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Ion and acid–base regulation in the freshwater mummichog (*Fundulus heteroclitus*): a departure from the standard model for freshwater teleosts

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Abstract

Ion and acid–base balance were examined in the freshwater-adapted mummichog (*Fundulus heteroclitus*) using a series of treatments designed to perturb the coupling mechanisms. Unidirectional Cl^- uptake (J_{in}^{Cl}) was extremely low whereas J_{in}^{Na} was substantial (three- to sixfold higher); comparable differences occurred in unidirectional efflux rates (J_{out}^{Cl}, J_{out}^{Na}). J_{in}^{Cl} was refractory to all treatments, suggesting that Cl^-/base exchange was unimportant or absent. Indeed, no base excretion or modulation of ion fluxes occurred for acid–base balance for up to 8 h after $NaHCO_3^-$ loading (injections of 1000 or 3000 nequiv.·g^{-1}). Acute environmental low pH (4.5) and amiloride (10^{-4} M) treatments caused concurrent inhibition of J_{in}^{Na} and net H^+ excretion (J_{net}^{H+}), indicating the presence of Na^+/H^+ exchange. J_{in}^{Na} was elevated and J_{net}^{H+} restored during recovery from both treatments, but this exchange did not appear to be dynamically adjusted for acid–base homeostasis. High external ammonia exposure (1 mmol·l^{-1}) initially blocked ammonia excretion (J_{net}^{Amm}) but had no effect on J_{in}^{Na}, whereas high pH (9.4) reduced both J_{net}^{Amm} and J_{in}^{Na}. Inhibition of J_{in}^{Na} by the low pH and amiloride treatments had no effect on J_{net}^{Amm}. These results indicate that ammonia excretion is entirely diffusive and independent of both Na^+ uptake and the protons that are transported via the Na^+/H^+ coupling. In addition, ureagenesis served as a compensatory mechanism during high external ammonia exposure, as a marked elevation in urea excretion partially replaced the inhibited J_{net}^{Amm}. In all treatments, changes in the Na^+–Cl^- net flux differential were consistent with changes in J_{net}^{H+} measured by traditional water titration techniques, indicating that the former can be used as an estimate of the acid–base status of the fish. Overall, the results demonstrate that the freshwater-adapted *F. heteroclitus* does not conform to the ion/acid–base relationships described in the standard model based on commonly studied species such as trout, goldfish, and catfish.

Keywords: Acid–base balance; Freshwater ion regulation; *Fundulus heteroclitus*; Mummichog; Killifish; Teleost; Ammonia excretion; Urea excretion

1. Introduction

Recently, we described unusual characteristics of the ion and acid–base transport system of the freshwater-adapted mummichog (killifish, *Fundulus heteroclitus*; [29]). These properties appear to fundamentally differ from the 'standard freshwater model' which is presented in most reviews based on work with other freshwater teleosts such as salmonids, goldfish, and catfish [10,11,16,19,25,36].

First, the turnover rate (i.e. unidirectional influx and efflux) of Na^+ was extremely high relative to other freshwater fish. Second, kinetic analysis of Na^+ uptake indicated a low affinity, high capacity system, independent of both ammonia excretion and Na^+ efflux (i.e.

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no exchange diffusion). Third, the turnover rate of Cl^- was extremely low (unidirectional influx was virtually zero at typical freshwater concentrations), and did not display saturation kinetics. Finally, the linkage between ion and acid–base transport appeared to be very different from that in other teleosts, where dynamic acid–base regulation is achieved by manipulation of Na^+ influx coupled to acidic equivalent excretion and Cl^- influx coupled to basic equivalent excretion. In contrast, when a systemic acidosis was induced by an intraperitoneal injection of 1000 nequiv.$\cdot g^{-1}$ HCl, the mummichog compensated by simultaneously attenuating Na^+ efflux and stimulating Cl^- efflux without altering either Na^+ or Cl^- influx rates. The resulting greater net Cl^- over Na^+ loss was associated with a net acid loss which was confirmed by direct measurement of acid–base fluxes using traditional titration methodology. This departs, for example, from HCl infusion studies on the rainbow trout in which Na^+ uptake was stimulated and Cl^- uptake reduced. These responses also constrain a net acid loss but do so via the direct coupling of the Na^+ and Cl^- uptake components to acidic and basic equivalent extrusion respectively [13].

These discrepancies encouraged us to further examine ion and acid–base regulation mechanisms and the nature of their coupling in freshwater-adapted *F. heteroclitus*. Systemic base loading (intraperitoneal $NaHCO_3$ injections at two dose levels) and a variety of environmental challenges (low pH, high pH, high external ammonia, amiloride exposure) known to disturb the mechanisms in characteristic fashion in 'standard' freshwater teleosts were employed. The results provide further evidence for a very different set of regulatory mechanisms in this species.

An additional goal was to determine if net Na^+ and Cl^- flux differential is a practical means for estimating acid–base balance in vivo. In our previous study [29] and in several other recent studies [11–16,24,25,30,40], net Na^+–Cl^- flux differentials have provided an alternate means of quantifying net acid–base balance in freshwater fish, independent of the traditional 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 or 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). In the present study, we concurrently measured both Na^+ and Cl^- fluxes *and* acid–base fluxes (by titration) under the various treatment conditions. The results provide further support for the utility of this approach, particularly under conditions where water chemistry confounds the titration approach (e.g. high pH, see Section 2).

2. Materials and methods

2.1. Holding conditions

Mummichogs, weighing 2.5–11.0 g, were collected from a brackish water estuary located near Antigonish, Nova Scotia, and were air-shipped to Hamilton, Ontario, Canada. Fish were held in 500-l fiberglass tanks containing 10% seawater at ambient temperature (18–20°C). The water was aerated and charcoal-filtered. Ten to 14 days prior to experimentation, 10–12 fish were placed in a tank containing 60 l of aerated, filtered and dechlorinated Hamilton tapwater with the following composition (all in mmol$\cdot l^{-1}$): Na^+, 0.6; Cl^-, 0.8; Ca^{2+}, 1.0; Mg^{2+}, 0.25; K^+, 0.04; titration alkalinity to pH 4.0 = 1.2; pH = 7.9–8.0. Fish were fed up to 4 days before the start of an experiment with a 1:2 mixture of Tetramin/Tetramarin.

2.2. Experimental protocols

The night prior to experiments, fish were weighed and placed in individual darkened 400-ml Nalgene beakers with lids. The fish were held in a static 350 ml volume with vigorous aeration for no longer than 6 h prior to experimentation. After the overnight settling period, the flux containers were drained, fresh water was added and control fluxes were initiated. The volume of water used in each study varied (see below) but water was changed every 3 h throughout the experimental period.

As detailed earlier [29], a series of tests were performed to see whether the water changeover procedures and/or the handling and needle insertion in the injection experiments caused any disturbance to the parameters measured. Briefly, we monitored ion and acid–base fluxes, two sensitive indicators of stress in fish. In each treatment, the chambers were periodically siphoned and refilled, a process which took less than 30 s. When this water changeover 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 significant effect on Na^+, Cl^-, and acid–base fluxes indicating that *F. heteroclitus* is not stressed by brief handling or air exposure. This contrasts with rainbow trout, in which a few seconds of handling alone can more than double Na^+ loss [8]. We are confident that our experimental methods minimized the potential for stress artifact.

2.3. Low pH

Control fluxes (pH 8.0) were initiated by the change of 300 ml of fresh water. After a 1-h settling period, isotope solution was added to each chamber (592 kBq of ^{24}Na and 111 kBq of ^{36}Cl) and allowed 10 min of mixing. Water samples (20 ml) were taken then (time 0) and 1.5 h later. Following the control period at circumneutral pH, the flux chambers were drained, flushed and refilled with 300 ml of water which had been titrated to pH 4.0 with 1 N H_2SO_4 and then vigorously aerated overnight prior to use. Following the isotope addition and 10 min mixing time, water samples (20 ml) were taken at 0 and 1.5 h. Following the 1.5-h sampling, water pH was measured using a Radiometer GK2401C pH electrode coupled to a PHM82 meter. A volume of 0.1 N H_2SO_4 sufficient to return the pH to 4.0 was added to each flux chamber, based on a predetermined titration curve for Hamilton tapwater in this pH range. The additional acid was allowed to mix for 10 min followed by water samples (20 ml) taken at 0 and 1.5 h again. Overall mean pH was 4.5. At the end of this 3 h of low pH exposure, the chambers were drained, flushed and filled with 300 ml fresh control water at circumneutral pH followed by isotope addition. Water samples (20 ml) were taken after 0, 1.5 and 3 h of recovery. All samples were subsequently analyzed for titratable acidity, ammonia, total Na^+, total Cl^-, ^{24}Na radioactivity, and ^{36}Cl radioactivity except at the 1.5-h point of control, experimental and recovery where the acid–base measurements were omitted.

Because of the large water volume used in this particular experiment, the change in titratable acidity value over the 1.5-h flux period was small and reduced the accuracy of the J_{net}^{TA} measurements. Therefore the beginning and end points were 0 and 3 h for J_{net}^{TA} during the control and recovery periods, but during the low pH exposure, where the pH was returned to 4.0 after 1.5 h, J_{net}^{TA} was calculated for each of the two fluxes and was averaged for the entire 3-h exposure as was J_{net}^{Amm}. From this J_{net}^{H+} was calculated.

2.4. High pH

The protocol was very similar to that for low pH (i.e. same time base, same radioisotopic additions and flushes), but with some minor modifications. Flux water volume was reduced to 200 ml per chamber and the experimental medium was freshwater which had been titrated to pH 10.0 using 1 N KOH and then aerated overnight prior to use. During the experiment, after 1.5 h of high pH exposure, water pH was measured and then an appropriate volume of 0.1 N KOH (again determined based on a pre-determined titration curve) was added to each chamber to bring the pH of the water back up to 10.0. Overall mean pH was 9.4. Titratable acidity (TA) and ammonia fluxes were measured at 1.5-h intervals during recovery, but TA measurements were excluded during the 3-h experimental period as tests indicated that they were confounded at high pH by the precipitation of carbonate salts as a result of CO_2 generation by the fish.

2.5. Amiloride

The protocol was again similar to that of the low pH exposure, but with the use of 200-ml volumes (as in the high pH study). The experimental medium was made by adding the appropriate amount of the potent Na^+ uptake antagonist amiloride HCl (Sigma) directly to Hamilton tapwater to achieve a 10^{-4} M solution. The medium was sonicated for 2 h to ensure dissolution of the amiloride and was aerated overnight. Water pH remained at 7.9. The flux protocol followed the same radioisotopic addition and flushing schedule as above but with sampling and all analyses at 0, 1.5, 3.0 h in each portion (control, experimental, recovery) of the regime.

2.6. High external ammonia

The experimental period was extended from 3 to 6 h for this study, water samples were taken every 1.5 h, and no recovery fluxes were performed. The experimental media contained 10^{-3} M total ammonia [T_{Amm}] (0.5 mmol·l^{-1} $(NH_4)_2SO_4$ in Hamilton tapwater) and was aerated overnight prior to use. The pH remained at 7.9. A 200-ml water volume was used throughout with water changes and radioisotopic additions at the beginning of the 3-h control period, and at 0 and 3 h during the experimental high [T_{Amm}] treatment.

2.7. $NaHCO_3$ injections

Intraperitoneal injections of 1000 and 3000 nequiv.·g^{-1} $NaHCO_3$ were used to induce systemic metabolic alkalosis. The experimental procedure for the 1000 nequiv.·g^{-1} series was identical to the earlier HCl injection series used to produce systemic metabolic acidosis, as described by [29]. Briefly, after the control radioisotopic flux period, the flux chambers were rinsed, and the fish were given an intraperitoneal injection of 1000 nequiv.·g^{-1} $NaHCO_3$ (7.1 μl·g^{-1} of a 140 mM $NaHCO_3$ solution) using a 50-μl gas-tight Hamilton syringe. The fish were then rinsed, blotted dry, inspected for visual signs of leakage (obviously leaking fish were rejected), and returned to their containers filled with fresh water, for a 15–20 min recovery period prior to the start of the experimental monitoring period (four 1-h flux measurements with rinsing and fresh radioisotopic additions at 0 and 2 h). A parallel control series (1000 nequiv.·g^{-1} NaCl injections, using 7.1

$\mu l \cdot g^{-1}$ of a 140 mM NaCl solution) was performed in parallel on a separate batch of fish, and has been presented earlier [29].

In light of the absence of a specific response to the 1000 nequiv.$\cdot g^{-1}$ $NaHCO_3$ load (see Section 3), a second series was performed with a threefold higher load, and a longer experimental monitoring period. After a 2-h control radioisotopic flux period, fish were injected with 3000 nequiv.$\cdot g^{-1}$ $NaHCO_3$ (7.1 $\mu l \cdot g^{-1}$ of a 420 mM $NaHCO_3$ solution) and rinsed as above prior to an 8-h experimental period (four successive 2-h flux measurements, with flushing and fresh radioisotope additions between each one). A 200-ml volume was used throughout.

2.8. Analytical methods and calculations

Ammonia concentrations (T_{Amm}) were determined by a micro-modification of the salicylate-hypochlorite assay [34], with specific adjustments to increase resolution of small changes against the high background T_{Amm} levels in the high external ammonia experiment [35]. Water samples from the high pH, high external ammonia exposures and the base injection series were assayed for urea nitrogen using the diacetyl monoxime assay modified for low urea concentrations [32]. Titratable alkalinity was determined by titration of 10-ml water samples with standardized HCl to pH 4.0 as described by McDonald and Wood [26]. The final minus the initial titratable alkalinity measurement for a flux period represents the total titratable base flux from the fish to the water, which is equivalent to the total titratable acid (TA) uptake into the fish from the water, and includes ammonia excreted as NH_3. The sum of the total ammonia flux (usually negative) and the total TA flux (usually positive), signs considered, represents the net acidic equivalent flux into the fish (if positive) or out of the fish (if negative). All measurements were made using a Radiometer GK2041C pH electrode coupled to a PHM82 pH meter. Titration acid (0.02 N) was dispensed using a Gilmont 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) and a coulometric titrator (Radiometer CMT-10), respectively. Water samples were assayed in duplicate for ^{24}Na radioactivity alone using a Packard 5000 series γ (gamma) counter. The ^{24}Na radioactivity was then allowed to decay to background levels (> 50 half-lives) before assaying for the β (beta) radioactivity of ^{36}Cl alone. Duplicate 2-ml water samples were prepared with 10 ml cocktail scintillant (ACS, Amersham) and counted for ^{36}Cl on a scintillation counter (LKB 1217 Rack Beta, Pharmacia-LKB).

Rates of uptake (J_{in}) of Na^+ and Cl^- (in nequiv.$\cdot g^{-1}\cdot$per h), as measured by disappearance of radioactivity from the water, were calculated from the following equation:

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

where *volume* is the water volume of the chamber (ml), cpm_1 and cpm_2 are the activities of the isotope (cpm$\cdot$ml^{-1}) at the start and end of the flux period respectively, and *SA* is the mean specific activity of the water (cpm$\cdot$nequiv.$^{-1}$)

The net ion flux (J_{net}) (in nequiv.$\cdot g^{-1}\cdot$per h), was calculated by:

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

where *volume* is the water volume of the chamber (ml), $[ion]_1$ and $[ion]_2$ are the water concentrations at the start and end of flux period (nequiv.$\cdot$ml^{-1}), respectively. The rate of efflux (J_{out}) was determined as the difference between J_{net} and J_{in}.

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

2.9. Statistical analysis

Results have been expressed as means $\pm$ S.E.M. (N) throughout. For multiple comparisons, analysis of variance (ANOVA) was applied, followed by Fisher's test of Least Significant Difference (LSD) in cases where the F-value indicated significance ($P \leq 0.05$).

3. Results

In all series, the fish were in approximate ion balance during the pre-exposure period (i.e. net fluxes of Na^+ and Cl^- not significantly different from zero), with unidirectional fluxes of Na^+ being approximately three- to sixfold higher than those of Cl^-. Ammonia and TA fluxes were comparable in magnitude to the unidirectional flux rates of Na^+.

3.1. Low pH

During both the first and second 1.5-h periods of exposure to pH 4.5, there was a 50% reduction of J_{in}^{Na}, while J_{out}^{Na} remained unchanged. The fish experienced a net loss of Na^+, significant in the second period (Fig. 1a). The small J_{in}^{Cl} was not inhibited by pH 4.5, but a significant net loss of Cl^- developed during the second 1.5 h at low pH due to elevated J_{out}^{Cl} (Fig. 1b). Upon return to circumneutral water, J_{in}^{Na} was significantly

elevated above control levels throughout the 3-h recovery period, and this alone was responsible for a substantial net Na^+ gain; J_{out}^{Na} again remained unchanged (Fig. 1a). In contrast, J_{in}^{Cl} did not change during recovery, whereas J_{out}^{Cl} remained elevated during the first recovery flux but was reduced in the second resulting in a positive J_{net}^{Cl} (Fig. 1b).

J_{net}^{Amm} remained unchanged throughout low pH exposure and recovery whereas J_{net}^{TA} increased significantly and J_{net}^{H+} became positive (i.e. net acid uptake) but not significantly different from zero during low pH treatment (Fig. 2a). When the fish were returned to control water at circumneutral pH, J_{net}^{TA} decreased to within control rates. A net acid uptake was indicated during the low pH exposure by the excess net Na^+ loss over Cl^- loss and during recovery a net acid loss indicated by the reversal of the strong ion flux differential (i.e. greater Cl^- over Na^+ loss) (Fig. 2b).

3.2. Amiloride exposure

External amiloride (10^{-4} M) reduced J_{in}^{Na} to a level approximately 40% of the control value resulting in a

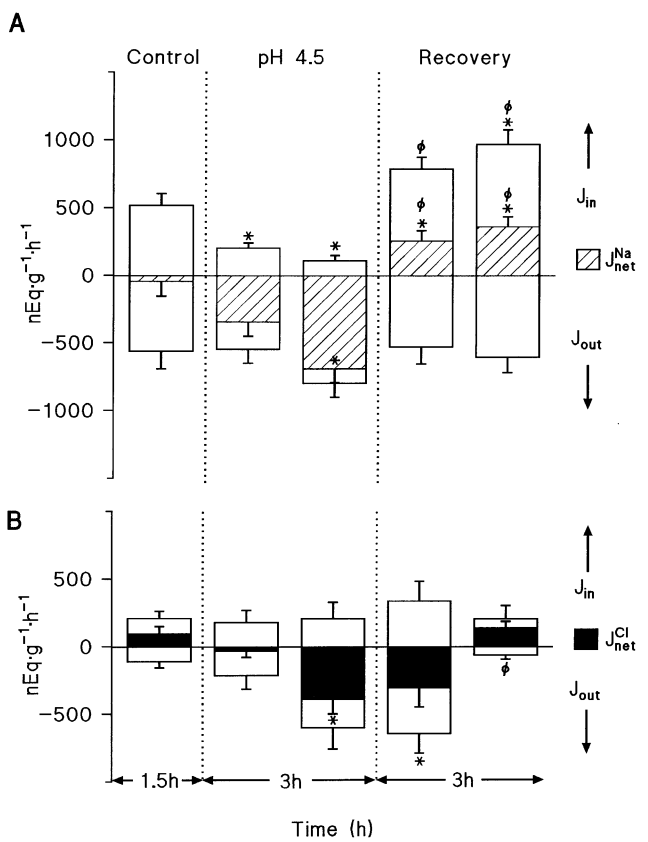

Fig. 1. The influence of low pH (pH 4.5) and subsequent return to circumneutral water upon whole body influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) of (A) Na^+ and (B) Cl^-. Means ± 1 S.E.M. $N = 15$ for Na^+ fluxes, $N = 8$ for Cl^-. *Denotes a significant difference from the control rates ($P \leq 0.05$). ϕ denotes a significant difference from the low pH treatment ($P \leq 0.05$).

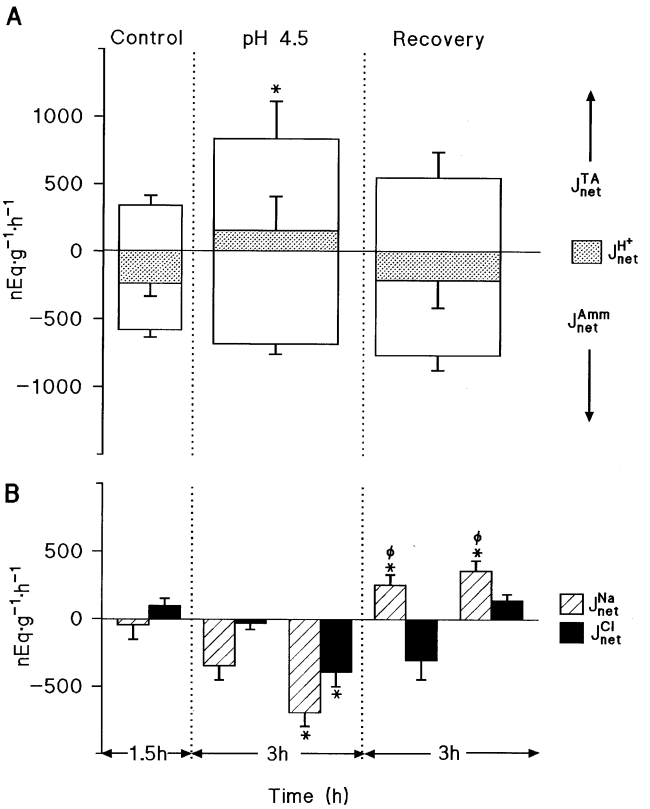

Fig. 2. The influence of low pH (pH 4.5) and subsequent return to circumneutral water upon whole body (A) net titratable acidity (J_{net}^{TA}), net acid movement (J_{net}^{H+}), net ammonia excretion (J_{net}^{Amm}) and (B) net flux (J_{net}) of Na^+ and Cl^-. Means ± 1 S.E.M. $N = 15$ for Na^+ fluxes, $N = 8$ for all other fluxes. *Denotes a significant difference from the control rates ($P \leq 0.05$). ϕ denotes a significant difference from the low pH treatment ($P \leq 0.05$).

significant net Na^+ loss (Fig. 3a). Upon return to control water, both J_{in}^{Na} and J_{net}^{Na} quickly resumed pre-exposure levels while J_{out}^{Na} did not vary throughout the experiment. Amiloride had no effect on the unidirectional Cl^- fluxes (Fig. 3b).

In addition to inhibiting Na^+ uptake (Fig. 3a), amiloride also significantly elevated J_{net}^{TA} and reversed net acid excretion (Fig. 4a). During recovery, J_{net}^{H+} rapidly returned to the negative pre-exposure levels. Although net acid movement was significantly modulated, J_{net}^{Amm} remained constant throughout the experiment (Fig. 4a). A net acid uptake during the amiloride treatment was also indicated by the greater net Na^+ loss over Cl^- loss (Fig. 4b). This difference between the net ion fluxes was reduced during the recovery period mirroring J_{net}^{H+} returning to a negative, control value (Fig. 4a).

3.3. High external ammonia

Exposure to a water ammonia concentration [T_{Amm}] of 1.0 mmol·l^{-1} lasted 6 h and over that time, neither Na^+ nor Cl^- unidirectional and net fluxes varied from

control values (Fig. 5a,b). In contrast, both J_{net}^{TA} and J_{net}^{Amm} were completely abolished but started to recover after 3 h of high external ammonia (Fig. 6b). By the final flux period (4.5–6 h), J_{net}^{TA} and J_{net}^{Amm} had approximately returned to control values. Over the entire exposure period, J_{net}^{H+} approximated J_{net}^{Amm} in amplitude, gradually becoming more positive (i.e. net acid gain), but none of these changes were significant (Fig. 6a). Similarly, there were no substantial differences between net Na^+ and Cl^- fluxes, though a net acid uptake was also indicated in the final flux period by net Na^+ loss exceeding net Cl^- loss (Fig. 6b). Urea-N excretion was immediately stimulated and continued to rise throughout the ammonia exposure (Table 1). Indeed the total amount of nitrogen excreted as urea (2 mol N/mol urea) approximated ammonia excretion by the final flux period (Fig. 6a). The ratio of J_{net}^{Amm} to J_{net}^{Urea} changed from 4:1 under control conditions to 1.3:1 after 6 h of high external ammonia exposure. Based on an estimate of the rise of internal ammonia (i.e. in the whole body fluids) due to inhibited J_{net}^{Amm} over the 6 h (Fig. 6a), the total urea-N excreted could effectively remove 25% of the buildup of internal ammonia-N.

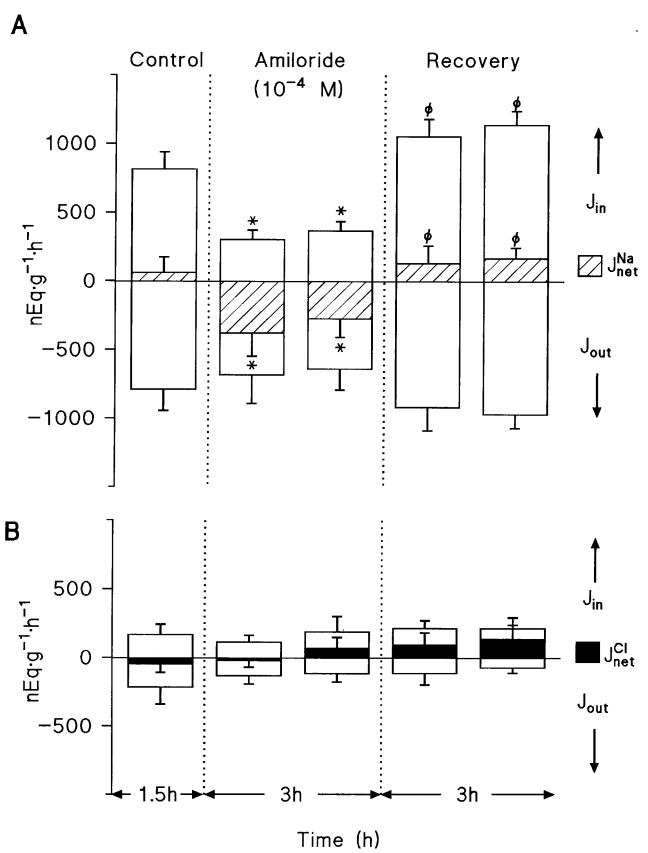

Fig. 3. The influence of amiloride treatment (10^{-4} M) and subsequent removal thereof upon whole body influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) of (A) Na^+ and (B) Cl^-. Means ± 1 S.E.M. $N = 8$. *Denotes a significant difference from the control rates ($P \leq 0.05$). ϕ denotes a significant difference from the amiloride treatment ($P \leq 0.05$).

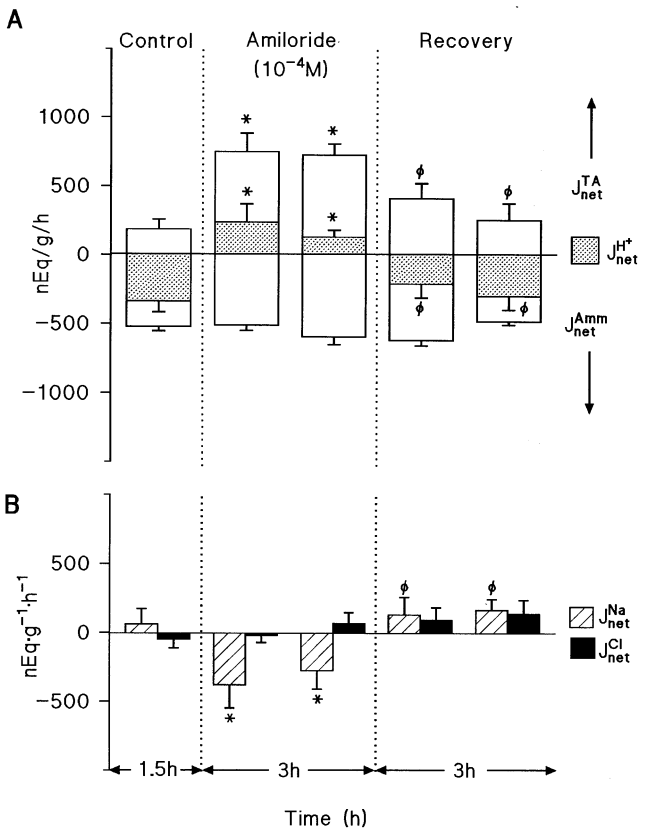

Fig. 4. The influence of amiloride (10^{-4} M) and subsequent return to circumneutral water upon whole body (A) net titratable acidity (J_{net}^{TA}), net acid movement (J_{net}^{H+}), net ammonia excretion (J_{net}^{Amm}) and (B) net flux (J_{net}) of Na^+ and Cl^-. Means ± 1 S.E.M. $N = 8$. *Denotes a significant difference from the control rates ($P \leq 0.05$). ϕ denotes a significant difference from the amiloride treatment ($P \leq 0.05$).

3.4. High pH

The responses to an average pH of 9.4 over 3 h (Fig. 7a,b) were similar to those seen at pH 4.5, in that both inhibited J_{in}^{Na} without altering J_{in}^{Cl}. Na^+ and Cl^- effluxes were not affected by high pH but a negative Na^+ balance did develop during the 3-h alkaline exposure (Fig. 7a). During the first 1.5 h of recovery, J_{net}^{Na} became positive and was significantly higher than control. This large net Na^+ gain was largely due to the significant reduction of J_{out}^{Na} at this time. By the second 1.5 h of recovery, all Na^+ flux components had returned to control values. Unlike pH 4.5 exposure (Fig. 1b), pH 9.4 treatment did not perturb J_{out}^{Cl} (Fig. 7b). However J_{in}^{Cl} exhibited a significant reduction during the second 1.5 h of recovery, resulting in a slightly greater net Cl^- loss.

Values for J_{net}^{TA} and J_{net}^{H+} have not been reported for the period of pH 9.4 exposure (Fig. 8a) due to the unusual water chemistry at this pH resulting in unreliable TA measurement (see Section 2). Relying on net Na^+ and Cl^- fluxes as indicators of acid–base status, there was no apparent disturbance during the high pH

treatment as net losses were equimolar (Fig. 8b). However, during recovery, the net Na^+ gain and net Cl^- loss indicated a substantial net acid loss which coincided with the significant and negative J_{net}^{H+} (Fig. 8b). J_{net}^{Amm} was significantly reduced during the first 1.5 h at high pH and completely inhibited during the second 1.5 h (Fig. 8a). In the recovery period, J_{net}^{TA} was significantly depressed throughout the 3 h, while J_{net}^{Amm} surpassed pre-exposure rates in the first 1.5 h. Urea-N excretion (Table 1) paralleled ammonia excretion (Fig. 8a) in that it was inhibited at pH 9.4 and stimulated upon return to circumneutral water.

3.5. $NaHCO_3$ injections

Intraperitoneal injection of 1000 nequiv.·g^{-1} $NaHCO_3$ produced responses (data not shown) virtually identical to those caused by intraperitoneal injection of 1000 nequiv.·g^{-1} NaCl (reported in Fig. 5 [29]). In both series, Na^+ and Cl^- net losses occurred during the first 2–3 h after injection, entirely due to elevated effluxes; J_{in}^{Na} and J_{in}^{Cl} remained constant. However there was no change in J_{net}^{H+}, in accord with negligible differences between net Na^+ and net Cl^- losses over the 4-h post-injection period.

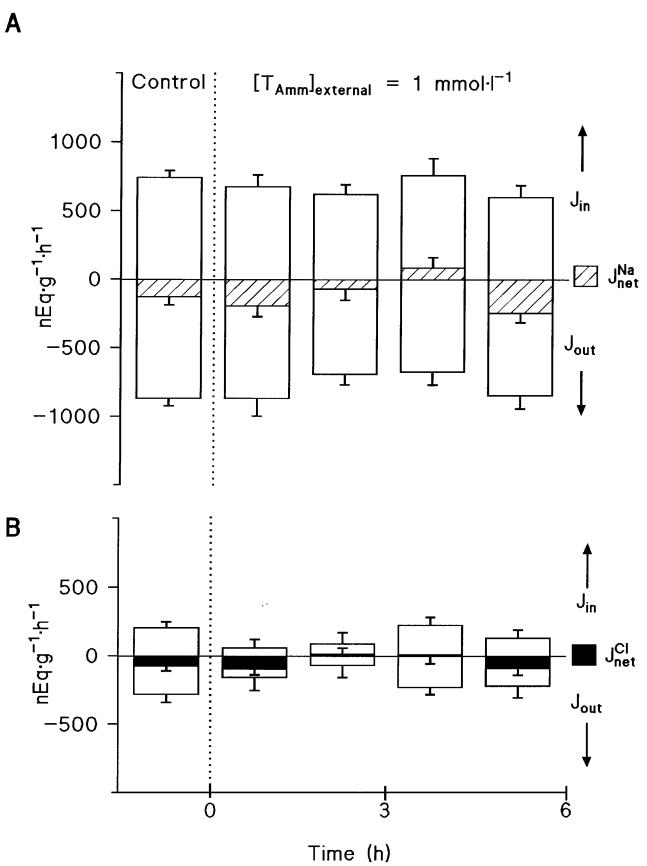

Fig. 5. The influence of high external ammonia (HEA) ([T_{Amm}] = 1 mmol·l^{-1}) upon whole body influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) of (A) Na^+ and (B) Cl^-. Means ± 1 S.E.M. $N = 8$. *Denotes a significant difference from the control rates ($P \leq 0.05$).

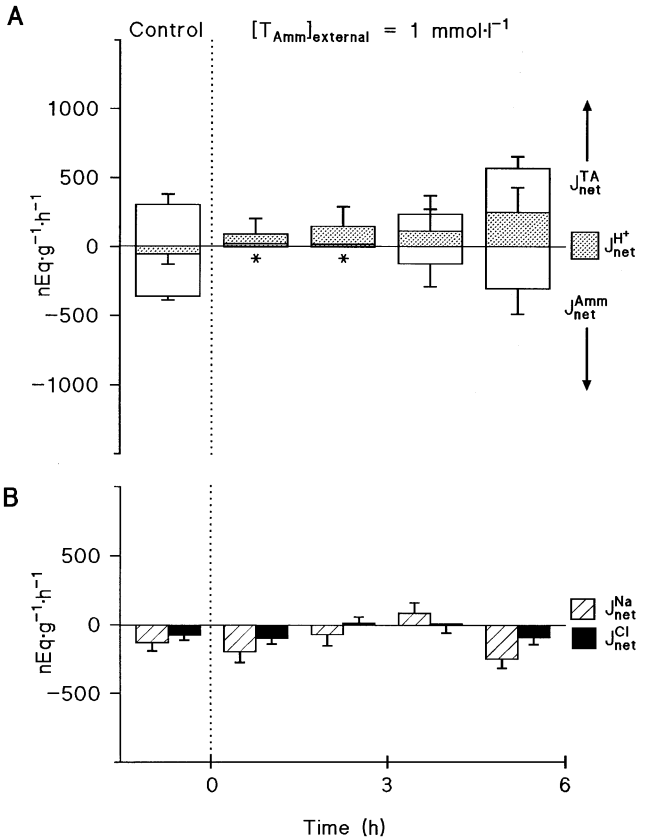

Fig. 6. The influence of high external ammonia (HEA) ([T_{Amm}] = 1 mmol·l^{-1}) upon whole body (A) net titratable acidity (J_{net}^{TA}), net acid movement (J_{net}^{H+}), net ammonia excretion (J_{net}^{Amm}) and (B) net flux (J_{net}) of Na^+ and Cl^-. Means ± 1 S.E.M. $N = 8$. *Denotes a significant difference from the control rates ($P \leq 0.05$). Note that during the first two fluxes at HEA, J^{TA} is not included (see text).

In order to confirm this lack of specific response to metabolic alkalosis, a second experiment was performed, with a threefold higher intraperitoneal $NaHCO_3$ load (3000 nequiv.·g^{-1}) and a longer (8 h) post-injection monitoring period. The pattern of response was again similar with unchanged J_{in}^{Na} and J_{in}^{Cl} (Fig. 9a,b). J_{out}^{Na} was again elevated during the first 2 h post-injection, resulting in significant Na^+ loss (Fig. 9a); similar, though non-significant changes occurred in J_{out}^{Cl} (Fig. 9b). J_{net}^{TA} and J_{net}^{Amm} remained unchanged after this high dose of $NaHCO_3$, and again there was no significant change in J_{net}^{H+} (Fig. 10a). The latter was in agreement with the fact that Na^+ and Cl^- losses remained virtually equal throughout the 8-h monitoring period (Fig. 10b).

In the absence of net base excretion, an alternate route for HCO_3^- removal could be via urea production [1]. However this was ruled out as urea-N excretion rate measured in the 3000 nequiv.·g^{-1} $NaHCO_3$ injection experiment was unchanged throughout the 8 h following injection (data not shown).

4. Discussion

The present study, in conjunction with our previous findings [29], reveals an ion/acid–base coupling in freshwater *F. heteroclitus*. However, several key features of this coupling depart from that described by the currently accepted ion transport model as for 'standard' freshwater teleosts (see Section 1).

The first major finding was the confirmation of an association between Na^+ uptake and the excretion of acidic equivalent by (1) the concurrent inhibition of J_{in}^{Na} and J_{net}^{H+} during low pH (Figs. 1 and 2) and amiloride exposures (Figs. 3 and 4) and (2) the rapid recovery of both fluxes during the post-exposure periods. Although this does not depart from the general freshwater model [16], our results support the presence of a Na^+/H^+ exchanger, rather than the other proposed mechanism of a Na^+ channel coupled to a H^+–ATPase which has recently been favored for fish such as freshwater salmonids [10,16,19,31]. In the amphibian epithelium, a system known to possess the Na^+ channel/H^+–ATPase arrangement, amiloride, which blocks the Na^+ uptake site on channels and exchangers [17], completely blocked Na^+ influx while H^+ flux remained unaltered by doses less than 5×10^{-4} M [6]. This suggests that these two processes are uncoupled when the Na^+ uptake is modulated by amiloride. However, when H^+ pumping is disrupted, Na^+ influx is diminished [31]. In contrast, exposure of *F. heteroclitus* to 10^{-4} M amiloride inhibited Na^+ uptake *and* H^+ excretion significantly (Figs. 3 and 4), a result which supports a mechanism in which these two fluxes are intimately connected (i.e. an exchanger). Also, the similarity in inhibitory patterns of low pH (Figs. 1 and 2) and amiloride (Figs. 3 and 4) exposures suggests that external protons, like amiloride, target the Na^+ uptake site directly (i.e. competitive inhibition). In doing so, both Na^+ and H^+ fluxes would cease. A recent study using an immunodetection assay identified a Na^+/H^+ exchanger in the branchial epithelium of seawater *F. heteroclitus* [3]. This transporter utilizes the inward Na^+ gradient in seawater to excrete protons in response to an acidosis. It is conceivable that this exchanger is maintained in the freshwater state and functions as the Na^+ uptake mechanism with the basolateral Na^+/K^+-ATPase and intracellular pH regulatory mechanisms maintaining the appropriate activity gradients. It would be of great interest to confirm the presence of the Na^+/H^+ exchanger by immunodetection in the freshwater mummichog.

The second major finding was confirmation of our earlier conclusion [29] that the freshwater *F. heteroclitus* is incapable of modulating either this Na^+ uptake/acidic equivalent excretion mechanism or a Cl^- uptake/basic equivalent mechanism so as to achieve dynamic acid–base balance. In most 'standard' freshwater teleosts, metabolic acidosis is corrected by elevat-

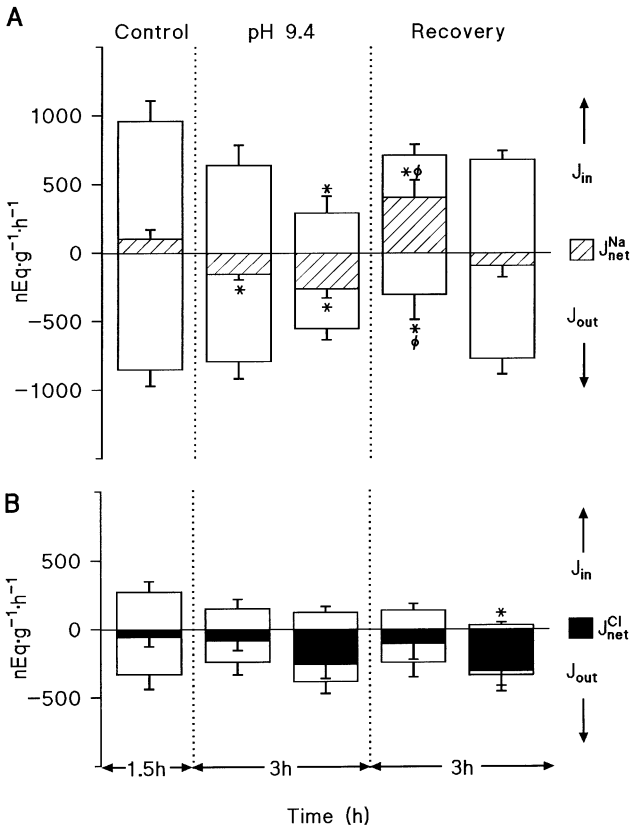

Fig. 7. The influence of high pH (pH 9.4) and subsequent return to circumneutral water upon whole body influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) of (A) Na^+ and (B) Cl^-. Means ± 1 S.E.M. $N = 8$. *Denotes a significant difference from the control rates ($P \leq 0.05$). ϕ denotes a significant difference from the high pH treatment ($P \leq 0.05$).

Table 1
Urea-N excretion rates (N nmol·g^{-1}·per h) from the high external ammonia (HEA) ($N = 8$) and high pH exposure ($N = 8$)[a]

		Urea-N excretion rates (N nmol·g^{-1}·per h)
High external ammonia exposure		
Control flux		89.5 ± 3.8
HEA	0–1.5 h	137.0 ± 10.8*
	1.5–3 h	127.6 ± 11.0*
	3–4.5 h	163.0 ± 14.3*
	4.5–6 h	234.1 ± 26.0*
High pH exposure and recovery		
Control flux		63.5 ± 9.3
pH 9.4	0–1.5 h	38.6 ± 21.1
	1.5–3 h	−25.0 ± 20.2*
Recovery	0–1.5 h	100.7 ± 14.4**
	1.5–3 h	89.8 ± 12.8**

[a] Note that J_{net}^{Urea} was measured in all experiments but only high pH and HEA exposure induced any changes. Mean ± S.E.M.
* Significantly different from control excretion rates ($P \leq 0.05$).
** Significantly different from the rates at pH 9.4 ($P \leq 0.05$).

ing J_{in}^{Na} and inhibiting J_{in}^{Cl} (effecting net acid excretion), while metabolic alkalosis is compensated by inhibiting J_{in}^{Na} and elevating J_{in}^{Cl} (effecting net base excretion; [10–13,16,24,25,31,40]). In the freshwater mummichog, neither J_{in}^{Na} nor J_{in}^{Cl} were altered during either metabolic acidosis [29] or metabolic alkalosis (Fig. 9a,b). Indeed metabolic acidosis was corrected by differential modulation of J_{out}^{Na} and J_{out}^{Cl} [29], whereas metabolic alkalosis was simply not corrected at all within the time frame (up to 8 h) of the experiments (Fig. 10a). These observations suggest that the Na^+ uptake/acidic equivalent excretion mechanism plays a 'housekeeping' role, rather than a role in dynamic acid–base balance, and that a Cl^- uptake/basic equivalent excretion mechanism is small or non-existent.

Both rainbow trout [13] and goldfish [4,5,21] compensated for a $NaHCO_3$ loading by stimulating Cl^- uptake and coupled base extrusion across the gill epithelium. However, two species of euryhaline eel (*Anguilla anguilla*, *Anguilla rostrata*), like the mummichog, lack an appreciable Cl^- uptake component and their

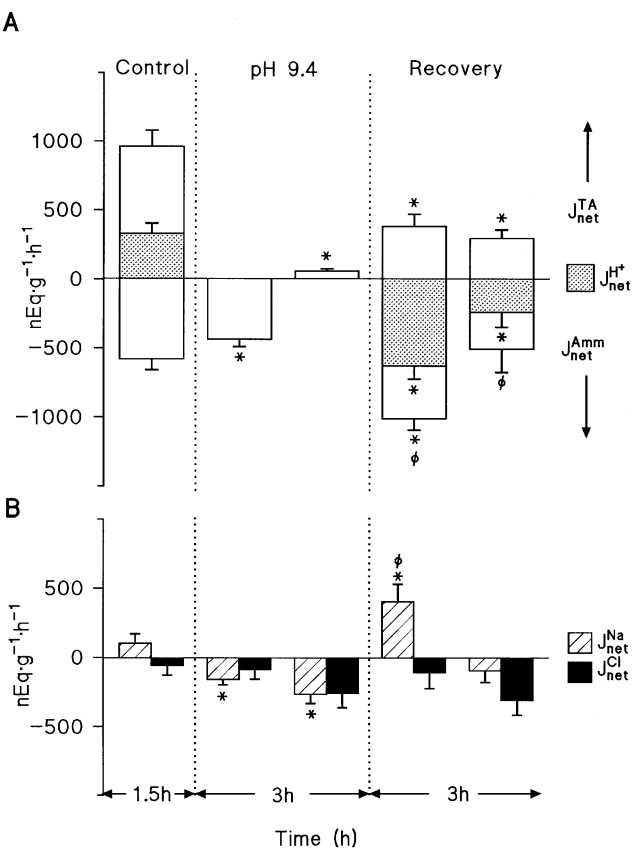

Fig. 8. The influence of high pH (pH 9.4) and subsequent return to circumneutral water upon whole body (A) net titratable acidity (J_{net}^{TA}), net acid movement (J_{net}^{H+}), net ammonia excretion (J_{net}^{Amm}) and (B) net flux (J_{net}) of Na^+ and Cl^-. Means ± 1 S.E.M. $N = 8$. *Denotes a significant difference from the control rates ($P \leq 0.05$). ϕ denotes a significant difference from the high pH treatment ($P \leq 0.05$). Note that J_{net}^{TA} and J_{net}^{H+} were not measured during high pH (see Section 2).

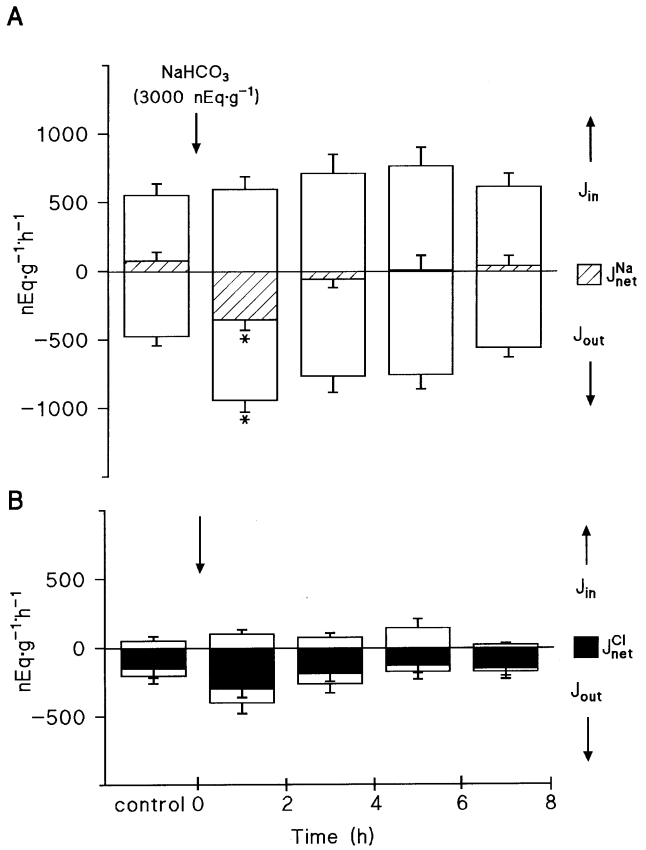

Fig. 9. The effect of an intraperitoneal injection of 3000 nequiv.·g^{-1} $NaHCO_3$ on whole body influx (J_{in}), efflux (J_{out}) and net flux (J_{net}) of (A) Na^+ and (B) Cl^-. Means ± 1 S.E.M. $N = 8$. *Denotes a significant difference from the control rates ($P \leq 0.05$).

ability to regulate acid–base status is limited by the absence of this Cl^-/HCO_3 coupling. Nevertheless these eels do compensate for certain acid–base disturbances by modulating Cl^- efflux in conjunction with Na^+ unidirectional fluxes [14]. For instance, following $NaHCO_3$ infusion, *A. rostrata* modulated branchial Cl^- efflux and Na^+ uptake to constrain a greater net Na^+ loss over Cl^- loss, and hence a net base excretion [9]. But *F. heteroclitus* distinguishes itself further in that it is incapable of correcting a systemic alkalosis (Fig. 10) using either Cl^- or Na^+ fluxes.

The third major finding was the complete uncoupling of ammonia excretion from Na^+ uptake. The low pH and amiloride treatments, and the recovery periods thereafter induced changes in Na^+ influx (Figs. 1a and 3a) without concurrent changes in J_{net}^{Amm} (Figs. 2a and 4a) whereas the high external ammonia exposure modulated J_{net}^{Amm} (Fig. 6a) while J_{in}^{Na} remained undisturbed throughout the 6 h (Fig. 5a). These results concur with the Michaelis–Menten saturation kinetics of Na^+ uptake which were completely independent of ammonia excretion as external Na^+ concentration was increased [29]. Remarkably, not only is ammonia excretion separate from Na^+ uptake but the outward NH_3 diffu-

sional gradient does not appear to be maintained by protons extruded by Na^+/H^+ transport. This departure was evident in the exposure to pH 4.5 (Fig. 2a), amiloride (Fig. 4a) and high external ammonia (Fig. 6a) in which J_{net}^{H+} became positive (i.e. net acid uptake rather than efflux) yet ammonia excretion continued. The ammonia diffusional gradient may rely upon the hydration of CO_2 by carbonic anhydrase located outside of the gill epithelium (i.e. gill boundary layer; [35]).

These Na^+–acid–ammonia relationships in *F. heteroclitus* depart from the general patterns shared among 'standard' freshwater species. Significant reductions of both J_{in}^{Na} and J_{net}^{Amm} during low pH exposure were measured in rainbow trout (*Oncorhynchus mykiss*; [18,23,41]), brown trout (*Salmo trutta*; [27]), goldfish (*Carassius auratus*; [20]), and also in amphibian skin [7], an effect attributed to ammonia excretion coupled to Na^+ uptake by either direct Na^+/NH_4^+ exchange or diffusion trapping by protons provided by H^+-coupled Na^+ uptake. Na^+–ammonia coupling was also evident in the responses of rainbow trout to 10^{-4} M amiloride [35,41] and of both goldfish [20,21] and rainbow trout [2,35] to high external ammonia levels.

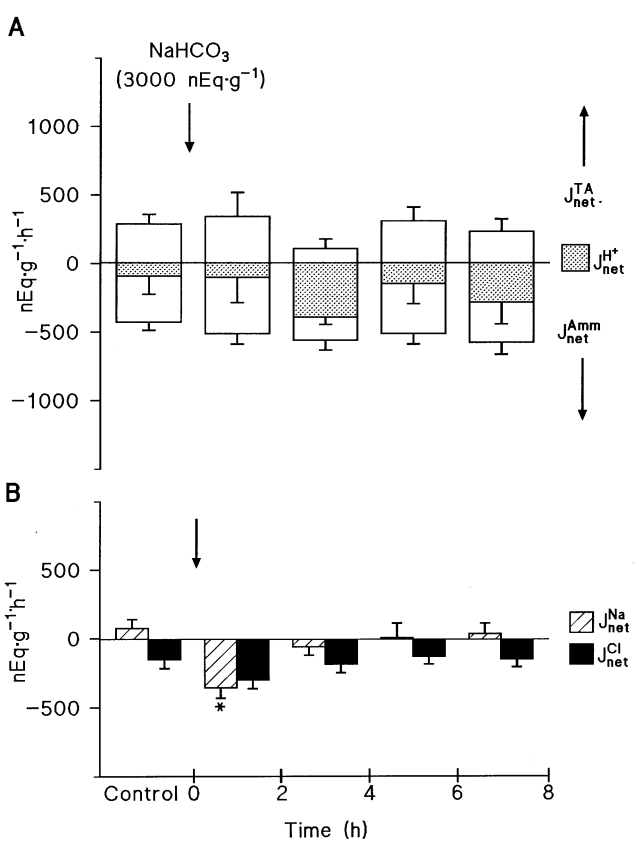

Fig. 10. The effect of an intraperitoneal injection of 3000 nequiv.·g^{-1} $NaHCO_3$ on whole body (A) net titratable acidity (J_{net}^{TA}), net acid movement (J_{net}^{H+}), net ammonia excretion (J_{net}^{Amm}) and (B) net flux (J_{net}) of Na^+ and Cl^-. Means ± 1 S.E.M. $N = 8$. *Denotes a significant difference from the control rates ($P \leq 0.05$).

Wilson et al. [35] attributed the recovery of J_{net}^{Amm} during high external ammonia exposure to enhanced boundary layer acidification for NH_3 diffusion trapping. In the mummichog, ammonia appeared to be excreted in the un-ionized form (NH_3) as evident by the equivalent recovery of J_{net}^{TA} (Fig. 6a). However, restoration of J_{net}^{Amm} was not due to increased boundary layer acidification as a net H^+ uptake occurred and indeed tended to increase throughout the exposure (Fig. 6a). Presumably, the resumption of NH_3 excretion was solely attributable to the restoration of the ΔP_{NH_3} gradient as plasma ammonia levels increased.

Another intriguing characteristic of *F. heteroclitus* was the immediate and significant increase of J_{net}^{Urea} coincident with the blockade of J_{net}^{Amm} during high external ammonia exposure (Table 1, Fig. 8a). J_{net}^{Urea} eventually reached about 80% of control ammonia excretion rates, and over 6 h accounted for about 25% of the 'missing' ammonia-N excretion which was presumably built up within the body fluids. J_{net}^{Urea} was also elevated after high pH exposure (Table 1), again a time of presumed high internal ammonia levels because of the reduction in J_{net}^{Amm} caused by pH 9.4 (Fig. 8a). However in this case, the increased urea excretion could alternately be explained as compensation for the inhibition of J_{net}^{Amm} (of unknown mechanism) which had also occurred during high pH exposure. Stimulated urea production could result from uricolysis or arginolysis [37]. However it is tempting to speculate whether the enzymes for a functional ornithine–urea cycle (OUC) are present in *F. heteroclitus*. The only two other teleosts known to initiate an immediate increase in urea excretion in response to ammonia loading are the Lake Magadi tilapia (*Oreochromis alcalicus grahami*; [33,39]) and the gulf toadfish (*Opsanus beta*; [28]) both of which are unusual in possessing a functional OUC. This pathway is not expressed in most adult teleosts [37]. However, the possibility that urea synthesis by the OUC served as a mechanism to void excess base [1] was ruled out by the $NaHCO_3$ loading which did not stimulate urea excretion.

Finally, the results of both this study and our earlier investigation [29] have presented a convincing agreement between net H^+ movement, as determined by titration methodology (Figs. 2a, 4a, 6a, 8a and 10a) and the differential net Na^+ and Cl^- fluxes (Figs. 2b, 4b, 6b, 8b and 10b). In each of the different experimental manipulations, the disparity between net Na^+ and net Cl^- fluxes concurred, at least qualitatively, with the net J_{net}^{H+} determined by titration. This finding is in general agreement with a number of other studies [11–14,16,25,40]. Clearly the fluxes of Na^+ and Cl^-, the two major extracellular ions, are those which are most important in constraining net acid–base fluxes between the fish and its environment, although under some unusual circumstances, other ions may also become

involved (e.g. K^+ fluxes during prolonged low pH exposure; [23,26]). Therefore in situations where water titration assays may not provide reliable measurements (e.g. the high pH medium—see Section 2), net acid–base status can be estimated satisfactorily by the disparity in the major cation and anion net fluxes. We feel that the measurement of Na^+ and Cl^- net flux disparity is a valid, practical, and methodologically independent alternative to titration techniques for following acid–base fluxes in vivo in freshwater teleosts.

To conclude, the in vivo ionoregulatory mechanisms of freshwater-acclimated *F. heteroclitus* clearly depart from the current model for ion transport in 'standard' freshwater teleosts. This conclusion agrees with recent in vitro studies on the opercular epithelium of freshwater-adapted *F. heteroclitus* where results again clearly differed from the expected 'standard' picture [22,38]. This should not be surprising in view of the fact that only a handful of the 10 000 different freshwater species have so far been examined, the majority of which are salmonids and cyprinids. Branchial morphological studies have been invaluable in elucidating the 'standard' model for such species [10]; their use is clearly called for in developing an alternate model for the freshwater killifish. As we extend our research to less commonly studied species, the need for alternate models may become even more apparent.

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