisky, '84; Pequeux et al., '88; Wood and Marshall, '94). This "leakiness" may then require a large uptake to maintain Na^+ balance. In contrast, there is virtually no Cl^- uptake, and efflux is small (~250 nEq/g/h). Consequently, under these control conditions the fish were in negative Cl^- balance. This may explain why Hannah and Pickford ('81) reported a disparity of approximately 40 mEq/l between Na^+ and Cl^- plasma levels in fresh water *F. heteroclitus*. The situation appears similar to that of euryhaline eels (*Anguilla anguilla* and *A. rostrata*) which also lack Cl^- uptake while in fresh water and exhibit a significant concentration difference of about 40 mEq/l between plasma Na^+ and Cl^- (Farrell and Lutz, '75; Bornancin et al., '77; Hyde and Perry, '87). The na-

ture of the "missing anions" remains unknown, although an unusually high negative charge on plasma protein is one possibility. In the present study, plasma [HCO_3^-] was measured at 4.7 mEq/l (Fig. 3B), which would not contribute greatly to narrowing the anion-cation disparity. In both eels and mummichogs, dietary Cl^- is presumably required to maintain Cl^- balance in fresh water.

Na^+ and Cl^- uptake kinetics

The discrepancy between Na^+ and Cl^- kinetic properties suggests that entirely different mechanisms may be responsible for uptake of these ions. The kinetic parameters determined for *F. heteroclitus* indicate a high capacity (2,258 nEq/g/h) but low affinity (high K_m = 1,723 μmol/l) Na^+ uptake system. Potts and Evans ('67) also reported a high K_m (2,000 μmol/l) which approximates the present study and confirms that a low affinity transport mechanism is present in the mummichog. If Cl^- uptake saturates at all, it does so at a much higher external salt concentration which is well beyond the freshwater range (i.e., > 2% sea water).

Saturable ion uptake is suggestive of a carrier-mediated process, which is probably the case for Na^+ absorption in *F. heteroclitus*. However, Cl^- uptake did not reach a maximum within freshwater [NaCl] values, suggesting a mechanism other than carrier transport is probable. Cl^- uptake kinetics performed on amphibian epithelium in vitro show a curve similar to Figure 1B, with saturation occurring at ~60 μmol/l Cl^- (Harck and Larsen, '86). Concurrent with this pattern of influx, Cl^- efflux increases in the amphibian epithelium in vitro preparation presumably because both fluxes occur through a common pathway (i.e., apical channels). This contrasts to the trend observed in *F. heteroclitus* in vivo, where J^{Cl}_{out} remained unchanged (Fig. 2A) while J^{Cl}_{in} increased (Fig. 1B), which suggests these fluxes occur at different sites.

Changes in the in vivo transepithelial (TEP) could influence Na^+ and Cl^- uptake kinetics. As external NaCl concentration increases, TEP could become less negative (inside relative to outside), which would reduce the driving force for Na^+ uptake and enhance the gradient for Cl^- uptake. This could explain in part the observed leveling off of J^{Na}_{in} and the linear increase of J^{Cl}_{in} (Fig. 1). Such a result would require a considerable change in TEP. In vitro studies using isolated *F. heteroclitus* opercular membrane have indicated that the very negative TEP (–30 to –60 mV) (Wood and Marshall, '94) can be reduced by an increase in mucosal NaCl levels (W.S. Marshall, unpublished results). However, in vivo TEPs measured in other fresh water euryhaline fish are only slightly negative. For instance, euryhaline gobies (*Gillichthys mirabilis*), when transferred to 5% seawater from full-strength seawater, develop a TEP of about –30 mV, but over 24 h acclimation the TEP approaches a new steady state at zero (Iwata and Bern, '85). Furthermore, in vivo TEP measured in goldfish and trout, in the presence of external Ca^{2+}, were unaffected when NaCl levels were varied between 0.1 and 100 mM (Kerstetter et al., '70; Eddy, '75), a range far greater than tested in the present study (Fig. 1). Thus, it is uncertain exactly how external NaCl levels would influence TEP for *F. heteroclitus* in vivo.

It becomes evident that mummichog ion absorption properties depart from what has been established for other fresh water fish. In general, freshwater species possess higher affinity, lower capacity ion uptake mechanisms than the mummichog. In adult rainbow trout, for example, Na^+ and Cl^- uptake, despite being independent processes, have virtually identical K_m values (~100 μmol/l NaCl) which are both saturated within a much lower $[NaCl]_{ext}$ range and have similar J_{max} values (~350 nEq/g/h) (Goss and Wood, '91). The goldfish (*Carassius auratus*) also displays a relatively high affinity and low capacity uptake for Na^+ (K_m = 300 μmol/l, J_{max} = 650 nEq/g/h) (Maetz, '72, '73) and Cl^- (K_m = 100 μmol/l, J_{max} = 500 nEq/g/h) (DeRenzis and Maetz, '73; DeRenzis, '75).

It has been suggested that the affinity of the ion uptake mechanism is correlated with the adaptability of a particular species to fresh water (Potts and Parry, '64; Evans, '73). Our results with the mummichog in conjunction with other studies using other species do not support this assertion. Although *Fundulus heteroclitus* can withstand a rapid transfer to freshwater and reside in fresh water (Griffith, '72), this fish has a low affinity for Na^+ (1,723 ± 223 μEq/l) and Cl^- (> 4,700 μEq/l) uptake. Both the sailfin molly (*Poecilia latipinna*), another euryhaline teleost that has been known to reside in freshwater bodies (high in hardness) in Florida (Evans, '73), and the stenohaline freshwater minnow (*Phoxinus phoxinus*) (Frain, '87) possess low affinities for Na^+ (molly: K_m = 8,000 μmol/l; minnow: K_m = 3,000 μmol/l; no data for Cl^-). Instead, other factors must be considered when assessing the ability to adapt to fresh water. Evans ('84) proposed that a reduction in the efflux component (i.e., reduction in permeability or surface area) and/or an increase in uptake by increasing the number of carriers (i.e., J_{max}) potentially has a larger role in maintaining ion bal-

ance when euryhaline fish enter fresh water than does increasing the affinity (i.e., decrease K_m).

Although Na^+ and Cl^- influxes were stimulated by the increasing external [NaCl] (Fig. 1A, B), the efflux rates of both ions remained unchanged (Fig. 2A). These results indicated that carrier-mediated exchange diffusion (Na^+/Na^+ and Cl^-/Cl^-) is not functioning in the mummichog. Although earlier studies examining exchange diffusion were artifactual due to changes in TEP, more recent studies that eliminated this problem provided strong evidence for exchange diffusion in rainbow trout (Goss and Wood, '90a,b). As for the mummichog, Na^+ and Cl^- effluxes are assumed to occur by simple diffusion through paracellular channels, as described in the freshwater ion transport model (Goss et al., '92; Marshall, '85; McDonald et al., '89).

Figure 2B presents evidence that ammonia excretion in *F. heteroclitus* is unaltered by changes in external [NaCl] and is therefore not directly coupled to Na^+ uptake (i.e., no Na^+/NH_4^+ exchange). In contrast, Wright and Wood ('85) and McDonald and Prior ('88) found a 1:1 correlation between Na^+ and ammonia excretion under certain conditions in freshwater rainbow trout. However, Maetz ('73), using goldfish, and Kersetter et al. ('70), using rainbow trout, varied $[Na^+]_{ext}$ and found no change in ammonia excretion. More recent studies on freshwater trout (Heisler, '90; Wilson et al., '94) suggest that Na^+/NH_4^+ exchange does not occur, that any linkage between Na^+ uptake and ammonia excretion is indirect, and that ammonia is excreted by NH_3 diffusion across the gills. This is likely the case in *F. heteroclitus*.

Injection studies

It is clear that a metabolic acidosis was induced by the HCl injection, as indicated by significant drops in both pH_a and $[HCO_3^-]$ (Fig. 3), and that approximately 50% of the acid load was eliminated within the 4 h following injection (based on the net H^+ fluxes determined by titration) (Fig. 4B). However, since water titration cannot distinguish between acid excretion and base uptake (McDonald and Wood, '81), the acidosis could have been corrected by either or both in vivo. To get an indication of which corrective measure was taken, we used the strong ion difference (SID) theory (Stewart, '83) as a tool to assess acid-base status independently of the water titration method. The way in which this is performed is to compare the differential ion fluxes. Briefly, Na^+ and Cl^- are the major strong cation and anion, respectively, that have appreciable fluxes across the branchial membrane. If a disparity between the Na^+ and Cl^- net fluxes exists, this indicates a charge imbalance which in freshwater fish has been equated to the net flux of acidic equivalents (i.e., $J^{H+}_{net} = J^{Cl}_{net} - J^{Na}_{net}$). A strong correlation has been reported between J^{H+}_{net}, as measured by titration, and the difference between the net Na^+ and Cl^- fluxes (Wood et al., '84; Kirschner, '96). By this reasoning, the greater rate of Cl^- loss over Na^+ loss that was measured following HCl injection (Fig. 4A) represents a net H^+ excretion. This acid excretion predicted by SID is in agreement with the J^{H+}_{net} that was measured by water titration (Fig. 4B). Furthermore, in the control series (NaCl injection), net Na^+ and Cl^- fluxes were not dissimilar (Fig. 5A), suggesting that an acid-base disturbance was not induced. This is confirmed by the net acid-base fluxes in which J^{H+}_{net} did not vary (Fig. 5B). We have found further agreement between the net Na^+ and Cl^- fluxes and net H^+ movement in subsequent studies using *Fundulus heteroclitus* (M.L. Patrick and C.M. Wood, unpublished results). Many other studies have reported similar accord between the two approaches for other freshwater fish (Wood et al., '84; Hyde and Perry, '87, '89; McDonald and Prior, '88; Perry et al., '87; McDonald et al., '89; Goss and Wood, '90a,b; Goss et al., '92; Kirschner, '96).

The application of SID in the present study not only tells us that a link does exists between ion and acid-base regulation in *Fundulus heteroclitus* but that the nature of this coupling departs from the established freshwater model. The net Na^+ gain and Cl^- loss following HCl injection were strictly due to the differential adjustment in the efflux components; Na^+ influx did not increase, and Cl^- influx remained negligible (Fig. 4A). In those freshwater fish that have been examined, acidosis is corrected by a stimulation of Na^+ uptake coupled in some way to H^+ excretion (see Introduction) and/or by an inhibition of Cl^- uptake coupled to HCO_3^- (base) excretion (reviewed by McDonald et al., '89; Wood, '91; Goss et al., '92; Kirschner, '96; Potts, '94). Instead, the mummichog excreted half of the acid load by differential modulation of Na^+ vs. Cl^- efflux (Fig. 4A). Ion efflux modulation for acid-base correction has been seen in rainbow trout but only in response to alkalosis, not acidosis (Goss et al., '92). The mechanisms for the control of differential efflux of Na^+ and Cl^- are yet to be defined in fish gill epithelia. However, Madara ('88) reported that tight junctions

of leaky epithelia can alter charge selectivity and structure, which in turn varies the resistance to paracellular ion flow. Therefore, selective regulation of the tight junction in gill epithelia would allow for the appropriate ion to diffuse through more easily. In the case of HCl-induced acidosis, anion permeability would be selectively elevated, whereas, in the case of NaCl loading, anion and cation permeability would be increased equally. This ability to differentially adjust Na^+ and Cl^- effluxes implies that a second connection between ion and acid-base regulation exists in addition to the well-known link between ion uptake and acid-base fluxes (see Introduction).

HCl injection stimulated both net ammonia and net H^+ excretion (Fig. 4B) without a concomitant increase of Na^+ uptake (Fig. 4A). It seems probable that NH_3 diffusive efflux was stimulated by an increase in the ammonia diffusion gradient across the gill due to local ammonia trapping by the H^+ excretion into the external medium immediately adjacent to the gill (i.e., boundary layer) (Wilson et al. '94). Enhanced ammonia production due to the disturbance by the experimental procedures may also have played a role, as increased ammonia excretion was also seen in the NaCl-injected group (Fig. 5B). Finally, it remains possible that the way in which acid was excreted through the paracellular route was via the passive diffusion of NH_4^+ (e.g., McDonald et al., '89), because mummichogs maintain a rather large cation permeability, as evidenced by the large Na^+ effluxes (Figs. 2, 4A, 5A). This high cation permeability may extend to include NH_4^+ and allow the predicted NH_4^+ diffusive loss. Experimentally, it would be very difficult to separate H^+ movement plus diffusion trapping of NH_3 from NH_4^+ diffusion; in terms of acid-base balance, they are functionally equivalent.

The metabolic alkalosis (increased blood pH and plasma HCO_3^-) (Fig. 3) exhibited by NaCl-injected fish was presumably an effect of disturbance, but its exact cause is unknown. Measurement of acid-base fluxes in this treatment (Fig. 5B) suggested that the environment was neither a source nor a sink for this base load, so it was presumably of internal origin. Interestingly, we have found that freshwater mummichogs have little ability to clear base loads (M.L. Patrick and C.M. Wood, unpublished results), in contrast to their effective clearance of an acid load seen in the present study.

Ionoregulatory mechanisms of the fresh water–adapted Fundulus heteroclitus

In conclusion, it would appear that the fresh water mummichog does not fit the current freshwater ion transport model which is based on experiments with salmonids, goldfish, and catfish. These differences include 1) a very low affinity but high capacity Na^+ uptake system; 2) the absence of a saturable Cl^- uptake system and indeed the absence of Cl^- uptake at the acclimation $[Cl^-]_{ext}$ level of 1.0 mmol/l, which is a typical freshwater concentration; 3) the absence of linkage between Na^+ and Cl^- influx and efflux components; 4) the markedly higher efflux of Na^+ than Cl^-; 5) the lack of coupling of Na^+ and Cl^- influxes to acid-base regulation; and 6) an ability to modulate Na^+ and Cl^- effluxes for the purpose of acid-base correction during acidosis. These very different characteristics of the mummichog's ionoregulation may reflect its estaurine habitat and strong euryhaline capabilities. The differences described here certainly suggest that a single model of ion transport is insufficient to describe the underlying mechanisms utilized by a wide variety of teleosts that are capable of adaptation to fresh water.

ACKNOWLEDGMENTS

The authors thank Dr. Richard J. Gonzalez for his helpful comments on the manuscript. This study was supported by NSERC operating grants to C.M.W. and W.S.M. and an NSERC foreign researcher award and grants from the Swedish Environmental Protection Agency and the C.F. Lundström Foundation to P.P. C.M.W. held the W.F. James Chair of Pure and Applied Science at St. Francis Xavier University during the initial stages of this research.

LITERATURE CITED

Bornancin, M., G. DeRenzis, and J. Maetz (1977) Branchial Cl transport, anion-stimulated ATPase and acid-base balance in *Anguilla anguilla* adapted to freshwater: Effects of hyperoxia. J. Comp. Physiol. [B], *117*:313–322.

Denoncourt, R.F., J.C. Fisher, and K.M. Rapp (1978) Freshwater population of the mummichog, *Fundulus heteroclitus*, from the Susquehanna River drainage in Pennsylvania. Estuaries, *1*:269–272.

DeRenzis, G. (1975) The branchial chloride pump in the goldfish *Carassius auratus*: Relationship between Cl^-/HCO_3^- and Cl^-/Cl^- exchanges and the effect of thiocyanate. J. Exp. Biol., *63*:587–602.

DeRenzis, G., and J. Maetz (1973) Studies on the mechanism of chloride absorption by the goldfish gill: Relation with acid-base regulation. J. Exp. Biol., *59*:339–358.

Eddy, F.B. (1975) Effects of calcium on gill potentials and on

sodium and chloride fluxes in the goldfish, *Carassius auratus*. J. Comp. Physiol., *96:*131–142.

Epstein, F.H., A. Manitius, E. Weinstein, A.I. Katz, and G.E. Pickford (1969) Sodium and potassium activated adenosine triphosphatase in kidneys of *Fundulus heteroclitus* adapted to fresh and salt water. Yale J. Biol. Med., *41:*388–393.

Evans, D.H. (1973) Sodium uptake by the sailfin molly, *Poecilia latipinna*: Kinetic analysis of a carrier system present in both fresh-water-acclimated and sea-water-acclimated individuals. Comp. Biochem. Physiol. A, *45:*843–850.

Evans, D.H. (1984) The roles of gill permeability and transport mechanisms in euryhalinity. In: Fish Physiology. W.S. Hoar and D.J. Randall, eds. Vol. 10, Academic Press, New York, pp. 239–283.

Evans, D.H., J.B. Claiborne, L. Farmer, C. Mallery, and E.J. Krasny (1982) Fish gill ionic transport: Methods and models. Biol. Bull., *163:*108–130.

Farrell, A.P., and P.L. Lutz (1975) Apparent anion imbalance in the fresh water adapted eel. J. Comp. Physiol., *102:*159–166.

Frain, W.J. (1987) The effect of external sodium and calcium concentrations on sodium fluxes by salt-depleted and non-depleted minnows *Phoxinus phoxinus* (L.). J. Exp. Biol., *131:*417–425.

Gonzalez, R.J., and D.G. McDonald (1992) The relationship between oxygen consumption and ion loss in a freshwater fish. J. Exp. Biol., *163:*317–332.

Gonzalez, R.J., C.H. Mason, and W.A. Dunson (1989) Anomalous tolerance to low pH in the estaurine killifish, *Fundulus heteroclitus*. Comp. Biochem. Physiol. [C], *94:*169–172.

Goss, G.G., and C.M. Wood (1990a) Na^+ and Cl^- uptake kinetics, diffusive effluxes and acidic equivalent fluxes across the gills of rainbow trout. II. Responses to bicarbonate infusion. J. Exp. Biol., *152:*549–571.

Goss, G.G., and C.M. Wood (1990b) Na^+ and Cl^- uptake kinetics, diffusive effluxes and acidic equivalent fluxes across the gills of rainbow trout. I. Responses to environmental hyperoxia. J. Exp. Biol., *152:*521–547.

Goss, G.G., and C.M. Wood (1991) Two substrate kinetic analysis: A novel approach linking ion and acid-base transport at the gills of the freshwater trout *Oncorhynchus mykiss*. J. Comp. Physiol. [B], *161:*635–646.

Goss, G.G., S.F. Perry, C.M. Wood, and P.L. Laurent (1992) Mechanisms of ion and acid-base regulation at the gills of freshwater fish. J. Exp. Zool., *263:*143–159.

Griffith, R.W. (1972) Studies on the Physiology and Evolution of Killifishes of the Genus Fundulus. Ph.D. thesis, Yale University, New Haven, CT.

Hannah, G.S., and G.E. Pickford (1981) Diurnal peaks in the hematocrit and in serum sodium in the killifish, *Fundulus heteroclitus*, and their absence in serum potassium and chloride: A lack of correlation. Comp. Biochem. Physiol. A, *70:*157–159.

Harck, A.F., and E.H. Larsen (1986) Concentration dependent of halide fluxes and selectivity of the anion pathway in toad skin. Acta Physiol. Scand., *304:*289–304.

Heisler, N. (1990) Mechanisms of ammonia elimination in fishes. In: Animal Nutrition and Transport Processes 2. Transport, Respiration, and Excretion: Comparative and Environmental Aspects, Comparative Physiology. J.P. Truchot and B. Lahlou, eds. Darger, Basel, pp. 137–151.

Hossler, F.E., G.M. Musil, K.J. Karnaky, and F.H. Epstein (1985) Surface ultrastructure of the gill arch of the killifish, *Fundulus heteroclitus*, from seawater and freshwater, with special reference to the morphology of apical crypts of chloride cells. J. Morphol., *185:*377–386.

Hyde, D.A., and S.F. Perry (1987) Acid-base and ionic regulation in the american eel (*Anguilla rostrata*) during and after prolonged aerial exposure: Branchial and renal adjustments. J. Exp. Biol., *133:*429–447.

Hyde, D.A., and S.F. Perry (1989) Differential approaches to blood acid-base regulation during exposure to prolonged hypercapnia in two freshwater teleosts: The rainbow trout (*Salmo gairdneri*) and the American eel (*Anguilla rostrata*). Physiol. Zool., *62:*1164–1186.

Iwata, M., and H.A. Bern (1985) Responses to salinity and the effect of prolactin on whole animal transepithelial potential in the Gobiid teleost, *Gillichthys mirabilis*. Gen. Comp. Endocrinol., *60:*434–440.

Karnaky, K.J. (1980) Ion-secreting epithelia: Chloride cells in the head region of *Fundulus heteroclitus*. Am. J. Physiol., *238:*33–39.

Karnaky, K.J. (1986) Structure and function of the chloride cell of *Fundulus heteroclitus* and other teleosts. Am. Zool., *26:*209–224.

Kerstetter, R.H., L.B. Kirschner, and D.A. Rafuse (1970) On the mechanism of sodium ion transport by the irrigated gills of rainbow trout, *Salmo gairdneri*. J. Gen. Physiol., *56:*342–359.

Kirschner, L.B. (1997) Extrarenal mechanisms in hydromineral and acid-base regulation in aquatic vertebrates. In: The Handbook of Physiology. W.H. Dantzler, ed. American Physiological Society, Bethesda, MD, pp. 577–622.

Klawe, W.L. (1957) Common mummichog and Newt in a lake on Digby Neck, Nova Scotia. Canadian Field Naturalist, *71:*154–155.

Madara, J.L. (1988) Tight junction dynamics: Is paracellular transport regulated? Cell, *53:*497–498.

Maetz, J. (1972) Branchial sodium exchange and ammonia excretion in the goldfish *Carassius auratus*. Effects of ammonia-loading and temperature changes. J. Exp. Biol., *56:*601–620.

Maetz, J. (1973) Na^+/NH_4^+, Na^+/H^+ exchanges and NH_3 movement across the gill of *Carassius auratus*. J. Exp. Biol., *58:*255–275.

Marshall, W.S. (1985) Paracellular ion transport in trout opercular epithelium models of osmoregulatory effects of acid precipitation. Can. J. Zool., *63:*1816–1822.

McDonald, D.G., and E.T. Prior (1988) Branchial mechanisms of ion and acid-base regulation in the freshwater rainbow trout, *Salmo gairdneri*. Can. J. Zool., *66:*2699–2708.

McDonald, D.G., and C.M. Wood (1981) Branchial and renal acid and ion fluxes in the rainbow trout, *Salmo gairdneri*, at low environmental pH. J. Exp. Biol., *93:*101–118.

McDonald, D.G., Y. Tang, and R.G. Boutilier (1989) Acid and ion transfer across the gills of fish: Mechanisms and regulation. Can. J. Zool., *67:*3046–3054.

Michal, G. (1985) Determination of Michaelis constants and inhibitor constants. In: Methods of Enzymatic Analysis, H.U. Bergmeyer, ed. Verlag Chemic, Weiheim, pp. 86–103.

Nemenyi, P., S.K. Dixon, N.B.J. White, and M.L. Hedstrom (1977) Statistics from Scratch. Holden-Day, Inc., San Francisco.

Pequeux, A., R. Gilles, and W.S. Marshall (1988) NaCl transport in gills and related structures. In: Advances in Comparative and Environmental Physiology. R. Greger, ed. Springer-Verlag, Heidelberg, pp. 1–73.

Perry, S.F., S. Malone, and D. Ewing (1987) Hypercapnic acidosis in the rainbow trout, *Salmo gairdneri*. I. Branchial ion fluxes and blood acid-base status. Can. J. Zool., *65:* 888–895.

Pickford, G.E., R.W. Griffith, J. Torretti, E. Hendlez, and F.H. Epstein (1970) Branchial reduction and renal stimulation of (Na^+, K^+)-ATPase by prolactin in hypophysectomized killifish in freshwater. Nature, *228:*378–379.

Potts, W.T.W. (1994) Kinetics of sodium uptake in freshwater animals: A comparison of ion-exchange and proton pump hypotheses. Am. J. Physiol., *266:*R315–R320.

Potts, W.T.W., and D.H. Evans (1966) The effects of hypophysectomy and bovine prolactin on salt fluxes in freshwater-adapted *Fundulus heteroclitus*. Biol. Bull., *131:* 362–368.

Potts, W.T.W., and D.H. Evans (1967) Sodium and chloride balance in the killifish *Fundulus heteroclitus*. Biol. Bull., *133:*411–425.

Potts, W.T.W., and G. Parry (1964) Osmotic and Ionic Regulation in Animals. Pergamon Press, Macmillan, New York.

Samaritan, J.M., and R.E. Schmidt (1982) Aspects of the life history of a freshwater population of the mummichog, *Fundulus heteroclitus* (Pisces: Cyprinodontidae), in the Bronx River, New York, U.S.A. Hydrobiologia, *94:*149–154.

Scott, W.B., and E.J. Crossman (1973) Freshwater fishes of Canada. Journal of the Fisheries Research Board of Canada, *184:*630–639.

Silva, P., R. Solomon, K. Spokes, and F.H. Epstein (1977) Ouabain inhibition of gill Na^+-K^+-ATPase: Relationship to active chloride transport. J. Exp. Zool., *199:*419–426.

Stewart, P.A. (1983) Modern quantitative acid-base chemistry. Can. J. Physiol. Pharmacol., *61:*1444–1461.

Verdouw, H., C.J.A. Vanechteld, and E.M.J. Dekkers (1978) Ammonia determinations based on indophenol formation with sodium salicylate. Water Research, *12:*399–402.

Wilson, R.W., P.M. Wright, R.S. Munger, and C.M. Wood (1994) Ammonia excretion in freshwater rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification: Lack of evidence for Na^+/NH_4^+ exchange. J. Exp. Biol., *191:*37–58.

Wood, C.M. (1991) Branchial ion and acid-base transfer in freshwater teleost fish: Environmental hyperoxia as a probe. Physiol. Zool., *64:*68–102.

Wood, C.M., and W.S. Marshall (1994) Ion balance, acid-base regulation, and chloride cell function in the common killifish *Fundulus heteroclitus*, a freely euryhaline estaurine teleost. Estuaries, *17:*34–52.

Wood, C.M., M.G. Wheatley, and H. Hobe (1984) The mechanisms of acid-base and ionregulation in freshwater rainbow trout during environmental hyperoxia and subsequent normoxia. III. Branchial exchanges. Respir. Physiol., *55:*175–192.

Wright, P.A., and C.M. Wood (1985) An analysis of branchial ammonia excretion in the freshwater rainbow trout: Effects of environmental pH change and sodium uptake blockade. J. Exp. Biol., *114:*329–353.

Zadunaisky, J.A. (1984) The chloride cell: The active transport of chloride and the paracellular pathways. In: Fish, Physiology, Volume 10, W.S. Hoar, and D.J. Randall, eds. Academic Press, New York, pp. 129–176.