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Intraspecific divergence of ionoregulatory physiology in the euryhaline teleost *Fundulus heteroclitus*: possible mechanisms of freshwater adaptation

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Summary

We examined intraspecific variation in ionoregulatory physiology within euryhaline killifish, *Fundulus heteroclitus*, to understand possible mechanisms of freshwater adaptation in fish. Pronounced differences in freshwater tolerance existed between northern (2% mortality) and southern (19% mortality) killifish populations after transfer from brackish water (10 g l^{-1}) to freshwater. Differences in Na^+ regulation between each population might partially account for this difference in tolerance, because plasma Na^+ was decreased for a longer period in southern survivors than in northerns. Furthermore, northern fish increased Na^+/K^+-ATPase mRNA expression and activity in their gills to a greater extent 1–14 days after transfer than did southerns, which preceded higher whole-body net flux and unidirectional influx of Na^+ at 14 days. All observed differences in Na^+ regulation were small, however, and probably cannot account for the large differences in mortality. Differences in Cl^- regulation also existed between populations. Plasma Cl^- was maintained in northern fish, but in southerns, plasma Cl^- decreased rapidly and remained low for the duration of the experiment. Correspondingly, net Cl^- loss from southern fish remained high after transfer, while northerns eliminated Cl^- loss altogether. Elevated Cl^- loss from southern fish in freshwater was possibly due to a persistence of seawater gill morphology, as paracellular permeability (indicated by extrarenal clearance rate of PEG-4000) and apical crypt density in the gills (detected using scanning electron microscopy) were both higher than in northern fish. These large differences in the regulation of Cl^- balance probably contributed to the marked differences in mortality after freshwater transfer. Glomerular filtration rate and urination frequency were also lower in southerns. Taken together, these data suggest that northern killifish are better adapted to freshwater environments and that minimizing Cl^- imbalance appears to be the key physiological difference accounting for their greater freshwater tolerance.

Key words: killifish, gene expression, gill morphology, Na^+/K^+-ATPase, ion flux, kidney, evolution.

Introduction

The common killifish, *Fundulus heteroclitus*, inhabits brackish water estuaries and salt marshes along the eastern coast of North America. The species distribution is latitudinal, from Newfoundland to Florida, and thus spans a cline of environmental temperatures. Correspondingly, many previous studies have investigated thermal adaptations in populations across the range. Differences between populations include latitudinal differences in glycolytic enzyme expression and activity (Powers et al., 1986; Pierce and Crawford, 1996), endocrinology (DeKoning et al., 2004; Picard and Schulte, 2004), metabolism (Podrabsky et al., 2000), morphology and behaviour (Powers et al., 1993), and, as a result, these fish are sometimes divided into two subspecies, *F.h. macrolepidotus* (northern) and *F.h. heteroclitus* (southern). By contrast, few studies have assessed whether intraspecific physiological differences exist between populations of *F. heteroclitus* in response to other environmental factors (e.g. tidal cycle; DiMichele and Westerman, 1997).

Species within the genus *Fundulus* are suggested to have arisen from brackish water ancestors, and there is substantial variation in both the salinity of their native habitats (ranging from freshwater to seawater) and their salinity tolerance (Griffith, 1974). Intraspecific differences in salinity tolerance and distribution also appear to exist within some *Fundulus* species. For example, northern populations of *F. heteroclitus* have higher fertilization success and larval survival in hyposmotic salinities than southern populations (Able and Palmer, 1988). Furthermore, the proportion of northern genotypes increases in freshwater habitats, even at latitudes and temperatures that are typical for the southern subspecies (Powers et al., 1993). It is therefore likely that molecular or physiological differences exist within *F. heteroclitus* that form the basis for variation in freshwater tolerance.

Past habitat availability may have selected for differences in freshwater tolerance between northern and southern individuals of *F. heteroclitus*. After previous glaciation events, new freshwater and estuarine habitats would have opened due to glacial retreat from previously ice-covered areas (Powers et al., 1986). *F. heteroclitus* populations able to colonize northern habitats therefore faced opportunities for freshwater invasion without competition from indigenous fish species, in contrast to the situation further south. The role of natural selection in freshwater invasion events has been explicitly demonstrated (Lee and Petersen, 2002), and numerous accounts attest to the selective advantage of euryhalinity (Lee and Bell, 1999). However, the physiological adaptations necessary for brackish water and marine fish to invade freshwater are unclear. Intraspecific comparison of the mechanisms maintaining ion balance in *F. heteroclitus* populations in freshwater may identify factors of selective importance for freshwater adaptation.

The hyposmotic nature of freshwater environments (typically <10 mOsmol l^{-1}) favours ion efflux from fish, because they maintain substantially higher body fluid osmolarity (300–350 mOsmol l^{-1}). The largest component of ion efflux in freshwater fish probably occurs across the gills due to their high surface area. Fish in freshwater therefore decrease the paracellular permeability across the gill epithelium, primarily by increasing the thickness of tight junctions between mitochondria-rich (MR) cells and neighbouring cells (Sardet et al., 1979; Ernst et al., 1980). Fish also decrease transcellular permeability by inactivating ion secretion pathways (Marshall et al., 1993, 1998, 2000; Scott et al., 2004).

To counteract ion efflux and maintain ionic homeostasis, fish in freshwater absorb ions across the gills (see reviews by Wood and Marshall, 1994; Perry, 1997; Evans et al., 1999; Marshall, 2002). Sodium absorption by killifish gills is likely to involve a basolateral Na^+/K^+-ATPase and an apical Na^+/H^+-exchanger (Patrick and Wood, 1999; Claiborne et al., 2002; Scott et al., 2004) but may also involve basolateral H^+-ATPase (Katoh et al., 2003). Unlike the majority of fish in freshwater, *F. heteroclitus* does not actively absorb chloride and thus maintains chloride balance through unique and yet undefined mechanisms (Patrick and Wood, 1999).

The objective of the present study was to compare the ionoregulatory ability of individuals from northern and southern populations of *F. heteroclitus* after direct salinity transfer. The ionoregulatory ability of each population was assessed by measuring survival, plasma ions, mRNA expression, protein activity, ion flux, paracellular permeability, gill morphology and aspects of renal function after transfer from near-isosmotic brackish water (10 g l^{-1}) to freshwater.

Materials and methods

Experimental animals

Adult killifish (*Fundulus heteroclitus* L.) of the northern subspecies (*F.h. macrolepidotus*) were captured from Hampton, NH, USA. Adults of the southern subspecies (*F.h. heteroclitus*) were captured from either Whitney Island, FL, USA (salinity transfer and flux experiments) or New Brunswick, GA, USA (microscopy experiment). For the salinity transfer and microscopy studies, fish were maintained in indoor holding facilities in synthetic brackish water (10 g l^{-1}; Deep Ocean; Energy Savers, Carson, CA, USA) made up in dechlorinated Vancouver city tap water ([Na^+], 0.17 mmol l^{-1}; [Cl^-], 0.21 mmol l^{-1}; hardness, 30 mg l^{-1} as $CaCO_3$; pH 5.8–6.4) in static filtered glass aquaria. Before sampling, fish were maintained for at least 30 days in this brackish water at an ambient temperature of 21–24°C and a 14 h:10 h L:D photoperiod. Fish were fed commercial trout chow (PMI Nutrition International, Brentwood, MO, USA; 2.2% calcium, 0.8% chloride, 0.5% sodium, 0.5% potassium, 0.2% magnesium) at an approximate daily ration of 1–2% of body mass. Treatment of animals was conducted according to University of British Columbia and McMaster University animal care protocols #A01-0180 and #02-10-61, respectively.

Salinity transfer experiment

Some of the data collected in this salinity transfer experiment have been previously reported for the northern subspecies alone (Scott et al., 2004). Eight northern and southern control fish were sampled after acclimation to 10 g l^{-1}, after which fish from each population were quickly transferred by net to aquaria containing freshwater (0 g l^{-1}; composition as above) or brackish water (10 g l^{-1}). Individual fish were subsequently sampled by netting at 3 h, 8 h, 24 h, 96 h, 14 days and 30 days after transfer from brackish water. The fish were stunned by cephalic blow, blood samples were collected in heparinized capillary tubes from the severed caudal peduncle, and the fish were then killed by rapid decapitation. Blood was centrifuged at 13 000 ***g*** for 10 min, and plasma was frozen in liquid nitrogen. Second and third gill arches were immediately frozen in liquid nitrogen. All tissues were stored at –80°C until analysed.

Total RNA extraction and reverse transcription

Total RNA was extracted from tissues (~20 mg) using Tripure isolation reagent (Roche Diagnostics, Montreal, QC, Canada) following the manufacturer's instructions. RNA concentrations were determined spectrophotometrically, and RNA integrity was verified by agarose gel electrophoresis [~1% agarose:Tris-acetate EDTA (w/v)]. Extracted RNA samples were stored at –80°C following isolation. First-strand cDNA was synthesized by reverse transcribing 3 μg total RNA using 10 pmoles of oligo(dT_{18}) primer and 20 U RevertAid H Minus M-MuLV reverse transcriptase (MBI Fermentas Inc., Burlington, ON, Canada) following the manufacturer's instructions.

Real-time PCR analysis of gene expression

Primers for killifish Na^+/K^+-ATPase α_{1a} (accession no.

AY057072), cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (accession no. AF000271), $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1; accession no. AY533706) and elongation factor 1α (EF1α, expression control; accession no. AY430091) were designed using Primer Express software (version 2.0.0; Applied Biosystems Inc., Foster City, CA, USA) and are reported in Scott et al. (2004). Gene expression was quantified using quantitative real-time PCR (qRT-PCR) on an ABI Prism 7000 sequence analysis system (Applied Biosystems Inc.). PCR reactions contained 1 μl of cDNA, 4 pmoles of each primer and Universal SYBR green master mix (Applied Biosystems Inc.) in a total volume of 21 μl. All qRT-PCR reactions were performed as follows: 1 cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min (set annealing temperature of all primers). PCR products were subjected to melt curve analysis to confirm the presence of a single amplicon, and representative samples were electrophoresed to verify that only a single band was present. 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 1:49 starting cDNA copies for all templates.

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. Results were then standardized to EF1α, a gene for which mRNA expression in the gills does not change following salinity transfer (data not shown), and were expressed relative to the matched brackish water controls within each time point. All samples were run in duplicate (coefficients of variation were $\leqslant$10%). Because significant changes in gene expression due to freshwater transfer were observed in the northern population only at 24 h, 96 h and 14 days post-transfer by Scott et al. (2004), gene expression was only quantified at these time points (and pre-transfer) in the southern population.

Na^+/K^+-ATPase activity

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 (1993). For this assay, second and third gill arches were homogenized in 500 μl of SEI buffer (150 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} imidazole, pH 7.3) containing 0.1% Na-deoxycholate and centrifuged at 5000 ***g*** for 30 s at 4°C. Supernatants were immediately frozen in liquid nitrogen and stored at –80°C until analysed. ATPase activity was determined in the presence or absence of 0.5 mmol l^{-1} ouabain using 10 μl supernatant thawed on ice and was normalized to total protein content (measured using the bicinchoninic acid method; Sigma-Aldrich, Oakville, ON, Canada). All samples were run in triplicate (coefficients of variation were always $\leqslant$10%). Na^+/K^+-ATPase activity, measured as ouabain-sensitive ATPase activity, is expressed as μmol ADP mg^{-1} protein h^{-1}.

Freshwater flux experiments

Killifish were held at McMaster University for at least 30 days before experimentation in 10 g l^{-1} brackish water (made up in dechlorinated Hamilton city tap water) at room temperature (~21°C) and were maintained at a 14 h:10 h L:D photoperiod. Freshwater was prepared to approximate Vancouver city tap water by mixing appropriate amounts of dechlorinated Hamilton water and reverse-osmosis water (measured final composition: [Na^+], 0.17 mmol l^{-1}; [Cl^-], 0.20 mmol l^{-1}). Static polyethylene flux chambers were fitted with a lid and aeration line and were wrapped in black plastic to minimize disturbance of fish. Each chamber contained 270 ml of freshwater and 37 kBq of either ^{22}Na (NEN Life Science Products Inc., Boston, MA, USA) or ^{36}Cl (ICN Biomaterials, Irvine, CA, USA) isotope at the start of each flux period.

Unidirectional and net Na^+ flux rates and net Cl^- flux rates were measured over the first 8 h after transfer to freshwater and at 1 and 4 days post-transfer in one experiment, and also at 8 and 14 days post-transfer in a second experiment. Unidirectional Na influx was measured by monitoring the disappearance of ^{22}Na isotope from the water. In the first experiment, 10 fish from each population were transferred to individual freshwater flux chambers containing isotope, and duplicate water samples (2×5 ml for ^{22}Na radioactivity measurements) were taken every 30 min for the first 8 h after transfer. Additional flux measurements were made 1 and 4 days after transfer on the same fish. For these flux periods, duplicate water samples were taken every hour for 4 h after the addition of isotope. In the second experiment, 10 killifish from each population were first transferred to static aerated freshwater tanks for 7 days and then moved to flux chambers. Flux periods at 8 and 14 days after transfer were then conducted as before, with water samples being taken every hour for 4 h after the addition of isotope on days 8 and 14. In both experiments, water in the flux chambers was replaced between flux periods with clean freshwater (containing no radioactivity, at least once daily) to remove waste and excess isotope.

Unidirectional Cl^- influxes from *F. heteroclitus* in freshwater are normally extremely low and cannot be determined by measuring the disappearance of isotope from the water. They must instead be measured by quantifying isotope appearance in the fish, which is a more sensitive technique (Patrick et al., 1997; Wood and Laurent, 2003). Unidirectional Cl^- influx was therefore determined 8 days after freshwater transfer by measuring whole-animal uptake of ^{36}Cl. Eight killifish from each population were first held in static aerated freshwater tanks for 8 days, then moved to flux chambers containing isotope. Water samples were taken immediately after the addition of ^{36}Cl (just before fish were added), and fish and water were sampled 4 h later. At the end of both ^{22}Na and ^{36}Cl experiments, fish were rinsed by allowing live animals to ventilate their gills (for at least 5 s) in freshwater containing no radioactivity and were then sacrificed. Radioactivity (counts per minute, c.p.m.) was

determined in each fish and in each water sample, as were total water Na^+ and Cl^- concentrations.

Unidirectional Na^+ influx rates ($J_{in,Na}$) in μmol kg^{-1} h^{-1} were calculated as:

$$J_{in,Na} = \frac{\dot{\Sigma}}{SA \times M}\,, \quad (1)$$

where $\dot{\Sigma}$ is the slope of the regression line of radioactivity *versus* time (in c.p.m. h^{-1}), SA is the mean external specific activity (in c.p.m. μmol^{-1}) and M is the body mass (in kg). Unidirectional Cl^- influx rates ($J_{in,Cl}$) were calculated as:

$$J_{in,Cl} = \frac{\Sigma_{fish}}{SA \times M \times t}\,, \quad (2)$$

where Σ_{fish} is the total radioactivity in the fish (in c.p.m.) and t is the flux period duration (in h).

Mean net Na^+ and Cl^- flux rates ($J_{net,ion}$) in μmol kg^{-1} h^{-1} were calculated as:

$$J_{net,ion} = \frac{[\text{ion}]_i - [\text{ion}]_f}{M \times t}\,V\,, \quad (3)$$

where $[\text{ion}]_i$ and $[\text{ion}]_f$ are the concentrations of Na^+ or Cl^- in the water at the start and end of the flux period (in μmol l^{-1}), respectively, and V is the mean flux chamber volume (in litres). By conservation of mass, unidirectional efflux rates ($J_{out,ion}$) in μmol kg^{-1} h^{-1} were calculated as:

$$J_{out,ion} = J_{net,ion} - J_{in,ion}\,. \quad (4)$$

Because the above calculations assume there is no ‘backflux’ of radioisotope from the fish into the surrounding media, which can be a significant source of error, internal specific activity within the fish must remain low compared with external specific activity of the medium. At the end of each flux experiment, the ratio of internal to external specific activity was therefore verified to be <10% (Kirschner, 1970).

A method adapted from Curtis and Wood (1991) was employed to study the diffusive permeability of the fish to a paracellular permeability marker 8 days after freshwater transfer. The technique monitors the appearance of radiolabelled polyethylene glycol ([^{3}H]PEG-4000) in the external water relative to the radioactivity of a terminal plasma sample, from which PEG-4000 clearance rates can be calculated. Radioactivity appearing in discrete pulses represents bouts of urination from the urinary bladder (renal PEG-4000 clearance), whereas radioactivity not appearing in pulses represents diffusion across the gills and body surface (extra-renal PEG-4000 clearance). [^{3}H]PEG-4000 is the marker of choice for glomerular filtration rate in fish (Beyenbach and Kirschner, 1976), and its renal clearance rate is considered equivalent to the glomerular filtration rate (GFR). On day 7, approximately 16 h prior to measurements on day 8, killifish were injected intraperitoneally with 1 μl g^{-1} of [^{3}H]PEG-4000 (NEN Life Science Products) in 140 mmol l^{-1} NaCl (111 kBq μl^{-1}) and left in their individual containers for the label to equilibrate overnight throughout the extracellular compartment. On day 8, the water was changed, the volume set to 250 ml, the aeration set to produce good mixing, and the fish left to settle for a further 60 min. Thereafter, a 1 ml water sample was drawn from each fish container at exactly 5-min intervals for the next 6–7 h, after which a blood sample was taken and plasma separated as described earlier.

Plots of total water [^{3}H] radioactivity against time revealed clear step-wise increases attributable to bouts of urination, and the sum of all radioactivity (c.p.m.) appearing in these pulses (by renal excretion) was subtracted from the total appearance (c.p.m.) over the 6–7 h period to yield extrarenal excretion (c.p.m.). Dividing each of these values by plasma radioactivity (c.p.m. μl^{-1}), time (h) and body mass (kg) yielded the renal clearance rate of PEG-4000 (equivalent to GFR; in ml kg^{-1} h^{-1}) and the extrarenal clearance rate of PEG-4000 (in ml kg^{-1} h^{-1}), the latter providing an index of the diffusive permeability of the fish to a paracellular permeability marker. As terminal urine samples for radioactivity counting could not be obtained from these small fish, urine flow rate could not be determined (cf. Curtis and Wood, 1991), but urination frequency (bursts h^{-1}) could be calculated.

Ion and radioactivity measurements

Sodium concentrations of plasma and water samples were determined using flame atomic absorption spectrophotometry (SpectrAA-220FS; Varian, Mulgrave, VC, Australia) with Fisher Scientific (Nepean, ON, Canada) certified standards. Chloride concentrations of plasma and water samples were measured colorimetrically (Zall et al., 1956) with Radiometer (Copenhagen, Denmark) certified standards. ^{22}Na radioactivities in fish and water samples were determined using a Minaxi Autogamma 5000 counter (Packard Instruments, Downers Grove, IL, USA). For ^{36}Cl radioactivities in whole fish, animals were digested in three volumes of 1 mol l^{-1} HNO_3 at 60°C for 48 h. These samples were centrifuged, supernatants (1 ml) were added to 5 ml of an acid-compatible scintillation cocktail (Ultima Gold; Packard Bioscience, Meriden, CT, USA), and radioactivity was measured by scintillation counting (Rackbeta 1217; LKB Wallac, Turku, Finland). ^{36}Cl radioactivities in water samples (5 ml) were also measured by scintillation counting in 10 ml of scintillation fluid (ACS; Amersham, Piscataway, NJ, USA), and data were corrected for the slight difference in counting efficiencies between the two scintillation fluors. ^{3}H radioactivities in water (1 ml) and plasma (10–20 μl) samples were measured by scintillation counting in a standard volume ratio of 1 ml of water (or diluted plasma) to 5 ml ACS (quench was shown to be uniform across samples).

Scanning electron microscopy

Second and third gill arches of killifish from each population were sampled before (N=3) and 8 days after transfer to Vancouver freshwater (N=5), as described above. Gills were fixed for 24 h in 0.1 mol l^{-1} phosphate-buffered saline (pH 7.4) containing 2% paraformaldehyde and 2% glutaraldehyde.

After fixation, samples were post-fixed in 0.1 mol l^{-1} cacodylate buffer (pH 7.4) containing 1% OsO_4. Tissues were dehydrated progressively in ethanol (70, 85, 95 and 100%) for 10 min in each solution. Gill arches were dried in hexamethyldisilazane and sputter-coated with gold.

Images collected by scanning electron microscopy were analyzed using a method similar to Daborn et al. (2001). Random locations on the afferent-vascular edge of gill filaments were observed at 3000× magnification. Apical crypts, freshwater-type MR cells ('chloride cells') and 'intermediate' cells were counted for at least 10 different locations throughout the gills. Averages were calculated for each fish and expressed as density mm^{-2}.

Statistical analyses

Data are expressed as means ± S.E.M. Kruskal–Wallis *H* non-parametric analysis of variance (ANOVA) was used to determine overall differences as a function of time (for each salinity) for each variable except survival. Mann–Whitney *U* non-parametric comparisons were then used to compare between salinities (within populations at each time) or between populations (at each salinity and time). Survival between sampling times was compared using the one-tailed Fisher's exact test. Statistical analyses were conducted using SPSS version 10.0, and a significance level of $P<0.05$ was used throughout.

Results

Survival

There were pronounced differences between the survival of northern and southern killifish (Fig. 1). Individuals from the northern population had very low mortality after both freshwater (98% survival after 30 days) and brackish water control (100% survival) transfer. By contrast, southern individuals suffered substantial mortality after freshwater transfer compared with brackish water controls (94% survival), starting 5 days into freshwater and stabilizing after 11 days (81% survival). The mortalities experienced by brackish water-transferred northerns, brackish water-transferred southerns and freshwater-transferred northerns were statistically indistinguishable; significantly elevated mortality was detected between 4 and 14 day sampling times in freshwater-transferred southerns ($P<0.05$).

Plasma ions

The effect of freshwater transfer on plasma ions differed between populations in a manner consistent with the differences in mortality (Fig. 2). Freshwater transfer decreased plasma Na compared with brackish water controls in both populations, but this only

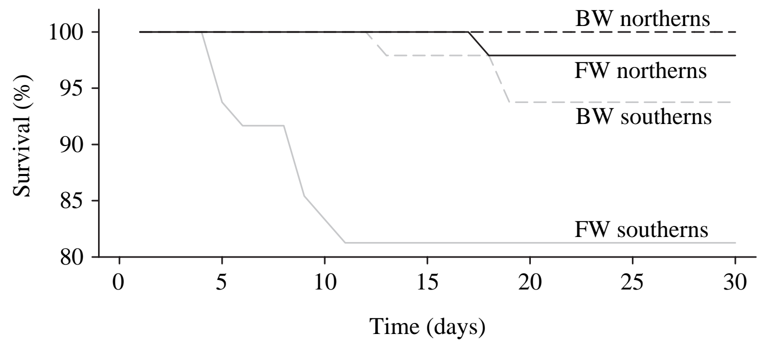

Fig. 1. Survival of northern (black) and southern (grey) killifish after transfer to either brackish water (BW; 10 g l^{-1}) or freshwater (FW).

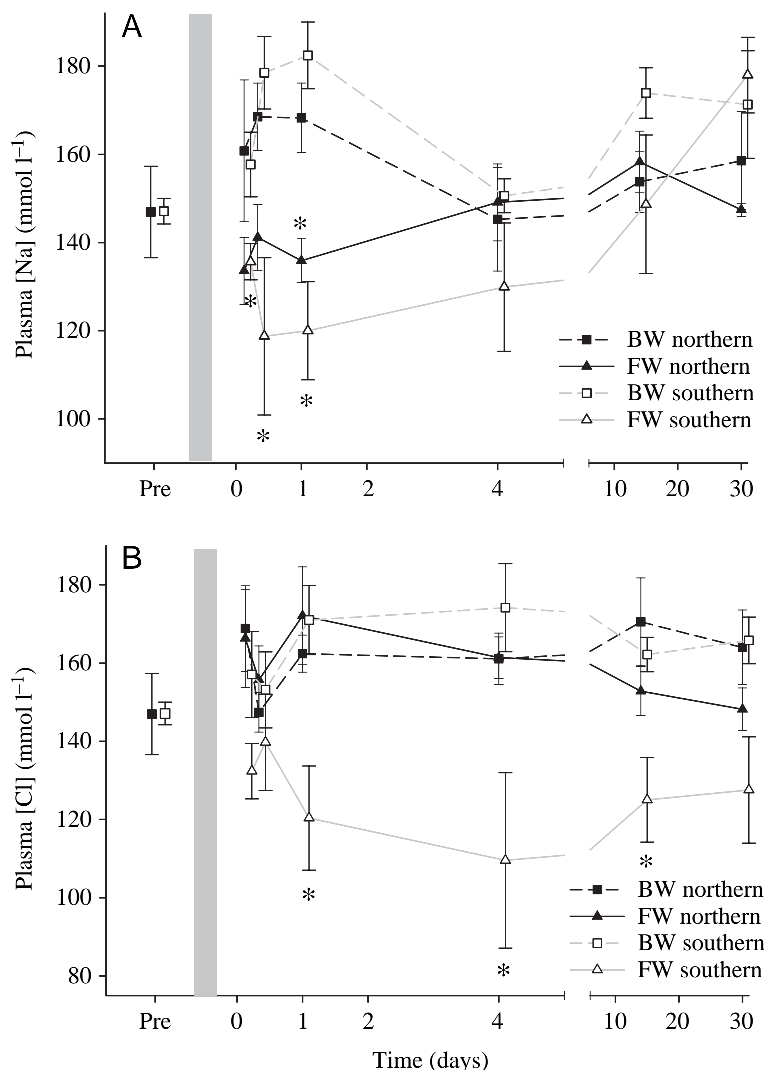

Fig. 2. Plasma sodium ($N>5$) (A) and chloride ($N>5$) (B) levels in northern (black) and southern (grey) killifish before (pre) and after transfer from brackish water (BW; 10 g l^{-1}) to either brackish water (squares) or freshwater (FW; triangles). Data are expressed as means ± S.E.M. *Significant difference from time-matched brackish water control ($P<0.05$). Plasma Na data for northern killifish have been previously reported (Scott et al., 2004).

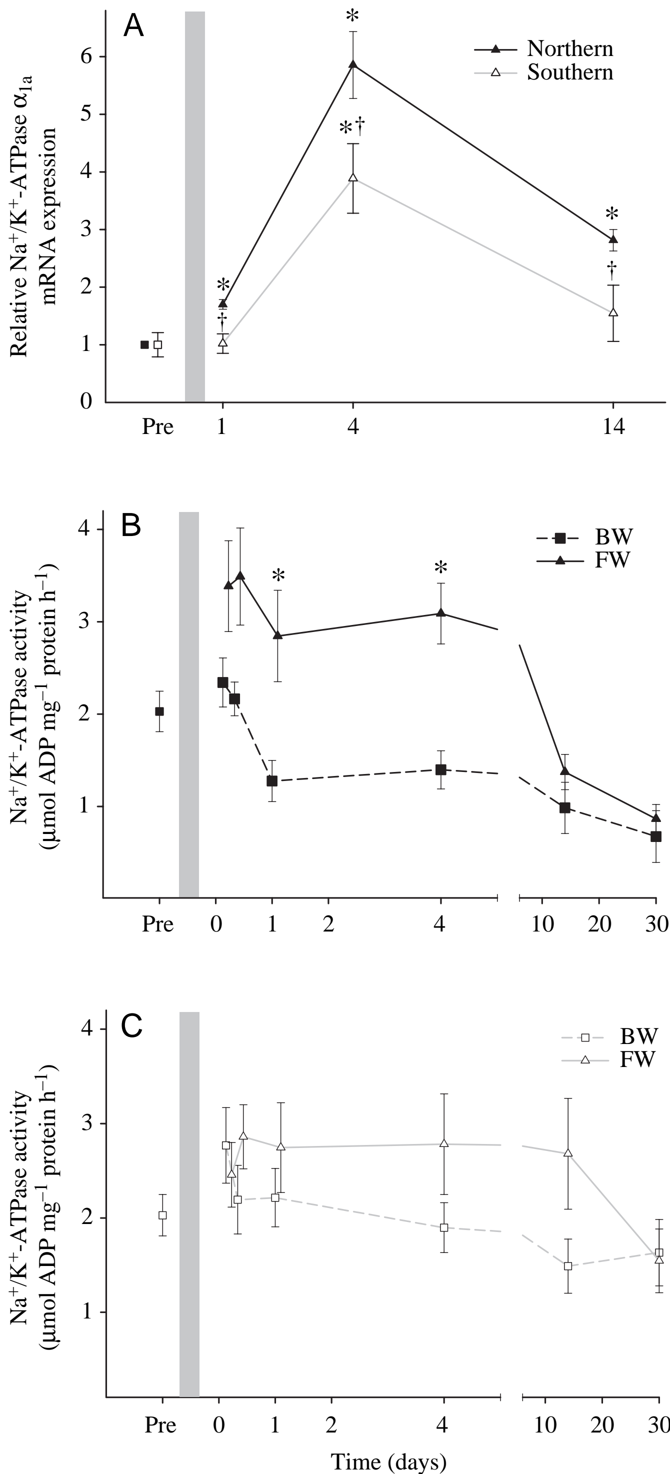

Fig. 3. Fold-change in Na^+/K^+-ATPase α_{1a} mRNA expression (N>7) (A) as well as Na^+/K^+-ATPase activity (N>6) in northern (B) and southern (C) killifish gills before (pre) and after transfer from brackish water (BW; 10 g l^{-1}; squares) to either brackish water or freshwater (FW; triangles). Expression data are standardized to elongation factor 1α and are relative to time-matched brackish water controls (see Materials and methods). Data are expressed as means ± S.E.M. *Significant difference from time-matched brackish water control. †Significant difference from northern population (P<0.05). Data for northern killifish are from Scott et al. (2004).

occurred 1 day after transfer of northern killifish while southern killifish had decreased plasma levels 3 h, 8 h and 1 day after transfer to freshwater (Fig. 2A). Plasma Na balance was re-established 4 days after transfer in both populations.

Differences in plasma Cl between populations after freshwater transfer were more pronounced than differences in plasma Na (Fig. 2B). Northern killifish maintained plasma Cl balance at all times after freshwater transfer. By contrast, southern killifish in freshwater had lower plasma Cl at 1, 4 and 14 days after transfer compared with brackish water controls.

Gene expression and protein activity

Na^+/K^+-ATPase regulation in the gills after freshwater transfer differed between northern and southern populations of *F. heteroclitus*. There was a prolonged increase in the relative Na^+/K^+-ATPase α_{1a} mRNA expression in individuals from the northern population, which peaked at 4 days in freshwater at nearly 6-fold above time-matched brackish water controls (Fig. 3A). Individuals from the southern population increased Na^+/K^+-ATPase α_{1a} expression to a lesser extent, to only 4-fold that of controls at 4 days after transfer. Unlike northern killifish, southern killifish did not increase expression at 1 or 14 days into freshwater.

Changes in Na^+/K^+-ATPase activity in the gills as a result of freshwater transfer also differed between populations. Activity increased 2-fold at 1 and 4 days following freshwater transfer in northern killifish (Fig. 3B), while no significant increases occurred after transfer in southern killifish (Fig. 3C). Activity also reached higher absolute levels in northern killifish (3.5±0.5 and 2.9±0.3 μmol mg^{-1} protein h^{-1} at 8 h in northerns and southerns, respectively).

Relative expression of the seawater ion transporters NKCC1 and CFTR decreased at 1 and 4 days after freshwater transfer in both populations, dropping to approximately 2.5- and 10-fold below brackish water controls 1 day after transfer, respectively (Table 1). Decreased expression of these genes persisted longer in southern individuals, however, remaining below that of controls 14 days after freshwater transfer.

Table 1. *Fold-changes in gill mRNA expression after freshwater transfer of seawater ion transporter genes measured by qRT-PCR*

		Time		
Gene	Population	24 h	96 h	14 days
NKCC1	Northern	0.44±0.06*	0.72±0.09*	1.34±0.21
	Southern	0.47±0.09*	0.55±0.15*	0.59±0.04*,†
CFTR	Northern	0.11±0.01*	0.59±0.07*	0.96±0.20
	Southern	0.14±0.03*	0.64±0.15*	0.38±0.08*,†

Values are means ± S.E.M. (N>7). qRT-PCR expression data are relative to time-matched brackish water control at the respective time point. *Significant difference from brackish water control at the respective time point (P<0.05). †Significant difference from northern population (P<0.05). Data for northern killifish are from Scott et al. (2004).

Ion fluxes and PEG-4000 clearance rates

There were only small differences in Na^+ fluxes between northern and southern populations following freshwater transfer. Both northern and southern fish decreased Na^+ efflux rapidly after freshwater transfer (Fig. 4A). Unidirectional efflux and negative net flux were significantly decreased 4–8 h after transfer compared with the 0–4 h flux period, and this decrease persisted until at least 14 days after transfer. Both populations also increased unidirectional Na^+ influx progressively after freshwater transfer. By 14 days after freshwater transfer, however, northern killifish had significantly higher unidirectional influx and net flux than southern killifish. In fact, northern fish increased Na^+ influx by 5-fold 14 days post-transfer compared with the 0–4 h flux period, while southerns increased influx by only 3.3-fold. Net flux was also slightly higher in northern fish after 96 h in freshwater.

We observed large differences in net Cl^- flux between northern and southern killifish (Fig. 4B). Although fish from both populations decreased Cl^- loss initially, northern killifish eliminated loss by 1 day after transfer and this was maintained after at least 14 days in freshwater. By contrast, southern killifish did not appear to decrease Cl^- loss below 100 μmol kg^{-1} h^{-1} and significantly differed from northern killifish at 1 and 14 days after transfer. Unidirectional Cl^- influx was small (less than 3% of unidirectional Na^+ influx; Fig. 4A) and was identical between populations at 8 days post-transfer (Table 2).

Patterns of PEG-4000 clearance differed substantially between killifish populations at 8 days post-transfer (Table 2). Extrarenal clearance rates, which represent the general paracellular permeability of the gills and body surface, were 3-fold higher in southern killifish. Renal clearance was higher in northern killifish, which had 1.6-fold greater glomerular filtration rates (i.e. renal PEG-4000 clearance rates) and 1.5-fold more frequent bursts of urination, although actual urine flow could not be calculated.

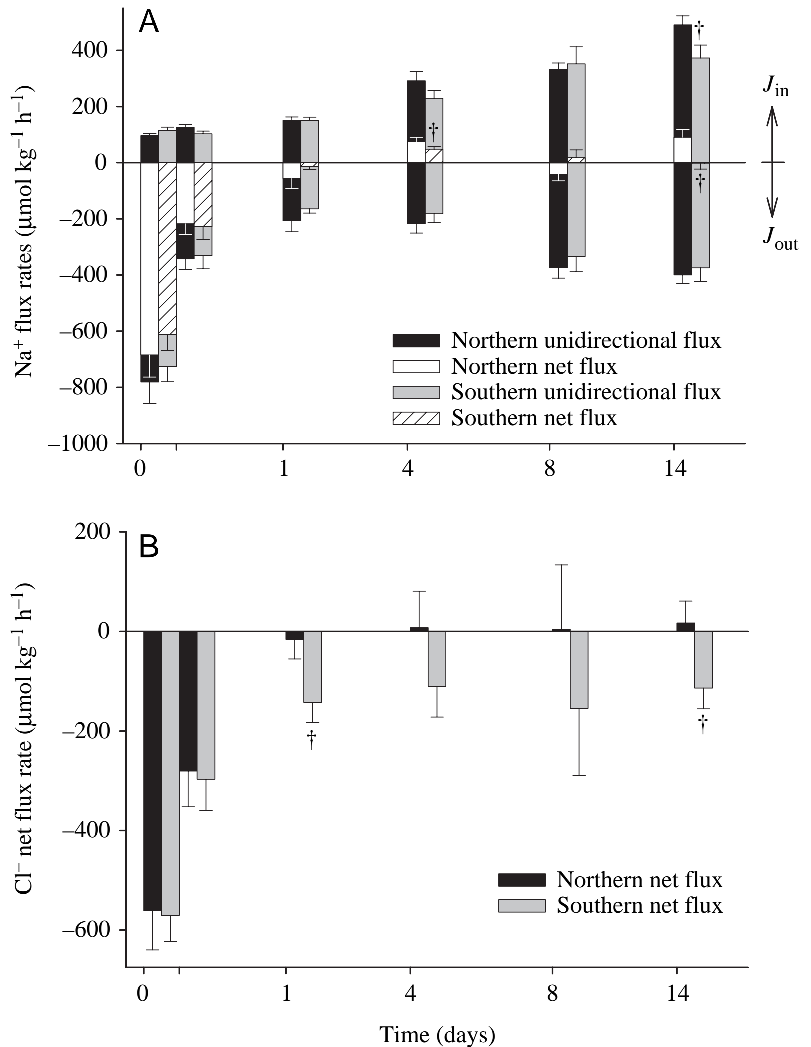

Fig. 4. Total (net) flux and unidirectional fluxes of Na^+ (A) and total Cl^- flux (B) in northern (black and white) and southern (grey and hatched) killifish after transfer from brackish water (10 g l^{-1}) to freshwater (N>9). Positive values represent influx (J_{in}). †Significant difference from northern population (P<0.05).

Table 2. *Unidirectional Cl^- influx, extrarenal and renal [^{3}H]PEG-4000 clearance rates and urination frequency 8 days after freshwater transfer*

	Northern population	Southern population
Cl^- influx (μmol kg^{-1} h^{-1})	9.2±2.3	6.6±1.7
ECR (ml kg^{-1} h^{-1})	1.3±0.2	4.3±0.3†
RCR (ml kg^{-1} h^{-1})	6.3±0.8	3.9±0.5†
Urination frequency (bursts h^{-1})	1.1±0.1	0.7±0.1†

Values are means ± S.E.M. (N=8). Unidirectional Cl^- influx was measured by monitoring the appearance of ^{36}Cl in the whole body of the fish. †Significant difference from northern population (P<0.05). ECR, extrarenal clearance rate (an index of paracellular permeability); RCR, renal clearance rate (glomerular filtration rate).

Gill morphology

The gills of both northern and southern killifish had a similar morphology in brackish water (Fig. 5A,B). Apical crypts were abundant in both populations (~2000 mm^{-2}), while freshwater-type MR cell density remained low (Fig. 6). 'Intermediate' cells, characterized by features that are midway between seawater (apical crypt) and freshwater (flat surface equipped with microvilli) morphologies were equally abundant between populations and salinities.

Northern and southern killifish had different gill morphologies after transfer to freshwater (Fig. 5C,D). Apical crypt density in northern killifish gills was 15-fold lower in

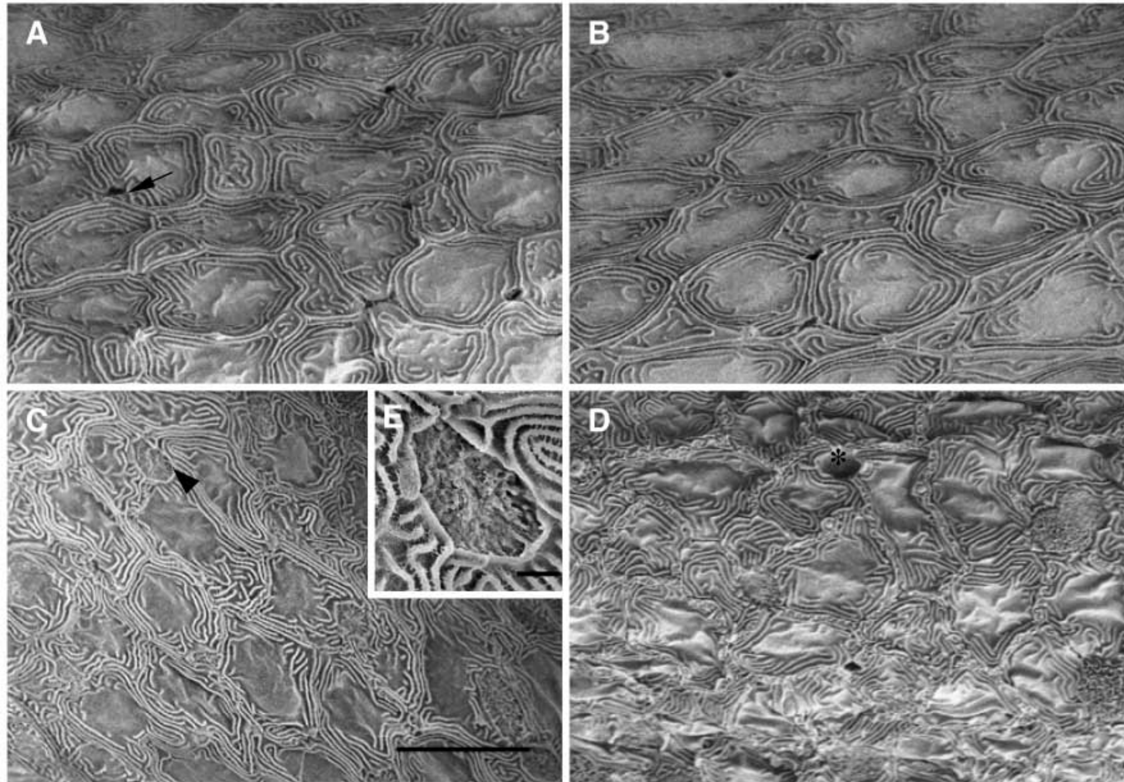

Fig. 5. Representative scanning electron micrographs of the gills of northern (A,C) and southern (B,D) killifish before (A,B) and 8 days after transfer from brackish water (10 g l^{-1}) to freshwater (C,D). Apical crypts (arrow), freshwater-type mitochondria-rich cells (arrowhead) and cells with intermediate morphology (asterisk) are apparent (scale bar, 10 μm). (E) Enlarged view of freshwater-type mitochondria-rich cell (scale bar, 1 μm).

freshwater than in brackish water, while southern killifish in freshwater had an apical crypt density only 3-fold lower than those in brackish water (Fig. 6). The abundance of freshwater-type MR cells increased significantly in both populations after freshwater transfer (~3000–4000 mm^{-2}).

Discussion

Killifish typically encounter fluctuations in environmental salinity on a daily and seasonal basis in their natural habitat and must therefore modulate ion flux across the gills to maintain ion balance (Marshall, 2003). In addition, we have observed substantial intraspecific variation between populations of *F. heteroclitus*, leading to large differences in freshwater tolerance. Individuals of the southern population suffered significantly greater mortality after prolonged freshwater transfer than did northerns. The eventual stabilization of mortality suggests that some southern individuals may be more freshwater tolerant than others, but even those southern individuals that survived experienced greater fluctuations in plasma ion levels (particularly plasma Cl^{-}) than did individuals of the northern population. The mechanistic differences between these populations that could have accounted for greater freshwater tolerance in northern killifish were numerous and suggest that the coordinated physiological response after freshwater transfer is somewhat reduced in the southerns. We speculate that northern killifish are better adapted to freshwater environments and that these identifiable differences between populations provide insight into the mechanisms of freshwater adaptation in fish.

Regulation of Na^{+} balance

There were small differences in the ability of northern and southern killifish populations to regulate Na^{+} balance after freshwater transfer. Plasma Na^{+} levels in individuals from the southern population decreased for a longer period after transfer when compared with those from the northern population, and this decrease appeared to be of greater magnitude. The cause of this difference is unclear, as Na^{+} fluxes did not differ between populations until 96 h after transfer. Differences in water flux across the gills (Robertson and Hazel, 1999), drinking rates (Potts and Evans, 1967) or renal function (see below) might have accounted for differences in plasma Na^{+} between populations.

Rapid re-establishment of plasma Na^{+} balance in northern killifish after freshwater transfer has been observed numerous times, demonstrating the excellent euryhalinity of these animals. Transfer from seawater to freshwater initially decreases plasma Na^{+} as early as 4 h after transfer, but pre-transfer seawater levels are quickly restored 1–2 days into

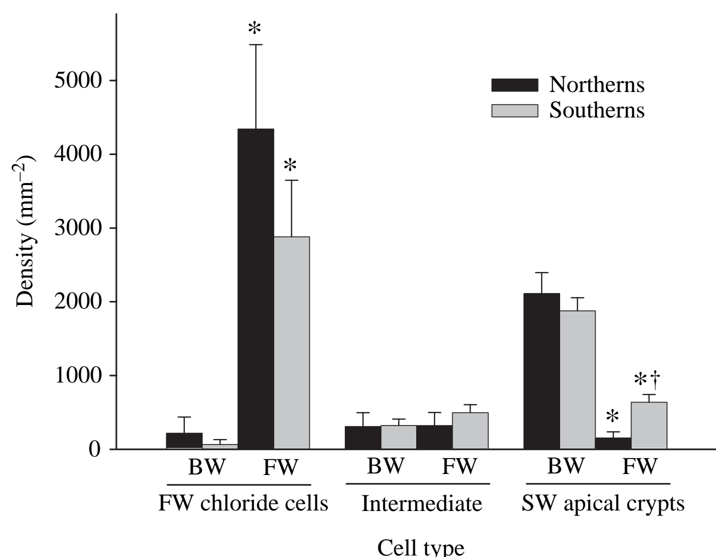

Fig. 6. Freshwater-type mitochondria-rich (chloride) cell, 'intermediate' cell and apical crypt density on the apical surface of northern (black) and southern (grey) killifish gills before (N=3) and 8 days after transfer from brackish water (10 g l^{-1}) to freshwater (N=5). Data are expressed as means ± S.E.M. *Significant difference from brackish water control. †Significant difference from northern population (P<0.05).

freshwater (Jacob and Taylor, 1983; Katoh and Kaneko, 2003). Northern killifish transferred from freshwater to seawater re-establish plasma Na^+ with equal rapidity (Jacob and Taylor, 1983; Marshall et al., 1999). We have observed similar results after transfer from near-isosmotic brackish water to either freshwater or seawater (Scott et al., 2004), suggesting that individuals of the northern population can freely move between freshwater and seawater environments.

Northern killifish increased both the relative mRNA expression (α_{1a}-isoform) and protein activity of gill Na^+/K^+-ATPase by a greater magnitude and for a longer duration after freshwater transfer than did southern killifish. Mitochondria-rich cells in killifish gills are known to proliferate after freshwater transfer (Katoh and Kaneko, 2003). An increase in the abundance of cells expressing high levels of Na^+/K^+-ATPase may have therefore contributed to the Na^+/K^+-ATPase upregulation in freshwater. Furthermore, differences in relative Na^+/K^+-ATPase expression between populations may have arisen from lower cell proliferation in southern fish after freshwater transfer. In support of this hypothesis, northern killifish gills had a high density of freshwater-type MR cells (as previously reported by Hossler et al., 1985), while density appeared to be lower in the gills of southern killifish in freshwater.

The regulation of ion flux across fish gills by changes in ion transporter expression is generally assumed but infrequently tested (e.g. Sullivan et al., 1995, 1996). In this regard, we speculate that the differences in Na^+/K^+-ATPase gene regulation between northern and southern killifish were at least partly responsible for the observed differences in Na^+ flux. Northern killifish had greater changes in Na^+/K^+-ATPase expression, which occurred concurrent with more positive net flux at 4 and 14 days and greater unidirectional Na^+ influx at 14 days after freshwater transfer. Therefore, these data provide evidence for how active ion flux can be modulated by ion transporter gene regulation.

Even though differences in Na^+/K^+-ATPase expression and activity appear to exist between *F. heteroclitus* populations after freshwater transfer, the resultant differences in Na^+ flux were small. Both populations initially suffered high Na^+ efflux, at rates comparable with other *Fundulus* species (Pang et al., 1974); interestingly, our initial rates in *F. heteroclitus* are intermediate between *F. diaphanus*, a freshwater species, and *F. majalis*, a seawater species. Efflux decreased rapidly after freshwater transfer, however, such that net loss was nearly eliminated by 24 h after transfer in both *F. heteroclitus* populations. Similarly rapid reductions in Na^+ efflux have been previously observed for *F. heteroclitus* (Motais et al., 1966; Potts and Evans, 1967; Pic, 1978; Wood and Laurent, 2003) as well as *F. kansae* (Potts and Fleming, 1971) after transfer from saline water to freshwater.

Along with reductions in passive Na^+ efflux, Na^+ influx increased progressively in both populations after freshwater transfer and, by 14 days, reached levels 3- to 5-fold above the initial influx. These influx rates are somewhat lower than previous reports in northern killifish, both shortly after transfer (Wood and Laurent, 2003) and after freshwater acclimation (Potts and Evans, 1966, 1967; Patrick and Wood, 1999). This difference is undoubtedly explained by the lower Na^+ levels in our freshwater (0.17 mmol l^{-1}) compared with these other studies (2–5-fold higher), because Na^+ influx is critically dependent on environmental Na^+ in this concentration range (Patrick et al., 1997). Taken together, the results discussed above suggest that differences in Na^+ regulation between populations of *F. heteroclitus* are small and are unlikely to account for the pronounced differences in mortality in freshwater.

Regulation of Cl^- balance

Northern and southern killifish populations differ significantly in their ability to regulate Cl^- in freshwater, which probably contributes to the large differences in mortality they experienced after transfer. Northern fish actually appear to regulate Cl^- levels more strictly than Na^+ levels, as plasma Cl^- was maintained for at least 30 days after freshwater transfer. This is in agreement with previous reports for northern killifish. For example, in a study by Jacob and Taylor (1983), transfer from seawater to freshwater decreased serum osmolality transiently, which was almost entirely accounted for by changes in serum Na^+. By contrast, we observed that southern killifish rapidly lost Cl^- balance after freshwater transfer, as plasma levels fell quickly after transfer and were not re-established. It is possible that these decreases in Cl^- levels were sufficient to cause mortality: similar decreases in plasma Cl^- levels have been observed in long-horned sculpin

(*Myoxocephalus octodecimspinosus*) after transfer to hyposmotic environments, and these decreases were associated with greater mortality (Claiborne et al., 1994). In addition, Cl^- imbalance would have created a 'strong ion' difference in the plasma of southern killifish (i.e. $[Na^+]>[Cl^-]$). To maintain charge neutrality, compensatory increases in plasma $[HCO_3^-]$ and pH may have occurred in southern fish, so the resulting blood alkalosis might have also contributed to the differences in mortality between populations.

The observed differences in plasma Cl^- between northern and southern killifish were probably due to differences in Cl^- flux. Because unidirectional Cl^- influx is extremely small in *F. heteroclitus* in freshwater (present study; Patrick et al., 1997; Patrick and Wood, 1999; Wood and Laurent, 2003), changes in total Cl^- flux after freshwater transfer are primarily dictated by changes in unidirectional Cl^- efflux. Shortly after transfer, individuals from both populations reduced passive Cl^- loss, which is consistent with previous results (Pic, 1978). Northern populations continued to decrease Cl^- efflux, such that Cl^- loss was eliminated by 24 h following freshwater transfer and remained negligible thereafter. This rapid elimination of Cl^- loss is likely to account for the ability of northern killifish to maintain plasma Cl^- balance in spite of negligible branchial uptake. Southern killifish did not eliminate Cl^- efflux, which remained consistently higher than 100 μmol kg^{-1} h^{-1}, so they were unable to preserve Cl^- balance.

In order to eliminate Cl^- efflux, killifish entering freshwater must eliminate both active and passive routes of Cl^- excretion. Active secretion of ions across fish gills as occurs in seawater involves a basolateral Na^+/K^+-ATPase, a basolateral $Na^+/K^+/2Cl^-$ cotransporter (NKCC1) and an apical cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (see reviews by Wood and Marshall, 1994; Perry, 1997; Evans et al., 1999; Marshall, 2002). One and four days after freshwater transfer, individuals from both populations decreased gill mRNA expression of the seawater transporters NKCC1 and CFTR. In fact, this suppression actually persisted for a longer duration in southern killifish, so differences in active Cl^- secretion are unlikely to account for the observed differences in Cl^- efflux.

Populations of *F. heteroclitus* appear to differ in their ability to eliminate passive Cl^- loss. The higher extrarenal clearance rate of PEG-4000 in southern killifish indicates that the paracellular permeability of their gills is higher; increased Cl^- loss may have therefore occurred through the paracellular pathway. The observed differences in gill morphology between populations are consistent with this hypothesis. A typical morphological feature of the gills of fish in seawater is the presence of apical crypts, which are formed by multicellular complexes of MR cells that share shallow junctions with high solute permeability. Both populations have equal abundance of apical crypts in brackish water, at densities approximately 7–8-fold lower than in seawater-acclimated animals (Hossler et al., 1985). However, southern killifish have significantly more apical crypts in freshwater and thus maintain morphological features of the seawater gill. After transfer of northern killifish to hyposmotic environments, apical crypts are either covered over by pavement cells (Daborn et al., 2001) or are widened to uncover freshwater-type MR cells equipped with microvilli (Katoh and Kaneko, 2003). Freshwater MR cells typically form tight junctions with neighbouring cells that have low paracellular permeability to solutes (Sardet et al., 1979; Ernst et al., 1980). These morphological transformations occur in conjunction with rapid reductions in Cl^- secretion (Daborn et al., 2001). More apical crypts on the gills of southern killifish therefore suggests that fewer crypts were covered and/or converted into freshwater-type MR cells; incidentally, there was a trend towards greater freshwater MR cell density in northern killifish.

As well as the structural reorganization of cell–cell junctions that occurs during transformation between seawater and freshwater gill morphologies, associated changes occur in the actin cytoskeleton within MR cells. These morphological alterations to cytoskeletal elements are important for the changes in transepithelial conductance that occur after salinity change (Daborn et al., 2001). Interestingly, interactions between actin and Na^+/K^+-ATPase appear essential for tight junction formation in epithelia (Rajasekaran and Rajasekaran, 2003), so differences in Na^+/K^+-ATPase gene regulation between killifish populations may be related to differences in Cl^- efflux through the paracellular pathway.

In addition to greater Cl^- efflux and paracellular permeability, southern killifish exhibited lower glomerular filtration rates and lower urination burst frequencies than did northerns in freshwater. High glomerular filtration rates and urination frequencies are characteristic of freshwater fish, while the opposite are characteristic of seawater fish (e.g. Hickman and Trump, 1969; Curtis and Wood, 1991; Sloman et al., 2004). These observations suggest that slower or incomplete acclimation of renal function may have been another factor contributing to the poorer ionoregulatory performance and survival of southerns in freshwater.

Possible mechanisms of freshwater adaptation

Our data suggest that southern killifish are less tolerant of freshwater transfer than are northerns because the coordinated response of their gills and kidney is less effective at maintaining ion balance. This may include small differences in Na^+ regulation, suggested by small differences between populations in plasma Na^+, Na^+/K^+-ATPase expression and activity in the gills, and ion flux and possibly gill MR cell abundance. A more convincing cause for differences in mortality, however, is the differences in Cl^- regulation. Southern killifish experience large decreases in plasma Cl^-, have significant Cl^- efflux and maintain a moderate density of apical crypts after freshwater transfer. By contrast, northern killifish maintain plasma Cl^-, eliminate Cl^- efflux, have lower paracellular permeability, have very few apical crypts in freshwater and have more typical freshwater renal function. Taken together, southern killifish seem to preserve some elements of seawater ionoregulatory physiology, while northerns are better able to make the necessary adjustments for freshwater acclimation.

A great deal of evidence suggests that northern populations of *F. heteroclitus* have evolved greater freshwater ionoregulatory ability than have southern populations. This is indicated by differences in distribution patterns (Powers et al., 1993), reproductive success (Able and Palmer, 1988) and adult survival (present study) in hyposmotic environments. These differences may have arisen from selection acting on pre-existing variability within *F. heteroclitus* or, possibly, from introgression of alleles from sympatric freshwater species (e.g. *F. diaphanus*; Dawley, 1992). Although the evolutionary pressures accounting for these differences in salinity tolerance are still uncertain, our data suggest that minimizing Cl^- imbalance was an essential evolutionary step allowing northern killifish to survive in freshwater. Freshwater acclimation was possible in northern killifish despite no apparent mechanism for branchial Cl^- uptake. These animals may instead be able to survive in freshwater habitats and maintain Cl^- balance by minimizing Cl^- efflux and meeting Cl^- demands through the diet (Wood and Laurent, 2003).

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