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# Plasticity of osmoregulatory function in the killifish intestine: drinking rates, salt and water transport, and gene expression after freshwater transfer

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## Summary

**We have explored intestinal function in the euryhaline killifish *Fundulus heteroclitus* after transfer from brackish water (10% seawater) to fresh water. Plasma $Na^+$ and $Cl^-$ concentrations fell at 12 h post-transfer, but recovered by 7 days. Drinking rate decreased substantially at 12 h (32% of control value) and remained suppressed after 3 and 7 days in fresh water (34 and 43%). By contrast, there was a transient increase in the capacity for water absorption measured across isolated intestines *in vitro* (3.3- and 2.6-fold at 12 h and 3 days), which returned to baseline after 7 days. These changes in water absorption could be entirely accounted for by changes in net ion flux: there was an extremely strong correlation ($R^2$=0.960) between water absorption and the sum of net $Na^+$ and net $Cl^-$ fluxes (3.42±0.10 μl water μmol$^{-1}$ ion). However, enhanced ion transport across the intestine in fresh water would probably not increase water uptake *in vivo*, because the drinking rate was far less than the capacity for water absorption across the intestine. The increased intestinal ion absorption after freshwater transfer may instead serve to facilitate ion absorption from food when it is present in the gut. Modulation of net ion flux occurred without changes in mRNA levels of many ion transporters ($Na^+/K^+$-ATPase $\alpha_{1a}$, carbonic anhydrase 2, CFTR $Cl^-$ channel, $Na^+/K^+/2Cl^-$ cotransporter 2, and the signalling protein 14-3-3a), and before a measured increase in $Na^+/K^+$-ATPase activity at 3 days, suggesting that there is some other mechanism responsible for increasing ion transport. Interestingly, net $Cl^-$ flux always exceeded net $Na^+$ flux, possibly to help maintain $Cl^-$ balance and/or facilitate bicarbonate excretion. Our results suggest that intestinal NaCl absorption from food is important during the period of greatest ionic disturbance after transfer to fresh water, and provide further insight into the mechanisms of euryhalinity in killifish.**

Key words: *Fundulus heteroclitus*, intestine, water absorption, ion flux, drinking rate, cortisol, Na/K-ATPase, NKCC, CFTR, carbonic anhydrase, fish.

## Introduction

The euryhaline killifish *Fundulus heteroclitus* lives in brackish water estuaries and salt marshes on the eastern coast of North America. The salinity of these habitats routinely fluctuates, so killifish must dynamically regulate ion balance in their natural environment. This species is exceptionally euryhaline, and can tolerate salinities ranging from fresh water to nearly four times sea water (Griffith, 1974). Killifish have therefore been used extensively in the past to understand the basic physiological mechanisms of osmoregulation in aquatic animals (reviewed by Wood and Marshall, 1994). More recently, killifish have been useful in understanding osmoregulatory plasticity, and are revealing many of the physiological mechanisms of euryhalinity in fish (Marshall, 2003).

For example, killifish rapidly reduce ion loss and activate ion uptake across the gills after freshwater transfer (Wood and Laurent, 2003). Ion loss is minimized by decreasing paracellular permeability (Karnaky, 1992; Scott et al., 2004b) and by suppressing active ion secretion *via* secretory ion transporters; the latter can occur by protein internalization (Marshall et al., 2002b), protein phosphorylation and inactivation (Marshall et al., 2000; Kültz et al., 2001), or suppression of ion transporter expression (Scott et al., 2004a). Ion uptake across the gills is known to be activated by increasing the expression and activity of absorptive ion transporters (Scott et al., 2004a; Scott et al., 2005a; Scott et al., 2005b), and by increasing cell proliferation (Katoh and Kaneko, 2003; Scott et al., 2005b) and differentiation (Marshall et al., 1999; Daborn et al., 2001; Katoh et al., 2001).

In contrast to gill function, much less is known about how intestinal function is modulated in response to freshwater transfer. Similar to other euryhaline fish, killifish acclimated to fresh water are known to have lower drinking rates than those

acclimated to seawater (Potts and Evans, 1967). This response presumably helps minimize potentially confounding water absorption in fresh water; however, little is known about the temporal pattern of the response, or whether water and ion transport across the intestine behave similarly. Regardless, killifish rapidly re-establish osmotic balance after transfer to fresh water (Jacob and Taylor, 1983; Scott et al., 2004a), so this modulation of intestinal function is effective.

Water absorption across the intestine of fish is primarily driven by transepithelial ion transport (Loretz, 1995; Schettino and Lionetto, 2003; Grosell et al., 2005). $Na^+$ and $Cl^-$ transport across the apical (luminal) surface of the intestine likely occurs through $Na^+/K^+/2Cl^-$ cotransporters (NKCC) and $Na^+/Cl^-$ cotransporters (but see Howard and Ahearn, 1988). Basolateral (serosal) $Na^+/K^+$-ATPase provides the electrochemical gradient for this process, and transports $Na^+$ across the basolateral surface. Apical $Cl^-$ absorption may also occur in exchange for $HCO_3^-$, which is formed by the hydration of $CO_2$ by carbonic anhydrase (CA) (Howard and Ahearn, 1988; Grosell et al., 2005). Chloride transport may also involve a CFTR $Cl^-$ channel (Marshall et al., 2002a).

The objective of the present study was to characterize intestinal function in the common killifish *Fundulus heteroclitus* after transfer to fresh water. Drinking rates, intestinal water and ion transport, $Na^+/K^+$-ATPase activity, plasma cortisol, and expression of genes potentially involved in ion transport were assessed at several times after transfer from brackish water to fresh water. Our initial hypotheses were that both drinking and intestinal ion and water transport would decrease after transfer, and that the latter would be coupled to changes in expression of the measured ion transport genes, as previously seen in the gills and opercular epithelium (Scott et al., 2004a; Scott et al., 2004b; Scott et al., 2005a; Scott et al., 2005b). Our results confirm some of these hypotheses but disprove others, and reveal surprising dissociations between drinking rate, fluid absorption and the molecular components of intestinal osmoregulation in killifish.

## Materials and methods

### *Experimental animals*

Adult killifish *Fundulus heteroclitus* L. (3–8 g body mass) were captured from estuaries near Antigonish, Nova Scotia, Canada. Fish were held in static charcoal-filtered Fiberglass tanks at a salinity of 10% seawater (3.5‰) made up with dechlorinated Hamilton, Ontario tap water ($[Na^+]$, 0.6 mmol $l^{-1}$; $[Cl^-]$, 0.7 mmol $l^{-1}$; $[Ca^{2+}]$ 1.0 mmol $l^{-1}$, $[Mg^{2+}]$ 0.2 mmol $l^{-1}$; hardness=120 mg $l^{-1}$ as $CaCO_3$; pH 8.0). Fish were maintained at room temperature (18–22°C) in a 14 h:10 h L:D photoperiod. Fish were fed once daily to satiation with a mix of commercial flakes (Wardley Total Tropical Gourmet Flake Blend, Hartz Mountain Corp., Secaucus, NJ, USA) and frozen brine shrimp (San Francisco Bay Brand, Newark, CA, USA). All animal care and experimentation was conducted according to McMaster University animal care protocol #02-10-61.

### *Salinity transfer protocol*

Fish were acclimated to a salinity of 10% seawater (brackish water) for at least 1 month before salinity transfer. Fish were transferred from brackish water to fresh water because (1) it is more environmentally representative of what killifish would naturally encounter in estuaries, (2) brackish water is the preferred salinity for killifish (Fritz and Garside, 1974), and (3) we sought to compare the results of the present work with previous studies using the same protocol. In experiments 1 and 3, drinking rates (experiment 1) and intestinal water and ion fluxes (experiment 3) were measured in fish before transfer, or 12 h, 3 days, or 7 days after transfer to fresh water. In experiment 2, the $Na^+$ and $Cl^-$ concentrations in the fluid phase of the gut contents were measured before and 12 h after transfer to fresh water. In experiment 4, mRNA expression in the whole intestine was measured 12 h, 3 days or 7 days after transfer either to brackish water (i.e. a sham treatment where animals were transferred to a new tank having the same salinity) or to fresh water. In experiment 5, $Na^+/K^+$-ATPase activity in intestinal segments as well as plasma cortisol were measured before and 12 h, 3 days, or 7 days after transfer either to brackish water or to fresh water. All fish transfers were made using a net. As feeding is known to be essential to keep the fish healthy after transfer to fresh water, in all series, feeding was continued until 24 h before the actual experiment, the same protocol as used previously (Wood and Laurent, 2003). The only exception was for one treatment in experiment 2 where an alternate protocol was evaluated (see below).

### *Experiment 1: drinking rates*

Drinking rates were measured in static polyethylene chambers containing 200 ml of the appropriate water. Chambers were fitted with a lid and aeration line and were wrapped in black plastic to minimize disturbance of the fish. The fish were allowed to settle for 2 h prior to measurement. At the start of each measurement period, approximately 8 μCi (0.29 MBq) of radiolabelled polyethylene glycol ($[^3H]$PEG-4000, 57.70 MBq $g^{-1}$; NEN Life Science Products Inc., Boston, MA, USA) was added to the chamber. A water sample (5 ml) was taken immediately for radioactivity measurements, as well as at 3 h and 6 h, when the experiment was ended. The fish was then killed with a lethal dose of tricaine methanesulfonate anaesthetic (0.8 g $l^{-1}$ MS-222; Syndel Laboratories, Vancouver, BC, Canada) neutralized with NaOH, the exact time was noted, and then the fish was removed from its chamber, rinsed in clean water, and weighed. A terminal blood sample was collected by caudal puncture using a modified 100 μl Hamilton syringe. Blood was transferred to lithium-heparinized capillary tubes, centrifuged at 500 ***g*** for 5 min, and the separated plasma used for $[^3H]$PEG-4000 radioactivity measurements. There was never any radioactivity in the plasma samples, indicating that $[^3H]$PEG-4000 was not absorbed but always stayed in the gastrointestinal tract. The gastrointestinal tract was then exposed by a ventral incision and ligated at both ends (anterior oesophagus and rectum) to prevent loss of contents. The entire gastrointestinal tract was removed,

weighed, and then digested in 0.8 ml of 2 mol $l^{-1}$ $HNO_3$ at 65°C for 48 h in a sealed vial. These samples were centrifuged, and supernatants (0.7 ml) were removed for radioactivity measurements. Drinking rate was calculated by determining the volume of external water taken into the tract from the radioactivity counts in the total tract digest and the reference water samples, and expressing this volume relative to the mass of the individual fish and the [$^3$H]PEG-4000 exposure time. The actual experimental period (approximately 6 h) was scheduled such that the nominal time (e.g. 12 h post-transfer) would be in the middle.

*Experiment 2: $Na^+$ and $Cl^-$ concentrations of the gut contents of killifish*

In experiment 2, fish were rapidly killed as in experiment 1. The whole intestinal tract was ligated immediately posterior to the oesophagus and at the anus, then removed. The entire gut contents were collected, and centrifuged at 10 000 ***g*** for 1 min. The supernatant was collected, and the volume of the supernatant and mass of the solid material were measured gravimetrically. $Na^+$ and $Cl^-$ concentrations were measured in the free supernatant. Ion concentrations were determined after either 24 h (when solid material was still present in the gut) or 3 days of starvation (when solid material remaining in the gut was much reduced or absent), to determine if the presence of food impacted the ionic composition of the gut fluids. In some fish, gut contents were too small to be extracted; data for such fish are not reported.

*Experiment 3: intestinal water transport and ion flux*

Before transfer to fresh water, and 12 h, 3 days and 7 days after transfer, fish were lightly anaesthetized in MS-222 (0.1 g $l^{-1}$), killed by a blow to the head, and then the blood was sampled, as described above. The plasma was frozen (–20°C) for later analysis of $Na^+$ and $Cl^-$ concentrations. The killifish lacks a distinct stomach (Babkin and Bowie, 1928), so the whole intestinal tract posterior to the oesophagus was removed. Heat-flared PE-50 polyethylene tubing was inserted and tied into the anterior end, and the tract was flushed thoroughly to clear any remaining chyme, using modified Cortland saline (Wolf, 1963). The composition, in mmol $l^{-1}$, was NaCl 133, KCl 5, $CaCl_2.2H_2O$ 1, $MgSO_4.7H_2O$ 1.9, $NaH_2PO_4.H_2O$ 2.9, glucose 5.5; pH 7.4. The sac was then filled with a 0.5 ml (exact volume) of this saline, which had been radiolabelled with 0.1 μCi $ml^{-1}$ (0.004 MBq $ml^{-1}$) $^{22}$Na (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA), the posterior end was tied closed, and the catheter was sealed with a pin. Saline (rather than brackish water or fresh water) was used in the sac to avoid passive water or ion movements due to osmotic gradients. Furthermore, the results from experiment 2, as well as those in previous studies (Shehadeh and Gordon, 1969), indicated that gut fluid *in vivo* is brought near isosmotic saline regardless of salinity or whether fish are starved or fed. A sample of the filling solution (1 ml) was taken for $^{22}$Na counting, and analysis of total $Na^+$ ion concentration ([$Na^+$]) and [$Cl^-$].

The sac was blotted on tissue paper in a standardized manner, weighed to 0.1 mg accuracy on an analytical balance, then suspended in a scintillation vial containing 11 ml of the same saline, but not radiolabelled. The external saline was continually bubbled with a humidified gas mixture containing 99.7% $O_2$ and 0.3% $CO_2$ (i.e. $P_{CO_2}$=2.25 mm Hg). The incubation period was 4 h, with sac mass recorded at 0 h, 2 h and 4 h, and samples of the external (serosal) solution (1 ml) taken at these same times. In addition, final samples were taken of the internal (mucosal) saline at 4 h for $^{22}$Na counting and analysis of total [$Na^+$] and [$Cl^-$]. The sac was cut open, and the gross area of the exposed epithelial surface determined by tracing its outline onto graph paper. Fluid transport was determined from changes in sac mass, and was linear over time. Net ion fluxes were calculated from measured changes in the net $Na^+$ and $Cl^-$ contents of the mucosal solution (volume × concentration) over the 4 h period. Unidirectional $Na^+$ fluxes were calculated from the appearance of $^{22}$Na counts in the serosal solution, and the specific activity (c.p.m. $nmol^{-1}$) for $Na^+$ of the mucosal solution. Unidirectional flux rate was calculated over the first 2 h period only to avoid the uncertainties of correcting for significant isotopic recycling in the second 2 h period. An exponential decay function was used to estimate the mean mucosal specific activity during the 2 h period. All flux rates were expressed as a function of surface area. A typical 4 g killifish had a gross intestinal surface area of about 10 $cm^2$.

*Experiment 4: intestinal gene expression*

In experiment 4, fish were killed by a blow to the head, followed by rapid decapitation, and the intestinal tract was removed and then immediately frozen in liquid nitrogen. Tissues were stored at –80°C until analyzed. RNA was extracted and reverse transcribed as previously described (Scott et al., 2004a; Scott et al., 2005a). Briefly, total RNA was extracted from tissues (approximately 20 mg) using Tripure isolation reagent (Roche Diagnostics, Montreal, QC, Canada). RNA concentrations were determined using a spectrophotometer and RNA integrity was verified by electrophoresis. Extracted RNA was stored at –80°C following isolation. First strand cDNA was synthesized by reverse transcribing 3 μg total RNA using 10 pmol oligo($dT_{18}$) primer and 20 i.u. RevertAid H Minus M-MuLV reverse transcriptase (MBI Fermentas, Burlington, ON, Canada).

Quantitative real-time PCR (qRT-PCR) analysis of gene expression has also been described previously (Scott et al., 2004a; Scott et al., 2005a; Scott and Schulte, 2005). Primer sequences for killifish cDNA, $Na^+/K^+$-ATPase $\alpha_{1a}$ (accession number AY057072) (Semple et al., 2002), cystic fibrosis transmembrane conductance regulator (CFTR) $Cl^-$ channel (acc. no. AF000271) (Singer et al., 1998), absorptive (apical) $Na^+/K^+/2Cl^-$ cotransporter 2 (NKCC2, acc. no. AY533707) and elongation factor 1α (EF1α, expression control; acc. no. AY430091) (Scott et al., 2004a), carbonic anhydrase 2 (CA2, acc. no. AY796057) (Scott et al., 2005a), and the signalling protein 14-3-3a (acc. no. AF302039) (Kültz et al., 2001) have been reported previously (Scott et al., 2004a; Scott et al., 2005a). These genes were chosen because we have previously

shown that they respond to salinity transfer in the gills and/or opercular epithelium of killifish. Quantification of gene expression by qRT-PCR was performed on an ABI Prism 7000 sequence analysis system (Applied Biosystems, Foster City, CA, USA). A randomly selected control sample was used to develop a standard curve for each primer set, and all results were expressed relative to these standard curves. Expression of each gene of interest was then standardized to expression of the EF1a gene, which does not change in the intestine following salinity transfer (data not shown), and were expressed relative to the 12 h brackish water control samples. All samples were run in duplicate (coefficients of variation were $\leqslant$10%). Control reactions were conducted with no cDNA template or with non-reverse transcribed RNA to determine the level of background or genomic DNA contamination, respectively. Genomic contamination was below 1:87 starting cDNA copies for all templates.

### *Experiment 5: Intestinal $Na^+/K^+$-ATPase activity and plasma cortisol*

In experiment 5, fish were rapidly killed as in experiment 1, intestines were removed, and blood was sampled and stored as described above. The intestinal tract was cut into anterior, middle, and posterior segments, which were immediately frozen separately in liquid nitrogen and then stored at –80°C until analyzed. $Na^+/K^+$-ATPase activity was determined by coupling ouabain-sensitive ATP hydrolysis to pyruvate kinase- and lactate dehydrogenase-mediated NADH oxidation as outlined by McCormick (McCormick, 1993), as we have previously reported (Scott et al., 2004a; Scott et al., 2005a). Plasma cortisol was determined by radioimmunoassay, as previously described (Scott et al., 2003).

### *Ion and radioactivity measurements*

Sodium and chloride concentrations were determined using flame atomic absorption spectrophotometry (SpectrAA-220FS, Varian, Mulgrave, VC, Australia) and coulometric titration (CMT-10 chloridometer, Radiometer, Copenhagen, Denmark), respectively. The only exception was in the assay of gut fluid samples, where the small volume obtained precluded use of the chloridometer, so chloride was measured by a colorimetric assay (Zall et al., 1956). The same certified NaCl standard (Radiometer) was used for all analyses. $^{22}Na$ radioactivities in mucosal and serosal saline samples were determined using a Minaxi Autogamma 5000 counter (Packard Instruments, Downers Grove, IL, USA). For [$^3$H]PEG-4000 radioactivities, 0.7 ml of the 2 mol $l^{-1}$ $HNO_3$ intestinal digest or 20 μl of plasma was added to 10 ml of an acid-compatible scintillation cocktail (Ultima Gold; Packard Bioscience, Meriden, CT, USA), and 5 ml water samples were added to 10 ml of an aqueous compatible cocktail (ACS; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Radioactivity was measured by scintillation counting (Rackbeta 1217; LKB Wallac, Turku, Finland). Quench was shown to be uniform across samples, and data were corrected for the slight difference in counting efficiencies between the two scintillation fluors.

### *Statistical analyses*

Data are expressed as means ± s.e.m. All data passed tests of normality and homogeneity of variance, so ANOVA (1-way or 2-way, where appropriate) was used to ascertain overall differences. In experiments 1–3, the effects of freshwater transfer were assessed by comparison with pre-transfer controls using Student–Newman–Keuls (SNK) *post-hoc* comparisons. Because gene expression, $Na^+/K^+$-ATPase activity and plasma cortisol levels can change as a result of handling the fish alone (Scott et al., 2004a), the effects of freshwater transfer in experiments 4 and 5 were assessed by comparing expression levels with time-matched brackish water controls using SNK *post-hoc* comparisons. The effects of handling in experiments 4 and 5 were assessed using SNK comparisons with 12 h brackish water controls or pre-transfer controls, respectively. All statistical analyses were conducted using Sigmastat version 3.0 and a significance level of $P<0.05$ was used throughout.

## Results

### *Drinking rates, plasma ions and cortisol after freshwater transfer*

Killifish drank significantly less after transfer to fresh water (Fig. 1). Before transfer from brackish water, the drinking rate of killifish was 1.32±0.22 ml $kg^{-1}$ $h^{-1}$, but 12 h after freshwater transfer the drinking rate decreased to 32% of the pre-transfer value. The reduced drinking rate persisted at 3 and 7 days after transfer, at 34% and 43% of the brackish water value.

Plasma [$Na^+$] and [$Cl^-$] were 143±5 and 142±7 mmol $l^{-1}$, respectively, in brackish water, but both fell significantly by about 20% at 12 h after transfer to fresh water (Fig. 2A). Thereafter, both recovered progressively, though the restoration of [$Cl^-$] appeared to be somewhat slower than that of [$Na^+$]. Plasma cortisol increased four- to fivefold at 12 h and 3 days after transfer to fresh water (Fig. 2B). This increase in cortisol was not due to handling, because there were no significant changes in plasma cortisol over time after brackish

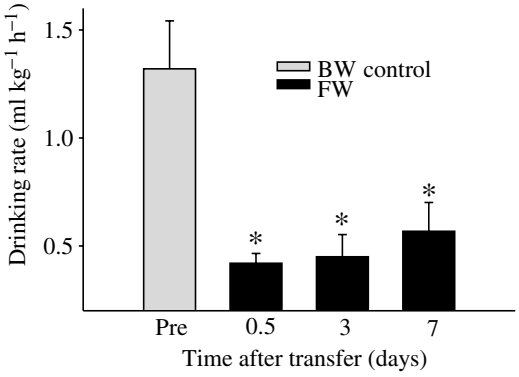


Fig. 1. Drinking rate of killifish decreases after transfer from brackish water (BW, 10% sea water; grey bars) to fresh water (FW; black bars). Values are means ± s.e.m. ($N\geqslant6$). *Significant difference from pre-transfer (Pre) brackish water control ($P<0.05$).

water to brackish water transfer, at least for the time points in this study.

### *Water and ion transport in the intestine after freshwater transfer*

The ion concentrations of gut fluids were high, ranging between 46–72% of the $Na^+$ concentration of physiological saline and 64–78% of the $Cl^-$ concentration (Table 1). Although freshwater transfer reduced [$Na^+$] of gut fluids (overall effect, determined by 2-way ANOVA), levels were still more than 100-fold higher than [$Na^+$] in imbibed freshwater. Furthermore, [$Cl^-$] of gut fluids did not change as a result of transfer to freshwater. The degree of starvation had no effect on the ion concentration of the gut fluids, though the solid mass of the gut contents was greatly reduced after 3 days of starvation. However, transfer to freshwater and prolonged starvation appeared to reduce the volume of gut fluids (all these comparisons relative to brackish water fish that had only been starved for 1 day; data not shown). Regardless, these results suggest that absorption of water and ions across the gut occurs from a mucosal solution that is closer in composition to extracellular fluids than to ingested water, and that this is not changed by the presence of food.

Table 1. *$Na^+$ and $Cl^-$ concentrations in the gut fluid of killifish*

| | Salinity | |
|---|---|---|
| | Brackish water | Fresh water |
| Killifish starved for 24 h | | |
| $Na^+$ (mmol $l^{-1}$) | 98.5±9.5 | 67.3±8.2 |
| $Cl^-$ (mmol $l^{-1}$) | 109.2±12.3 | 99.4±19.4 |
| Killifish starved for 3 days | | |
| $Na^+$ (mmol $l^{-1}$) | 80.3±10.2 | 62.0±6.9 |
| $Cl^-$ (mmol $l^{-1}$) | 91.5±8.5 | 90.1±13.2 |

Values are means ± s.e.m. ($N \geqslant 4$).

Fluid fractions of the gut contents were analyzed 12 h after transfer in freshwater killifish. The duration of starvation had no effect on gut ion concentrations. Freshwater transfer caused a significant reduction in gut [$Na^+$], when tested overall by 2-way ANOVA ($P<0.05$). There were no significant differences for any pair-wise comparisons.

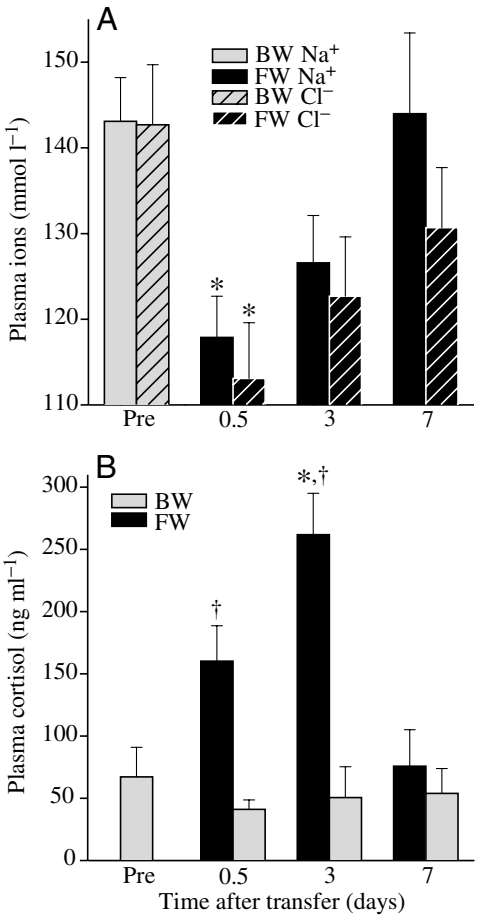


Fig. 2. (A) Plasma concentrations of $Na^+$ (solid bars) and $Cl^-$ (hatched bars) in killifish decrease after transfer from brackish water (BW, 10% seawater; grey bars) to fresh water (FW; black bars) and recover slowly thereafter. (B) Plasma cortisol concentrations increase transiently as a result of transfer to freshwater, compared to both pre-transfer and time-matched BW controls. Values are means ± s.e.m. ($N \geqslant 6$). *Significant difference from pre-transfer (Pre) brackish water control ($P<0.05$). †Significant difference between time-matched FW and BW groups.

Water absorption in killifish intestine increased transiently after transfer to freshwater (Fig. 3). Bulk water flow across isolated intestines was 0.00109±0.00031 ml $cm^{-2}$ $h^{-1}$ in brackish water, and increased by 3.3-fold at 12 h after transfer to freshwater. Bulk flow remained elevated (2.6-fold) 3 days after transfer, but by 7 days after transfer bulk flow was not significantly different from the brackish water value.

Similarly, net $Na^+$ and $Cl^-$ fluxes (inward direction) across the intestine were both elevated 12 h after transfer to freshwater (3.2- and 2.9-fold, respectively), and appeared to remain elevated at

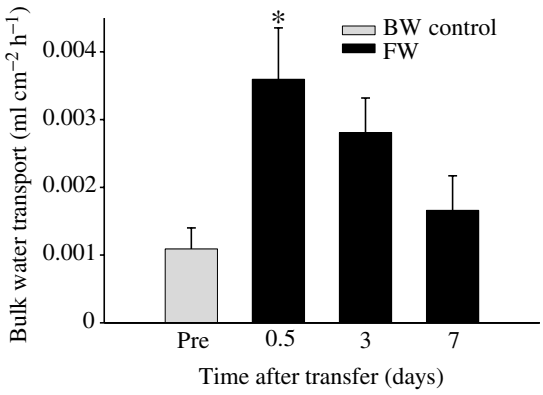


Fig. 3. Bulk water transport rate across the isolated intestine increases transiently in killifish, after transfer to fresh water (FW; black bars) from brackish water (BW, 10% seawater; grey bars). Net absorption occurs in the positive direction. Values are means ± s.e.m. ($N \geqslant 6$). *Significant difference from pre-transfer (Pre) brackish water control ($P<0.05$).

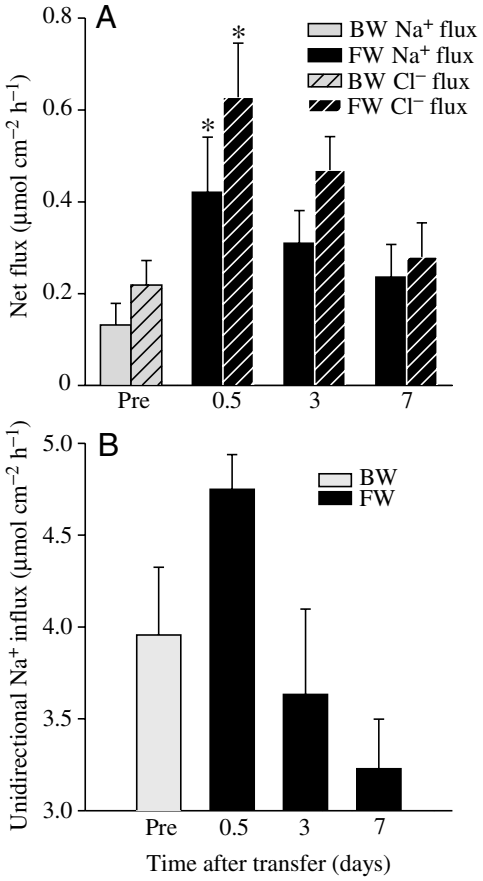


Fig. 4. (A) Net $Na^+$ (solid bars) and $Cl^-$ (hatched bars) flux across the isolated intestine increases transiently in killifish, after transfer to fresh water (FW; black bars) from brackish water (BW, 10% seawater; grey bars). (B) Unidirectional $Na^+$ influx across the isolated intestine does not change significantly after fresh water transfer, but the small non-significant increase is enough to account for the change of net $Na^+$ flux shown in A. Net absorption occurs in the positive direction. Values are means ± s.e.m. ($N \geqslant 6$). *Significant difference from pre-transfer (Pre) brackish water control ($P<0.05$). Net $Cl^-$ flux was found to be greater than net $Na^+$ flux by two-way ANOVA.

3 days (2.3- and 2.1-fold), before returning to brackish water levels at 7 days (Fig. 4A). Unidirectional $Na^+$ influx rates were 10–20-fold higher than net $Na^+$ flux rates (Fig. 4B). Although there were no statistically significant changes in unidirectional $Na^+$ influx rates after transfer, the slight non-significant increases observed are numerically large enough to account for the changes in net $Na^+$ flux rates. Net $Cl^-$ fluxes were greater than net $Na^+$ fluxes overall, as determined by 2-way ANOVA. Furthermore, the difference between net $Cl^-$ flux and net $Na^+$ flux appeared to increase transiently after transfer to freshwater: net $Cl^-$ flux exceeded net $Na^+$ flux by 0.086±0.049 μmol $cm^{-2}$ $h^{-1}$ in brackish water, and this increased to 0.207±0.062 μmol $cm^{-2}$ $h^{-1}$ and 0.158±0.053 μmol $cm^{-2}$ $h^{-1}$ at 12 h and 3 days post-transfer, and then returned to baseline levels (0.044± 0.028 μmol $cm^{-2}$ $h^{-1}$) by 7 days. However, these differences were not statistically significant.

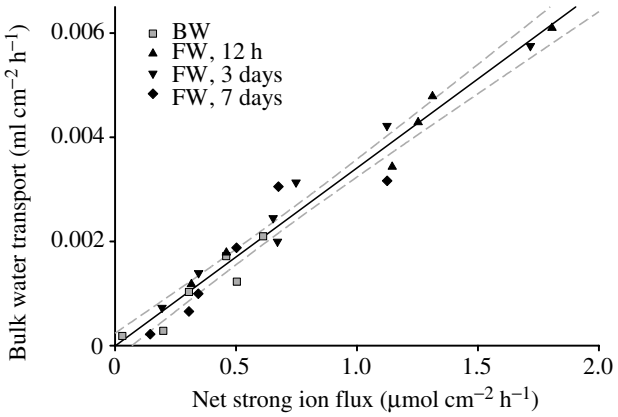


Fig. 5. Bulk water absorption rate correlated to net strong ion absorption rate (sum of net $Na^+$ and $Cl^-$ flux rate) across killifish intestine, in brackish water (BW, 10% seawater; grey squares) and at several time intervals after transfer to fresh water (FW; black triangles and diamonds). Bulk water transport was extremely well correlated to net ion transport ($r^2$=0.960, $P$<0.0001), with a slope of 0.00342±0.00010 ml water per μmol strong ion. Grey broken curves represent 95% confidence limits of the regression.

There was an extremely strong correlation (Fig. 5) between bulk water transport across the killifish intestine and net strong ion absorption rate (sum of net $Na^+$ and $Cl^-$ flux rates) ($r^2$=0.960). The slope of the regression, 0.00342±0.00010 ml water per μmol strong ion, indicates the relationship between the rates of water and ion absorption. Furthermore, the inverse of the slope indicates the 'apparent' strong ion concentration in the transported fluid, which was about 292 mmol $l^{-1}$.

### *Gene expression and $Na^+/K^+$-ATPase activity in the intestine after freshwater transfer*

Transfer to freshwater did not change the expression levels of any of the mRNAs analyzed in the intestine of killifish (Table 2). Levels of $Na^+/K^+$-ATPase $\alpha_{1a}$, $Na^+/K^+/2Cl^-$cotransporter 2 (NKCC2), and CFTR $Cl^-$ channel mRNAs all remained constant throughout the experiment, not changing as a result of either transfer to freshwater or time. Expression of carbonic anhydrase 2 (CA2) mRNA and mRNA for the signalling protein 14-3-3a were also unaffected by freshwater transfer (when compared to time-matched brackish water controls), but decreased throughout the experiment, suggesting that there could have been an effect of time (e.g. time after handling) on their expression.

The response of $Na^+/K^+$-ATPase activity to transfer to freshwater was analyzed in anterior, middle and posterior segments of the intestine (Fig. 6 and Table 3). Transfer to freshwater increased activity in the anterior portion of the intestine approximately 1.5-fold at 3 days after freshwater transfer (Fig. 6). By contrast, there were no effects of fresh water *per se* on activity in the middle or posterior segments (Table 3). There were significant effects of time on activity in

Table 2. *Gene expression after freshwater transfer in killifish intestine*

| Gene | Salinity | Time after transfer | | |
|---|---|---|---|---|
| | | 12 h | 3 days | 7 days |
| $Na^+/K^+$-ATPase $\alpha_{1a}$ | BW | 1.00±0.23 | 0.74±0.12 | 0.77±0.07 |
| | FW | 0.95±0.13 | 0.82±0.13 | 0.74±0.07 |
| NKCC2 | BW | 1.00±0.25 | 0.72±0.18 | 1.26±0.26 |
| | FW | 0.91±0.15 | 0.73±0.16 | 0.59±0.13 |
| CFTR | BW | 1.00±0.10 | 0.78±0.07 | 0.77±0.06 |
| | FW | 0.99±0.10 | 0.95±0.10 | 0.95±0.06 |
| CA2 | BW | 1.00±0.07 | 0.68±0.07* | 0.69±0.03* |
| | FW | 0.90±0.10 | 0.60±0.06* | 0.58±0.03* |
| 14-3-3a | BW | 1.00±0.19 | 0.82±0.07 | 0.56±0.03* |
| | FW | 1.24±0.15 | 0.68±0.07 | 0.67±0.06 |

Values are means ± s.e.m. ($N \geqslant 7$).

BW, brackish water control; FW, fresh water; NKCC2, $Na^+/K^+/2Cl^-$ cotransporters; CFTR, cystic fibrosis transmembrane conductance regulator; CA2, carbonic anhydrase 2; 14-3-3a, signalling protein 14-3-3a.

*Significant difference from 12 h BW group ($P<0.05$). There were no significant differences between FW and time-matched BW groups. qRT-PCR expression data are normalized to 12 h brackish water controls.

these segments, but the pattern of these differences were inconsistent between segments.

## Discussion

The ability of killifish to regulate water and ion homeostasis during environmental salinity fluctuations is exceptional, and depends on the concerted responses of many tissues, including gills, opercular epithelium, intestine and kidney. The mechanisms of euryhalinity at the gills and opercular epithelium are now reasonably well understood (Marshall, 2003; Scott et al., 2005a), but less is known about the intestine and kidney. In the present study we have enhanced our understanding of how intestinal function contributes to euryhalinity in killifish, and demonstrated that drinking rates and intestinal water and ion transport are dynamically and differentially regulated in response to freshwater transfer.

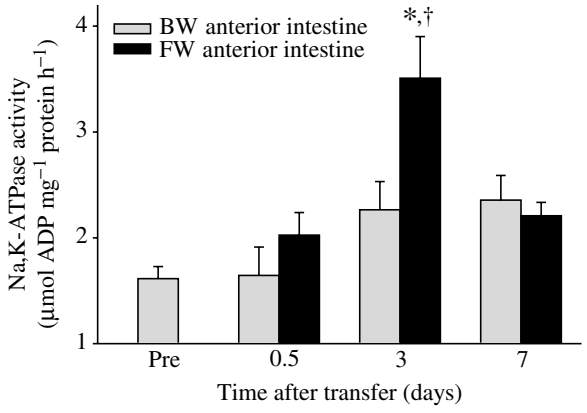


Fig. 6. $Na^+/K^+$-ATPase activity in the anterior segment of the intestine increases transiently in killifish, after transfer to fresh water (FW; black) from brackish water (BW, 10% seawater; grey). Values are means ± s.e.m. ($N \geqslant 8$). *Significant difference from pre-transfer ('pre') brackish water control ($P<0.05$). †Significant difference between time-matched FW and BW groups.

### *Plasticity of intestinal function after freshwater transfer*

Earlier reports indicated that killifish that are fully acclimated to fresh water drink at rates only 10–35% of those acclimated to seawater, and at rates 42% of those that are fully acclimated to near-isosmotic brackish water (40% seawater) (Potts and Evans, 1967; Malvin et al., 1980). Similar differences have been seen in other euryhaline species (e.g. Mozambique tilapia, *Oreochromis mossambicus*) (Lin et al., 2000). The results of the present study are in agreement with this pattern and with our original hypothesis: killifish transferred to fresh water reduced their drinking rate by 68% within 12 h, and drank only 32–43% as much as animals fully acclimated to 10% seawater over the next 7 days (Fig. 1). In some anadromous species (e.g. Atlantic salmon *Salmo salar* and Japanese eel *Anguilla japonica*), drinking rates are similar to those of killifish in seawater, but unlike in killifish, drinking appears to stop altogether in these species in fresh water (Fuentes and Eddy, 1997; Aoki et al., 2003). In the present study, the initial fall in drinking rate appears to recover slightly over time in fresh water. We are unaware of any previous studies examining the time course of drinking rates after transfer to fresh water, but drinking rates rose continually over time after transfer from fresh water to seawater in Atlantic salmon (Fuentes and Eddy, 1997).

After water was ingested, its composition in the gut was adjusted to levels that are reasonably close to the composition of extracellular fluids (Table 1). These results were expected based on previous studies in other fish species (Shehadeh and Gordon, 1969; Ando et al., 2003), and was true in both brackish water and fresh water, and regardless of whether or not food was present in the gut. When starved fish drink hyposmotic

Table 3. *$Na^+/K^+$-ATPase activity in middle and posterior segments of the killifish intestine*

| Segment | Salinity | Time after transfer | | | |
|---|---|---|---|---|---|
| | | Pre-transfer | 12 h | 3 days | 7 days |
| Middle | BW | 1.49±0.08 | 1.59±0.21 | 2.49±0.34* | 1.97±0.43 |
| | FW | | 2.13±0.14 | 1.78±0.19 | 2.44±0.26* |
| Posterior | BW | 2.21±0.37 | 1.46±0.26 | 2.89±0.47 | 3.71±0.61* |
| | FW | | 3.02±0.21 | 2.55±0.33 | 4.61±0.65* |

$Na^+/K^+$-ATPase activity (μmol $mg^{-1}$ protein $h^{-1}$) is expressed as means s.e.m. ($N \geqslant 8$).

BW, brackish water control; FW, fresh water.

*Significant difference from pre-transfer BW group ($P<0.05$). There were no significant differences between FW and time-matched BW groups.

water, salt is presumably secreted into the oesophagus or water is absorbed from it, before isosmotic fluid is reabsorbed in the intestine. Alternatively, when hyperosmotic water (or food) is consumed, water is added or salt is absorbed in the oesophagus (Ando et al., 2003). Therefore, although net absorption of water or ions from the environment depends on the composition of ingested water and food, the intestine absorbs water and ions from the fluid phase of chyme that is near-isosmotic. Clearly, ingested food is a much larger source of ions than ingested water when killifish are in fresh water.

In contrast to our initial hypothesis, and in contrast to the pattern in drinking rate, intestinal water absorption increased greatly after transfer to freshwater (Fig. 3). However, the response was transient, and had virtually ceased by 7 days. At 12 h post-transfer, the reduction in drinking rate to 32% of the brackish water value was coupled with a 3.3-fold increase in water absorption across isolated intestine. This might suggest that water absorption by the intestine *in vivo* falls more slowly than indicated by the drinking rate data alone, but another interpretation is more likely, and is discussed in a later section. Interestingly, eels acclimated to fresh water have similar intestinal water absorption rates to the killifish in this study, but in seawater-acclimated eel, intestinal water absorption rates are substantially higher (Ando, 1975; Aoki et al., 2003).

### *Contribution of the intestine to water and ion homeostasis in vivo*

Even though the ionic composition of ingested fluid is adjusted once it enters the gut (Table 1) (Shehadeh and Gordon, 1969), water absorption from the environment by the intestinal tract *in vivo* cannot exceed the drinking rate. An approximate but instructive comparison can be made between the drinking rates recorded *in vivo*, and the bulk water absorption rates measured *in vitro*. Assuming that a 4 g killifish has an intestinal surface area of about 10 $cm^2$ (see Materials and methods), the estimated absorption rate would be about 2.5 ml $kg^{-1}$ $h^{-1}$ in brackish water, not too far from the measured drinking rate of 1.32 ml $kg^{-1}$ $h^{-1}$, indicating that supply and absorptive capacity are approximately matched. However after 12 h in fresh water, the absorptive capacity is about 8.75 ml $kg^{-1}$ $h^{-1}$, far in excess of the measured drinking rate (supply rate) of 0.42 ml $kg^{-1}$ $h^{-1}$. This calculation does not take into account additional fluid on the supply side that may be secreted *in vivo* by the oesophagus, biliary and pancreatic systems, etc.; nevertheless, it suggests that the observed bulk water transport rates recorded *in vitro* after transfer to fresh water do not occur *in vivo*.

What is the meaning of this discrepancy? At 12 h post-transfer, plasma [$Na^+$] and [$Cl^-$] (Fig. 2), as well as whole body concentrations of these ions (Wood and Laurent, 2003) have fallen precipitously, but thereafter start to recover. Similar patterns have been seen in many previous studies on killifish transferred to fresh water (Jacob and Taylor, 1983; Wood and Laurent, 2003; Scott et al., 2004a; Scott et al., 2004b). We suggest that the increased net $Na^+$ and $Cl^-$ absorption, from chyme that would normally be present in the intestinal tract of animals that are feeding, is critical to ionic homeostasis at this time. In other words, killifish probably increase ion absorption to access the large reservoir of ions in food, not to absorb more water. Concurrent increases in the capacity for water absorption *in vitro* occur as a consequence of the increased ion absorption, but net water absorption from the environment would be minimal *in vivo* because of the reduced drinking rate (Fig. 1). Similar calculations to those outlined above indicate that the measured capacity for net ion absorption after 12 h in fresh water would amount to about 100 μmol $kg^{-1}$ $h^{-1}$ for $Na^+$ and 150 μmol $kg^{-1}$ $h^{-1}$ for $Cl^-$, when measured unidirectional uptake rates at the gills have dropped to about 700 and 0 μmol $kg^{-1}$ $h^{-1}$, respectively, and net gill fluxes of both ions remain negative (Wood and Laurent, 2003). Because ion supply rate from drinking is so low in fresh water, this capacity for intestinal ion absorption could only be realized *in vivo* in animals that are feeding. The role of food in the normal ionic homeostasis of fish is often overlooked, but has been recently highlighted by Marshall and Grosell (Marshall and Grosell, 2005) as an important area for future work. We know that feeding is essential to keep killifish healthy in fresh water (Wood and Laurent, 2003), as it is in salmonids when gill uptake mechanisms are impaired, such as during low pH exposure (D'Cruz and Wood, 1998). Indeed, gut uptake of $Cl^-$ must be critically important in killifish and other species (e.g. eel, *Anguilla* sp., and bluegill, *Lepomis macrochirus*) that lack branchial $Cl^-$ uptake in fresh water (reviewed by Tomasso and Grosell, 2005).

Even though drinking rate is reduced in fresh water, if water

uptake across the intestinal tract occurs *in vivo* as a result of heightened ion absorption, the kidney undoubtedly has the capacity to deal with it. Glomerular filtration rate (GFR) measured in fresh water in a previous study (Scott et al., 2004b) is approximately 15-fold higher than the drinking rates measured in the present study, and 4-fold higher than the sum of drinking rate and extrarenal clearance rate (an index of whole-body water permeability) (Scott et al., 2004b). Water excretion by the kidney therefore appears more than capable of maintaining total whole-body water content in fresh water.

*Molecular responses to transfer to freshwater*

In contrast to our initial hypothesis, the expression levels of a number of genes, inferred from mRNA levels, did not change after transfer from brackish to fresh water (Table 2). Although mRNA expression of two genes (those of carbonic anhydrase 2 and the signalling protein 14-3-3a) tended to decrease over time, this was not due to an effect of transfer to freshwater, but rather an effect of handling itself. Expression of $Na^+/K^+$-ATPase $\alpha_{1a}$, $Na^+/K^+/2Cl^-$ cotransporter 2, and CFTR $Cl^-$ channel remained constant in all fish throughout the experiment. This is markedly different from the patterns we have observed earlier in gills and opercular epithelium from killifish subjected to an identical brackish water to fresh water transfer (Scott et al., 2005a). In this previous study, mRNA expression of CFTR went down and that of 14-3-3a went up in both tissues, while carbonic anhydrase 2 expression changed in opposite directions in the two tissues. Note that mRNA levels of the absorptive $Na^+/K^+/2Cl^-$ cotransporter 2 were assayed in the intestine in the present study, so it cannot be compared with the downward mRNA response of the secretory $Na^+/K^+/2Cl^-$ cotransporter 1 reported in gills and opercular epithelium (Scott et al., 2005a).

In contrast to $Na^+/K^+$-ATPase $\alpha_{1a}$ expression, $Na^+/K^+$-ATPase activity increased in the anterior portion of the intestine after freshwater transfer (Fig. 6), and the time course of this increase was roughly similar to that of plasma cortisol (Fig. 2B). One plausible conclusion from these data is that the changes observed in intestinal ion transport rates are partly due to post-transcriptional regulation of $Na^+/K^+$-ATPase activity, and that these changes may be partly controlled by plasma cortisol. Post-transcriptional regulation is thought to be important in killifish gills and opercular epithelium (Marshall, 2003; Scott et al., 2005a), and cortisol is known to be important for several aspects of ion transport physiology in the intestine (Veillette et al., 1995; Lin et al., 2000). However, in the present study, the increase in $Na^+/K^+$-ATPase activity in the anterior portion of the intestine occurred later (at 3 days) than the largest rise in ion transport (at 12 h), suggesting that other molecular mechanisms must be important during the early stages of freshwater transition.

The molecular mechanisms responsible for changes in ion transport across killifish intestine in these early stages do not appear to include transcriptional regulation of the CA2, CFTR or NKCC2 isoforms measured in this study. Transcriptional regulation of other isoforms and/or ion transport proteins may instead be important after freshwater transfer. However, mRNA for all genes examined in the present study were expressed at high levels in the intestine (data not shown), which implies that they may still have an important role in the intestine. If these genes are important during freshwater transition, they could be regulated by post-transcriptional mechanisms. Understanding the molecular basis for ion transport in the killifish intestine deserves further study.

*Relative $Na^+$, $Cl^-$ and water transport rates in vitro*

The strong correlation between water transport rate and the net flux rates of $Na^+$ and $Cl^-$ (Fig. 5) suggest that changes in water absorption after freshwater transfer are entirely driven by transepithelial ion transport. Other mechanisms for changing the rate of water absorption, such as regulating the abundance or channel properties of aquaporins and thus intestinal water permeability, may therefore be less important in killifish after freshwater transfer. In eel, aquaporins were expressed at higher levels in the intestine of seawater-acclimated fish compared to those in fresh water (Aoki et al., 2003; Martinez et al., 2005), which has been correlated with a higher water absorption rate (Aoki et al., 2003). Unfortunately, the relative contributions of regulated water permeability and osmotic driving force (i.e. ion transport) to transepithelial water absorption are not known in these species.

Our data are in accord with the general view, based mainly on similar *in vitro* studies with seawater fish, that an isosmotic solution is absorbed across the intestine of fish (Loretz, 1995). The slope of the relationship in Fig. 5 of this study indicates that the strong ion concentration of the absorbed fluid was about 292 mmol $l^{-1}$ (see Results), which would make it nearly isosmotic to blood plasma and to the incubation saline (note this neglects the small contribution from the unmeasured cation/anion, as discussed below). A very similar value (272 mmol $l^{-1}$) may be estimated from the data of other researchers (Marshall et al., 2002a) for posterior intestine sacs of seawater-adapted killifish under similar 'symmetrical' conditions.

It is notable that net $Cl^-$ fluxes were always higher than net $Na^+$ fluxes, indicating that additional uptake of cation or efflux of anion must occur. Greater $Cl^-$ absorption across the intestine of killifish would obviously help to compensate for the inability of this species to actively absorb $Cl^-$ in fresh water (Patrick et al., 1997; Patrick and Wood, 1999). This suggestion is supported by the observation that the difference between net $Cl^-$ flux and net $Na^+$ flux appeared to increase after freshwater transfer. However, net $Cl^-$ fluxes are also higher than net $Na^+$ fluxes across the intestine of fresh water-acclimated flounder *Platichthys flesus* (Smith et al., 1975) and rainbow trout *Oncorhynchus mykiss* (Nonnotte et al., 1987), both of which can actively absorb $Cl^-$ at the gills, so this suggestion is uncertain. Furthermore, a recent review (Marshall and Grosell, 2005) has noted that an excess of $Cl^-$ over $Na^+$ absorption across the intestine is the normal pattern for a variety of marine species.

Although it may play a role in $Cl^-$ homeostasis *per se*, the difference between net $Cl^-$ flux and net $Na^+$ flux across the

intestine may simultaneously be a consequence of other physiological processes. For example, the intestines of seawater fish are known to secrete an appreciable amount of bicarbonate, which may be an important part of seawater ionoregulation (Wilson et al., 2002). Chloride is likely exchanged for bicarbonate at the luminal surface (Grosell et al., 2005), so there are probably two routes for intestinal $Cl^-$ absorption: bicarbonate linked (*via* $Cl^-/HCO_3^-$ exchange) and $Na^+$ linked (*via* NKCC and/or $Na^+$,$Cl^-$ cotransporters). The difference between intestinal $Cl^-$ and $Na^+$ absorption may therefore reflect the extra influx pathway for chloride, that is, $Cl^-/HCO_3^-$ exchange. This is normally thought of as a phenomenon peculiar to marine fish, but the present findings, together with a report of a great excess of [$Na^+$] over [$Cl^-$] and very high pH (9.0) in the rectal fluid of fresh water-adapted trout (Shehadeh and Gordon, 1969), suggest otherwise. The possible acid–base consequences of such a strategy for a fish in fresh water deserves future study.

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