The Journal of Experimental Biology 211, 2450-2459
Published by The Company of Biologists 2008
doi:10.1242/jeb.017947

Physiological and molecular mechanisms of osmoregulatory plasticity in killifish after seawater transfer

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Accepted 22 May 2008

SUMMARY

We have explored the molecular and physiological responses of the euryhaline killifish *Fundulus heteroclitus* to transfer from brackish water (10% seawater) to 100% seawater for 12 h, 3 days or 7 days. Plasma [Na^+] and [Cl^-] were unchanged after transfer, and plasma cortisol underwent a transient increase. Na^+/K^+-ATPase activity increased 1.5-fold in the gills and opercular epithelium at 7 days (significant in gills only), responses that were preceded by three- to fourfold increases in Na^+/K^+-ATPase α_{1a} mRNA expression. Expression of $Na^+/K^+/2Cl^-$ cotransporter 1, cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel, Na^+/H^+-exchanger 3 (significant in opercular epithelium only) and carbonic anhydrase II mRNA also increased two- to fourfold after transfer. Drinking rate increased over twofold after 12 h and remained elevated for at least 7 days. Surprisingly, net rates of water and ion absorption measured *in vitro* across isolated intestines decreased ~50%, possibly due to reduced salt demands from the diet in seawater, but water absorption capacity still exceeded the drinking rate. Changes in bulk water absorption were well correlated with net ion absorption, and indicated that slightly hyperosmotic solutions (≥298 mmol l^{-1}) were transported. There were no reductions in unidirectional influx of Na^+ from luminal to serosal fluid or intestinal Na^+/K^+-ATPase activity after transfer. Overall, our results indicate that gill and opercular epithelia function similarly at a molecular level in seawater, in contrast to their divergent function in freshwater, and reveal unexpected changes in intestinal function. As such they provide further insight into the mechanisms of euryhalinity in killifish.

Key words: *Fundulus heteroclitus*, gene expression, intestine, gills, opercular epithelium.

INTRODUCTION

The euryhaline killifish *Fundulus heteroclitus* lives in estuaries and salt marshes on the eastern coast of North America. Killifish must dynamically regulate their ion balance because of routine fluctuations in salinity in their natural environment. This species can tolerate changes in salinity from freshwater to nearly four times seawater (Griffith, 1974), and have therefore been used extensively to understand fish osmoregulation (Wood and Marshall, 1994), the mechanisms of physiological plasticity (Marshall, 2003; Scott et al., 2004a; Scott et al., 2005b) and the evolution of salinity tolerance (Scott et al., 2004b; Scott and Schulte, 2005; Singer et al., 2008).

When killifish move into seawater they rapidly increase active ion secretion by two secretory epithelia, the gills and opercular epithelium, to counteract passive salt loading (Marshall et al., 1999; Wood and Laurent, 2003; Prodocimo et al., 2007). The initial activation of ion secretion in opercular epithelium involves membrane trafficking and rapid activation of pre-existing ion transporters (Hoffmann et al., 2002; Marshall et al., 2002b), which may also be true for the gills (Towle et al., 1977; Mancera and McCormick, 2000). Longer-term modulation of ion secretion involves transcriptional increases in gill ion transporter abundance (Scott et al., 2004a) as well as changes in the morphology of both secretory epithelia (Hossler et al., 1985; Daborn et al., 2001; Katoh et al., 2001; Laurent et al., 2006).

While a fair amount is known about transcriptional regulation of ion transport in the gills of killifish transferred to seawater (Singer et al., 1998; Scott et al., 2004a; Scott and Schulte, 2005; Choe et al., 2006; Shaw et al., 2007), much less is known about the opercular epithelium. Gene expression differs greatly between these tissues after transfer of intact killifish to freshwater (Scott et al., 2005a). These molecular differences are probably the basis for divergent physiological mechanisms of ion transport in freshwater between these two organs (Wood and Marshall, 1994; Marshall et al., 1997; Patrick et al., 1997; Burgess et al., 1998; Patrick and Wood, 1999). However, the gills and opercular epithelium are thought to function similarly in seawater (Burns and Copeland, 1950; Karnaky et al., 1977), which has prompted wide-spread *in vitro* use of the thin opercular membrane as a model for understanding the function and regulation of ion secretion in the gills of marine fish (reviewed by Zadunaisky, 1984; Karnaky, 1986; Péqueux et al., 1988; Wood and Marshall, 1994; Marshall, 1995; Marshall and Bryson, 1998; Marshall and Singer, 2002; Marshall, 2003). It is therefore of interest to determine whether the molecular responses of these two tissues to seawater transfer are similar.

The intestine is essential for counteracting passive water loss in seawater fish (Potts and Evans, 1967). Water is ingested and absorbed across the intestine, following osmotic gradients that are created by transepithelial NaCl transport driven by the activity of basolateral Na^+/K^+-ATPase (Loretz, 1995; Schettino and Lionetto, 2003; Grosell et al., 2005). Some recent evidence suggests that this organ may dynamically regulate salt absorption in response to salinity change (Scott et al., 2006; Grosell et al., 2007), but little is

known about how intestinal plasticity contributes to euryhalinity in fish.

The first objective of the present study was to compare patterns of gene expression in the gills and opercular epithelium of killifish after transfer to seawater. Because of the apparently similar physiological functions of these tissues after transfer to seawater, we hypothesized that the expression of genes associated with ion secretion would increase in both (Hwang and Lee, 2007), in contrast to the divergent pattern of gene expression which we documented previously after transfer to freshwater (Scott et al., 2005a). Our second objective was to characterize intestinal function in killifish after seawater transfer by measuring drinking rates, intestinal water and ion transport rates and Na^+/K^+-ATPase activity. We initially hypothesized that all would increase, because of the increased importance of intestinal water absorption in osmoregulation in seawater. Some of our hypotheses were supported, whereas others were not, but in total our results cast further light on the physiological and molecular mechanisms underlying the osmoregulatory plasticity of this species.

MATERIALS AND METHODS

Experimental animals

Adult killifish (*Fundulus heteroclitus* L.; 2–5 g) were captured from estuaries near Antigonish, Nova Scotia. Fish were held in static charcoal-filtered fibreglass tanks at a salinity of 10% seawater (3.5 ppt), at room temperature (18–22°C) and 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). Animal care and experimentation was performed according to McMaster University animal care protocols #02-10-61 and #06-01-05.

Salinity transfer protocol

Fish were acclimated to a salinity of 10% seawater (brackish water) for at least 1 month before transfer. Six separate experiments were performed, all with sampling at various times (usually 12 h, 3 days and 7 days) after transfer from brackish water to 100% seawater. Transfer from brackish water to seawater was used because of its environmental relevance to estuaries, and because we sought to compare our results with previous studies using the same salinity transfer protocol (Wood and Laurent, 2003) and with parallel transfers from brackish water to freshwater (Scott et al., 2005a; Scott et al., 2006). Experiment 1 focused on gill and opercular epithelium Na^+/K^+-ATPase activities as well as plasma Na^+ and Cl^- concentrations. Experiment 2 determined intestinal Na^+/K^+-ATPase activities and plasma cortisol concentrations. Experiment 3 measured gill and opercular epithelium mRNA expression. Experiment 4 recorded drinking rates. Experiment 5 measured the ionic composition of the intestinal fluid. Experiment 6 involved *in vitro* measurements of intestinal water and ion fluxes. In experiments 1, 4 and 6, measurements were made before (i.e. brackish water) and at 12 h, 3 days and 7 days after transfer to 100% seawater. In experiments 2, 3 and 5, measurements were made before (i.e. brackish water) and at 12 h (note: this was the only post-transfer sampling point in experiment 5), 3 days and 7 days after transfer to both brackish water (i.e. handling control) and 100% seawater. The simultaneous brackish water handling control treatments were incorporated in these latter experiments as we expected that there was a greater chance that handling alone might disturb the parameters being monitored in these experiments.

All fish transfers were made using a net. At sampling, fish were rapidly sacrificed with either a lethal dose of tricaine methanesulfonate anaesthetic (0.8 g l^{-1} MS-222; Syndel Laboratories, Vancouver, BC, Canada; neutralized with NaOH) or by cephalic blow. Detailed procedures for most of these experiments have been published (Scott et al., 2004a; Scott et al., 2005a; Scott et al., 2006), so will be discussed only briefly here.

In experiments 1 and 2, gills (second and third arches), opercular epithelia, intestines (split into anterior, middle and posterior segments as described below), and plasma were sampled, frozen in liquid N_2 and stored at –70°C. Na^+/K^+-ATPase activities were determined by a method outlined previously (McCormick, 1993) and plasma cortisol was determined by radioimmunoassay, both as previously described (Scott et al., 2005a; Scott et al., 2006).

In experiment 3, gills and opercular epithelia were sampled, immediately frozen in liquid N_2, and stored at –70°C. Frozen tissues were subsequently transferred to ice-cold RNAlater-ICE (Applied Biosystems, Foster City, CA, USA) and returned to –70°C. RNA extraction, reverse transcription and measurements of gene expression using quantitative real-time PCR have been previously described in detail (Scott et al., 2004a; Scott et al., 2005a). Primer sequences for killifish Na^+/K^+-ATPase α_{1a} (accession number AY057072), $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1; Acc. no. AY533706), cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (Acc. no. AF000271), Na^+/H^+-exchanger 3 (NHE3; Acc. no. AY818825), carbonic anhydrase II (CAII; Acc. no. AY796057) and elongation factor 1α (EF1α; Acc. no. AY430091) have been previously reported (Scott et al., 2005a). EF1α was used as an expression control because its mRNA expression does not change following salinity transfer (Scott et al., 2004a). All samples were run in duplicate, and data are expressed relative to the pre-transfer brackish water control samples. Control reactions were conducted with no cDNA template or with non-reverse transcribed RNA to ensure that levels of background and genomic DNA contamination were low.

In experiment 4, drinking rates were measured as previously described (Scott et al., 2006). Fish were moved to static polyethylene chambers containing 200 ml of the appropriate water, and allowed to settle for 2 h. At the start of each measurement period, 8 μCi (0.29 MBq) of radiolabelled polyethylene glycol ([^{3}H]PEG-4000, 57.70 MBq g^{-1}; NEN Life Science Products Inc., Boston, MA, USA) was added to the chamber. Water samples (5 ml) were taken 0, 3 and 6 h later for radioactivity measurements. Fish were then killed with MS-222, rinsed in clean water, and weighed. Blood was collected by caudal puncture and the separated plasma was used for radioactivity measurements, to ensure that [^{3}H]PEG-4000 was not absorbed but always stayed in the gastrointestinal tract. The gastrointestinal tract was then exposed and ligated at both ends (anterior esophagus and rectum). The entire gastrointestinal tract was weighed, digested in HNO_3, and centrifuged. [^{3}H]PEG-4000 radioactivity in the supernatant was measured. Drinking rate is expressed as the volume ingested (from radioactivity counts of the tract digest and the water samples), relative to body mass and [^{3}H]PEG-4000 exposure time. The actual experimental period (approximately 6 h) was scheduled so that the nominal time (e.g. 12 h post-transfer) would be in the middle of this period. The selection of a 6 h period was based on a pilot time course study, which revealed that radioactivity did not appear in rectal fluid (sampled from the anal opening) prior to 8 h of [^{3}H]PEG-4000 exposure.

In experiment 5, ionic composition was measured in the anterior, middle and posterior segments of the intestine. The whole intestinal tract was ligated immediately posterior to the oesophagus and at the anus. Functional differences can exist along the length of the

intestine (Bucking and Wood, 2006), so the tract was also ligated to demarcate three anatomical segments: anterior, middle and posterior segments were upstream, between, and downstream, respectively, of the sharp caudo-rostral and rostro-caudal bends in the killifish intestine. The entire contents of each segment were collected and centrifuged at 10 000 ***g*** for 1 min. Na^+, Cl^-, Ca^{2+} and Mg^{2+} concentrations were measured in the free supernatant. This procedure was performed on fish that had been fasted for either 24 h (when solid material was still present in the gut) or for 3 d (when little or no solid material remained in the gut), to determine if the presence of food impacted the ionic composition of the gut fluids.

In experiment 6, intestinal water transport and ion flux rates were measured *in vitro* as described earlier (Scott et al., 2006), but using a 2 h flux period to avoid the isotopic recycling noted over longer periods in that study. At each time point, the whole intestinal tract was removed, the anterior end was cannulated with heat-flared PE-50 polyethylene tubing, and the tract was flushed thoroughly with a modified Cortland saline (composition in mmol l^{-1}: NaCl 133, KCl 5.0, $CaCl_2$ 1.0, $MgSO_4$ 1.9, NaH_2PO_4 2.9, glucose 5.5; pH 7.4) (Wolf, 1963). The sac was then filled with 0.5 ml of this saline containing radiolabelled ^{22}Na (0.1 μCi ml^{-1}=0.004 MBq ml^{-1}; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) and sealed. A sample of this filling solution (1 ml) was taken for ^{22}Na counting, and analysis of total [Na^+] and [Cl^-]. Saline (rather than brackish water or sea water) was used in the sac to avoid passive water or ion movements due to osmotic gradients. The sealed gut sacs were suspended in non-radioactive oxygenated Cortland saline (11 ml) for 2 h. Samples (1 ml) of the external (serosal) solution were taken and the sacs were weighed at 0 h, 1 h and 2 h. Final samples of the internal (mucosal) solution were taken at 2 h for ^{22}Na counting and analysis of total [Na^+] and [Cl^-]. Fluid transport rate was determined from the linear changes in sac mass over time. Net ion fluxes were calculated from the changes in mucosal Na^+ and Cl^- contents over time. Unidirectional Na^+ influx from mucosal to serosal solutions was calculated from the rate of ^{22}Na appearance in the serosal solution and the specific radioactivity of Na^+ of the mucosal solution. Efflux was calculated from the difference between influx and net flux rate in the same preparations. All flux rates were expressed as a function of the intestinal surface area (measured by tracings onto graph paper). A typical 4 g killifish had a gross intestinal surface area of about 10 cm^2. The apparent whole-animal capacity for water absorption was thus calculated by multiplying the measured flux rates by 10 cm^2 and dividing by 0.004 kg.

Ion and radioactivity measurements

Sodium concentrations were determined using flame atomic absorption spectrophotometry (SpectrAA-220FS, Varian, Mulgrave, VC, Australia). Chloride concentrations were measured by coulometric titration (CMT-10 chloridometer, Radiometer, Copenhagen, Denmark), with the exception of gut fluid samples, which were measured by colorimetric assay (Zall et al., 1956). High concentrations (100 mmol l^{-1}) of HCO_3^- or SO_4^{2-}, which may be present in gut fluids (Grosell, 2006), had no effect on the colorimetric assay (data not shown). ^{22}Na radioactivities were measured in a Minaxi Autogamma 5000 counter (Packard Instruments, Downers Grove, IL, USA). [^{3}H]PEG-4000 radioactivity was measured by scintillation counting (Rackbeta 1217; LKB Wallac, Turku, Finland): for plasma or tissue digests, 0.7 ml were added to 10 ml of an acid-compatible scintillation cocktail (Ultima Gold; Packard Bioscience, Meriden, CT, USA); for water samples, 5 ml was added to 10 ml of an aqueous compatible cocktail (ACS; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Quenching was determined using the external standard ratio method, and was found to be uniform across samples within each type of fluor. Data were corrected for the slight relative difference in counting efficiencies between the two scintillation fluors, which we observed by internal standardization.

Statistical analyses

Data are expressed as means ± s.e.m. ANOVA was used to ascertain overall differences (one, two or three factor, where appropriate). The effects of seawater transfer were assessed by pair-wise comparison with pre-transfer controls and/or time-matched brackish water controls. Holm–Sidak *post-hoc* comparisons were used in one-factor ANOVAs (gill and opercular epithelium Na^+/K^+-ATPase activities, drinking rate and intestinal fluid transport) for comparisons with pre-transfer brackish water controls. Student–Newman–Keuls *post-hoc* comparisons were used in two- (analyses of mRNA expression, plasma cortisol and intestinal ion transport data) and three- (analyses of gut fluid composition and intestinal Na^+/K^+-ATPase activity data) factor ANOVAs for comparisons with pre-transfer and/or time-matched brackish water controls. Least-squares linear regression was used to assess the relationships between bulk water transport and net strong ion flux. All statistical analyses were conducted using Sigmastat (version 4, Systat Software Inc., San Jose, CA, USA) and a significance level of $P<0.05$ was used throughout.

RESULTS

Plasma ions and cortisol after seawater transfer

Killifish maintained ion balance after transfer from brackish water to seawater, without any apparent increase in either plasma Na^+ or plasma Cl^- concentration (Table 1). Plasma cortisol increased two- to threefold at 12 h and 3 days after transfer (although the former

Table 1. Plasma Na^+, Cl^- and cortisol concentrations of killifish

		Time after transfer			
	Salinity	Pre-transfer	12 h	3 days	7 days
Plasma Na^+ (mmol l^{-1})	BW	143.1±5.8 (9)	–	–	–
	SW		145.6±2.2 (6)	131.7±9.9 (5)	138.5±10.5 (5)
Plasma Cl^- (mmol l^{-1})	BW	142.7±7.5 (9)	–	–	–
	SW		157.9±7.8 (6)	148.8±10.4 (5)	132.1±11.5 (5)
Plasma cortisol (ng ml^{-1})	BW	67.1±19.9 (7)	41.1±7.5 (10)	50.5±23.5 (9)	53.9±16.8 (7)
	SW		112.0±21.2 (8)	160.2±47.4* (10)	49.7±13.7 (8)

Data are expressed as means ± s.e.m. (*N*). *A significant pair-wise difference from time-matched brackish water control (assessed using a *post-hoc* test; $P \leq 0.05$). BW, brackish water control; SW, seawater.

was not significant), but returned to brackish water levels by 7 days (Table 1).

Na^+/K^+-ATPase activity and gene expression in the gills and opercular epithelium

Na^+/K^+-ATPase activity in the gills increased by approximately 1.5-fold by 7 days after transfer from brackish water to seawater, and there appeared to be a similar magnitude increase in the opercular epithelium (P=0.09 by ANOVA) (Fig. 1). Activity was unchanged at 12 h and 3 days. This increase in Na^+/K^+-ATPase activity at 7 days was preceded by a three- to fourfold increase in the expression of Na^+/K^+-ATPase α_{1a}-subunit mRNA, at 12 h (gills only) and 3 days (both tissues), that persisted 7 days after transfer in both organs (Fig. 2).

The expression of other genes important for active ion secretion in seawater were also upregulated after transfer in both gills and opercular epithelium. Expression of $Na^+/K^+/2Cl^-$ cotransporter isoform 1 (NKCC1) and cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel increased by two- to fourfold after seawater transfer (Fig. 2). In opercular epithelium the increase in CFTR mRNA occurred earlier (at 12 h) than the increase in NKCC1 mRNA. Carbonic anhydrase isoform II (CAII) expression increased by twofold after transfer, but this occurred earlier in opercular epithelium (at 3 days and 7 days) than in the gills (7 days; Fig. 2). In opercular epithelium, Na^+/H^+-exchanger isoform 3 (NHE3) mRNA increased by approximately twofold at 12 h, 3 days and 7 days after transfer; however, its expression in the gills increased only slightly (<1.5-fold, not significant) in seawater (Fig. 2). Overall, the patterns of mRNA expression after seawater transfer were similar between gills and opercular epithelium, but with some temporal differences.

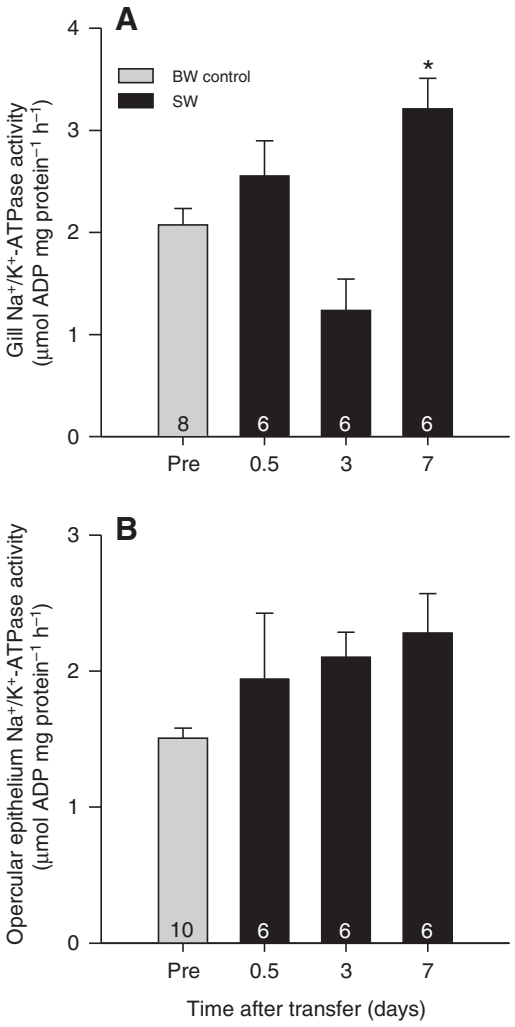

Fig. 1. (A) Na^+/K^+-ATPase activity increased in the gills of killifish after transfer from brackish water (BW; 10% seawater; grey bars) to seawater (SW; black bars). (B) Activity appeared to increase by a similar magnitude in the opercular epithelium, but this was not significant. Data are expressed as means ± s.e.m. (N shown within each bar). *Significantly different from pre-transfer (Pre) brackish water control (assessed using a *post-hoc* test; $P \leq 0.05$).

Drinking rates, water and ion transport, and Na^+/K^+-ATPase activity in the intestine

Drinking rates increased by 2.2-fold (from 1.3±0.2 ml kg^{-1} h^{-1}) early after transfer from brackish water to seawater (Fig. 3). Drinking declined slightly at 3 days and 7 days, such that it was not statistically different from brackish water controls, but appeared to remain elevated by approximately 1.6-fold on average.

The ionic composition of ingested seawater was reduced in the intestinal lumen. Luminal Na^+ and Cl^- concentrations were similar to or less than those in blood plasma (Table 1) or physiological saline, regardless of salinity, the presence of food in the gut or the site of measurement in the intestine (Table 2). Cl^- levels were higher than Na^+ levels in most gut fluid samples. Luminal Ca^{2+} concentration was also unaffected by salinity, food, or intestinal segment, but Mg^{2+} concentration increased following seawater transfer (Table 2). The apparent charge imbalance (measured cationic charge minus anionic charge) became more positive after seawater transfer, suggesting that unmeasured anions increased in concentration.

The transport of luminal water and ions, as assessed by gut sac experiments, decreased after transfer from brackish water to seawater. Bulk water absorption declined after transfer, to only 50% of the rate in brackish water (0.0044±0.0004 ml cm^{-2} h^{-1}) by 3 days and 7 days after transfer (Fig. 4A). Net Na^+ absorption declined similarly in seawater, to 40% of brackish water levels (from 0.58±0.06 μmol cm^{-2} h^{-1}) at 7 days (Fig. 4B). Net Cl^- absorption appeared to decline in seawater (from 0.72±0.06 μmol cm^{-2} h^{-1} in brackish water), but this decrease (to 70%) was not significant (Fig. 4B). Net Cl^- absorption was higher than net Na^+ absorption overall. There was a very good correlation between bulk water transport and net strong ion absorption rate (sum of net Na^+ and Cl^- flux rates; r^2=0.792; Fig. 5). The slope of this line was 0.00336±0.00030 ml water per μmol strong ion; its inverse was 298.0 mmol l^{-1}, indicating the 'apparent' strong ion concentration ($[Na^+]+[Cl^-]$) of the transported fluid.

The decline in intestinal water and ion transport after seawater transfer occurred without any statistically significant changes in unidirectional Na^+ flux rate from the mucosal to serosal solutions – i.e. 'influx' (Table 3). Na^+/K^+-ATPase activity in the anterior, middle and posterior segments of the intestine was similarly unaffected by transfer to seawater (Table 3). There were also no statistically significant changes in unidirectional Na^+ efflux rates (Table 3). Because unidirectional fluxes exceeded net fluxes by nearly tenfold, small undetected changes in unidirectional fluxes may have still contributed to the decline in Na^+ absorption.

DISCUSSION

The ability of killifish to maintain osmotic and ionic homeostasis during environmental salinity fluctuations depends on the

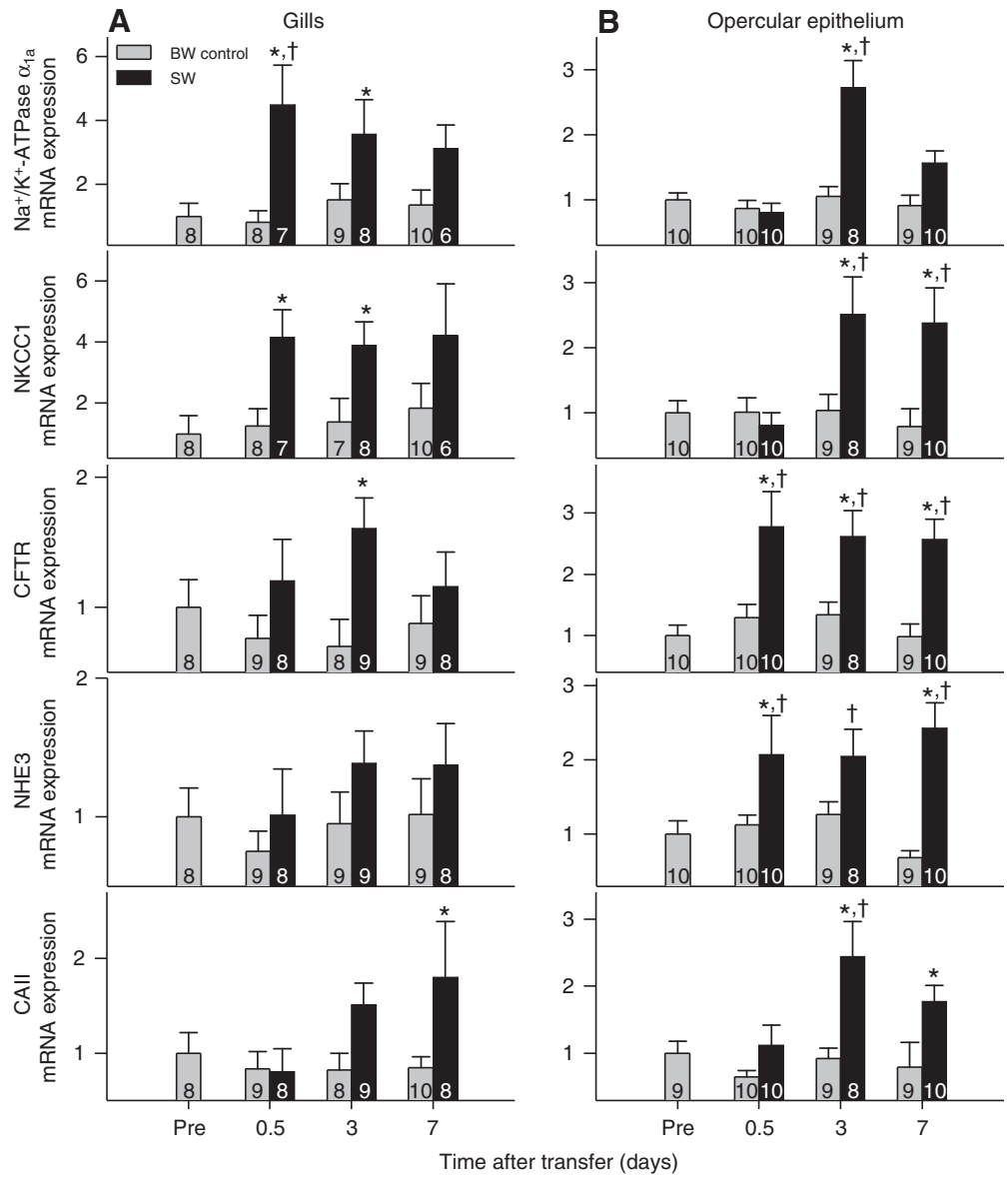

Fig. 2. Expression of genes involved in ion secretion increased in both gills (A) and opercular epithelium (B) after seawater transfer. Na^+/K^+-ATPase α_{1a} subunit, $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1), cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel, Na^+/H^+ exchanger isoform 3 (NHE3) and carbonic anhydrase isoform II (CAII) mRNA levels are shown after transfer from brackish water (10% seawater) to brackish water (BW; handling control, grey bars) or seawater (SW; black bars). Expression is relative to the expression of EF1α and is normalized to pre-transfer brackish water controls. Data are expressed as means ± s.e.m. (*N* shown within each bar). *Significantly different from time-matched brackish water controls ($P \leqslant 0.05$); †significantly different from pre-transfer (Pre) brackish water controls (assessed using a *post-hoc* test; $P \leqslant 0.05$).

Table 2. Ion concentrations in the gut fluid of killifish

	Fish starved for 24 h		Fish starved for 3 days	
Intestinal segment	BW	SW	BW	SW
Anterior				
$[Na^+]$	72.4±7.7 (7)	57.0±14.0 (6)	75.0±10.5 (7)	67.7±10.4 (7)
$[Cl^-]$	161.8±23.1 (7)	145.7±17.1 (6)	120.8±12.3 (7)	158.0±12.9 (7)
$[Ca^{2+}]$	4.6±0.5 (4)	7.9±2.5 (6)	12.1±7.9 (3)	10.6±4.5 (6)
$[Mg^{2+}]$	9.9±1.5 (4)	66.2±13.1* (6)	29.7±19.4 (3)	57.7±14.5 (6)
Middle				
$[Na^+]$	55.2±8.7 (7)	40.6±8.3 (6)	67.1±9.8 (7)	53.7±10.5 (6)
$[Cl^-]$	125.8±10.8 (7)	128.0±16.5 (6)	140.1±17.7 (7)	134.9±14.9 (6)
$[Ca^{2+}]$	4.1±0.7 (5)	2.9±1.4 (5)	4.6±2.1 (4)	11.4±2.3 (6)
$[Mg^{2+}]$	23.1±7.5 (5)	39.2±9.2 (5)	18.7±3.8 (4)	84.3±13.5* (6)
Posterior				
$[Na^+]$	56.4±17.4 (6)	45.1±6.7 (6)	72.0±6.8 (7)	46.8±11.5 (5)
$[Cl^-]$	103.5±23.9 (6)	163.8±19.1 (6)	144.4±18.3 (7)	113.4±25.4 (5)
$[Ca^{2+}]$	7.5±2.5 (3)	12.4±3.0 (4)	15.0±5.1 (3)	11.4±6.4 (3)
$[Mg^{2+}]$	31.6±9.5 (3)	116.3±30.2 (4)	26.0±12.2 (3)	72.0±34.8 (3)

Ion concentration (mmol l^{-1}) are expressed as means ± s.e.m. (*N*). Fluid fractions of the gut contents were analyzed 12 h after transfer in seawater killifish. Seawater transfer decreased $[Na^+]$ and increased $[Mg^{2+}]$ overall, but had no effect on $[Cl^-]$ or $[Ca^{2+}]$; neither duration of starvation nor intestinal segment had any overall effects on ion concentrations. *A significant pair-wise difference from brackish water control (within starvation treatment and segment; assessed using a *post-hoc* test; $P \leqslant 0.05$). BW, brackish water control; SW, seawater.

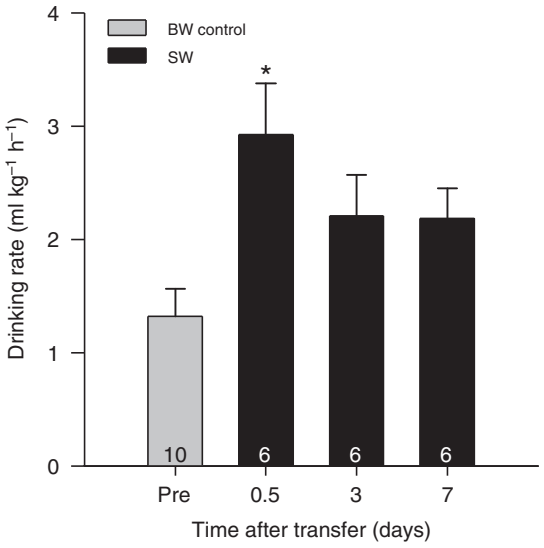

Fig. 3. Drinking rate of killifish increased after transfer from brackish water (BW, 10% seawater; grey bar) to seawater (SW; black bars). Data are expressed as means ± s.e.m. (*N* shown within each bar). *A significant difference from pre-transfer ('pre') brackish water control (assessed using a *post-hoc* test).

concerted responses of many tissues, including gills, opercular epithelium, intestine and kidney. The present study advances our understanding of how gene expression contributes to physiological plasticity in the gills and opercular epithelium of killifish after seawater transfer (Fig. 2). In particular, we confirm our original hypothesis that the patterns of gene expression in these two organs are very similar after transfer to seawater, consistent with their common function in salt secretion in the marine environment. We also confirm our hypothesis that plasticity of intestinal function is associated with euryhalinity in killifish, as shown by elevations in drinking rate (Fig. 3). However, intestinal Na^+/K^+-ATPase activity did not increase (Table 3) and intestinal water and ion transport rates *in vitro* (Figs 4 and 5) were reduced, in contrast to original predictions. Killifish maintain plasma ion levels after transfer from brackish water to seawater (Table 1) (Scott et al., 2004a; Scott et al., 2006; Scott and Schulte, 2005) and correct ionic imbalance shortly after transfer from freshwater to seawater (Marshall et al., 1999); our work provides important insight into this process, and clearly demonstrates the flexibility of osmoregulatory physiology in this species.

Gene expression and osmoregulatory plasticity

Within hours of transfer to seawater, Na^+ and Cl^- secretion by the gills of killifish increases dramatically (Wood and Laurent, 2003; Prodocimo et al., 2007). Our present results (Fig. 2A), as well as those in previous studies (Singer et al., 1998; Scott et al., 2004a; Scott and Schulte, 2005; Choe et al., 2006; Shaw et al., 2007), suggest that increases in the expression of ion transporter genes contribute to this response. Na^+/K^+-ATPase α_{1a}, NKCC1 and CFTR mRNA levels in the gills increased 12 h to 3 days after transfer of killifish to seawater, coinciding with subsequent increases in Na^+/K^+-ATPase activity at 7 days (Fig. 1). Similar results are observed after seawater transfer in other fish species, such as Atlantic salmon (*Salmo salar*), brown trout (*Salmo trutta*) and European sea bass (*Dicentrarchus labrax*) (Jensen et al., 1998; Singer et al., 2002; Tipsmark et al., 2002). Carbonic anhydrase II expression also increased in this and previous studies of fish gills after transfer to seawater (Boutet et al., 2006), and should increase the activity of this enzyme in the gills (Blanchard and Grosell, 2006). In current models of NaCl secretion by seawater-type mitochondria rich cells, carbonic anhydrase *per se* is not believed to be directly involved (Marshall and Bryson, 1998; Hirose et al., 2003; Marshall and Grosell, 2005), so the reason for this observation is uncertain. However, mitochondria-rich cell metabolism should be higher in seawater than in brackish water, so carbonic anhydrase could be important for excreting the elevated metabolic CO_2. Recent evidence for this function of carbonic anhydrase was provided in the shark rectal gland, which is rich in seawater-type mitochondria-rich cells (Shuttleworth et al., 2006).

Ion secretion across the opercular epithelium increases after seawater transfer (Zadunaisky et al., 1995; Marshall et al., 1999), which based on our current findings probably involves transcriptional regulation of ion transporters. In the opercular epithelium, as in the gills, mRNA levels of Na^+/K^+-ATPase, NKCC, CFTR and carbonic anhydrase increased after seawater transfer (Fig. 2B); interestingly, NHE3 expression increases in this tissue

Table 3. Unidirectional Na^+ fluxes and Na^+/K^+-ATPase activities in the killifish intestine

		Time after transfer			
Intestinal segment	Salinity	Pre-transfer	12 h	3 days	7 days
Unidirectional Na^+ influx					
Whole intestine	BW	5.03±0.20 (15)	–	–	–
	SW		4.65±0.13 (6)	5.57±0.25 (10)	5.18±0.38 (6)
Unidirectional Na^+ efflux					
Whole intestine	BW	4.46±0.21 (15)	–	–	–
	SW		4.18±0.10 (6)	5.16±0.22 (10)	4.94±0.33 (6)
Na^+/K^+-ATPase activities					
Anterior	BW	1.61±0.11 (10)	1.64±0.27 (10)	2.26±0.27 (10)	2.36±0.23 (10)
	SW		1.69±0.20 (8)	2.07±0.25 (10)	2.47±0.31 (10)
Middle	BW	1.49±0.08 (9)	1.59±0.21 (10)	2.49±0.34† (10)	1.97±0.43 (10)
	SW		1.62±0.19 (9)	2.37±0.23 (10)	2.11±0.22 (10)
Posterior	BW	2.21±0.37 (10)	1.46±0.26 (10)	2.89±0.47 (10)	3.71±0.61† (10)
	SW		1.85±0.10 (10)	2.27±0.18 (10)	4.01±0.41† (10)

Unidirectional Na^+ influxes (μmol cm^{-2} h^{-1}) and Na^+/K^+-ATPase activities (μmol mg protein^{-1} h^{-1}) are expressed as means ± s.e.m. (*N*). Salinity transfer had a significant overall effect on unidirectional Na^+ efflux across the intestine. Intestinal segment and time after transfer had significant overall effects on Na^+/K^+-ATPase activities, but salinity had no effect. †A significant difference from pre-transfer control (assessed using a *post-hoc* test; $P \leqslant 0.05$). BW, brackish water control; SW, seawater.

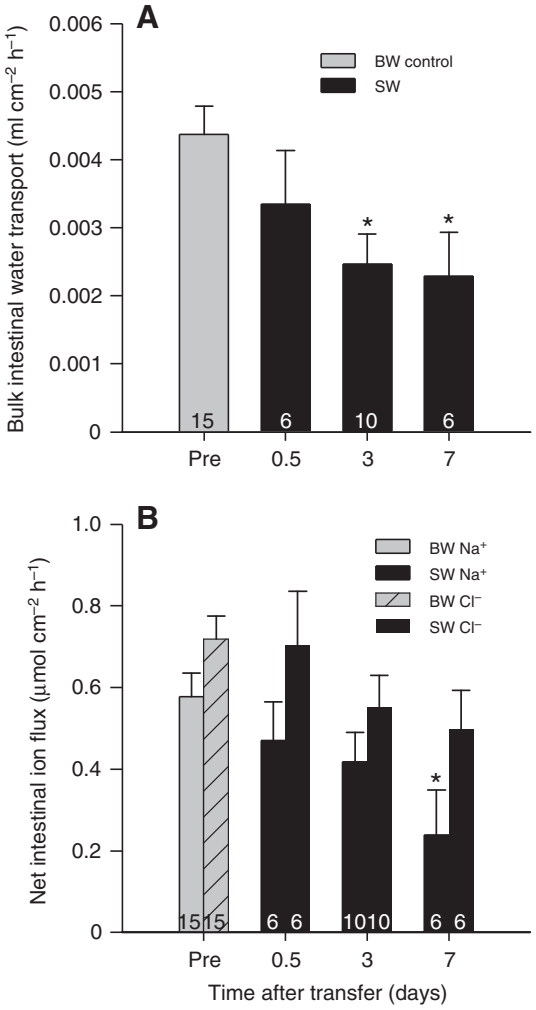

Fig. 4. Bulk water absorption rates (A) and net ion transport rates (Na^+, solid bars; Cl^-, hatched bars) (B) across the isolated killifish intestine decreased after transfer from brackish water (BW; 10% seawater; grey bars) to seawater (SW; black bars). Net absorption occurs in the positive direction. Data are expressed as means ± s.e.m. (*N* shown within each bar). *Significantly different from pre-transfer (Pre) brackish water control (assessed using a *post-hoc* test; $P \leqslant 0.05$). Net Cl^- flux was greater than net Na^+ flux overall (by two-way ANOVA).

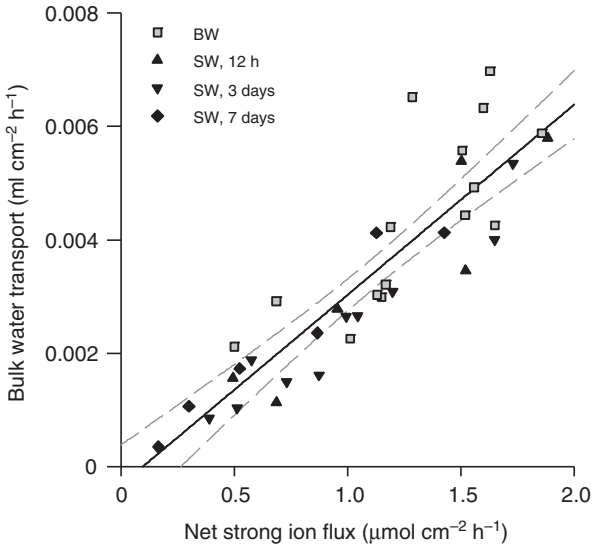

Fig. 5. Bulk water absorption rate correlated to net strong ion absorption rate (sum of net Na^+ and Cl^- flux rate) across killifish intestine, in brackish water (BW; 10% seawater; grey squares) and after transfer to seawater (SW; black triangles and diamonds). Bulk water transport was correlated to net ion transport (r^2=0.792, P<0.0001), with a slope of 0.00336±0.00030 ml H_2O μmol^{-1} strong ion. Grey dashed lines represent 95% confidence limits of regression.

after seawater transfer as well. NHE3 is probably important for acid–base regulation by the gills and opercular epithelium in seawater (Edwards et al., 2005), but in the gills it appears to be replaced, after transfer to freshwater, by NHE2, which may play dual roles in both Na^+ absorption and acid–base regulation (Edwards et al., 2005; Scott et al., 2005a). Nevertheless, the overall patterns of gene expression in the gills and opercular epithelium were very similar, consistent with their similarities in physiological function in seawater. This differs markedly to the situation in freshwater, where differences in gene expression between these tissues form a likely basis for divergent mechanisms of freshwater ion transport (Wood and Marshall, 1994; Marshall et al., 1997; Burgess et al., 1998; Patrick et al., 1997; Patrick and Wood, 1999; Wood and Laurent, 2003; Scott et al., 2004b, Scott et al., 2005a).

The temporal patterns of gene expression in the opercular epithelium were very similar to those found in the gills in previous studies of killifish (Singer et al., 1998; Scott et al., 2004a): CFTR increased very early after seawater transfer, followed by later increases in Na^+/K^+-ATPase and NKCC. By contrast, gills increased the expression of Na^+/K^+-ATPase and NKCC earlier than that of CFTR in the present study. The reason for this discrepancy is uncertain. Nevertheless, increases in ion secretion resulting from transcriptional regulation may not reach their full extent until both apical and basolateral ion transporters are expressed.

Endocrine signalling by cortisol is thought to regulate many physiological responses to seawater transfer, including events in both the gills (McCormick, 2001) and intestine (Veillette et al., 1995). Recent evidence suggests that cortisol-mediated signalling may directly regulate the expression of ion transport genes after seawater transfer in killifish, based on the structure and potential adaptive variation in the CFTR promoter (Singer et al., 2008) and the effects of glucocorticoid receptor antagonism on CFTR expression (Shaw et al., 2007). Cortisol may be similarly important for regulating gene expression and cell proliferation after freshwater transfer (Scott et al., 2004a; Scott et al., 2005b). In the current study, plasma cortisol levels in brackish water were similar to previous measurements in this species (DeKoning et al., 2004; Scott et al., 2006), and increased transiently after seawater transfer (Table 1). This elevation occurred before or concurrent to the changes in Na^+/K^+-ATPase, NKCC and CFTR expression in gills and opercular epithelium (Fig. 2). However, cortisol returned to resting levels after 7 days in seawater, when mRNA in the opercular epithelium was still elevated, so plasma cortisol elevation may not always be necessary for inducing ion transporter gene expression.

Plasticity of intestinal function after seawater transfer

Killifish that are fully acclimated to seawater have been shown to drink three- to five-times more than those acclimated to freshwater, and 20% more than those acclimated to near-isosmotic brackish water (40% seawater) (Potts and Evans, 1967; Malvin et al., 1980). Our present results show that the increase in drinking rate after

seawater transfer occurs rapidly (by 12 h), and although it declines slightly at later time points, the increase appears to persist for at least 7 days (Fig. 3). Drinking rate in seawater is therefore approximately twofold higher than in fish acclimated to 10% seawater and four- to sevenfold higher than in killifish transferred to freshwater (Scott et al., 2006). The present measurements of drinking rates in seawater killifish are lower than those reported previously on the same species (Potts and Evans, 1967; Malvin et al., 1980), perhaps reflecting less stress in the current measurements. Nevertheless, they are similar to those in other fish species acclimated to seawater (Webb and Wood, 2000), and tend to reach stable seawater rates more rapidly after transfer (Fuentes and Eddy, 1997; Aoki et al., 2003).

As we and others have previously shown (Shehadeh and Gordon, 1969; Ando et al., 2003; Scott et al., 2006), the composition of ingested fluid is adjusted early in the gastrointestinal tract, to osmolarities that are much less than seawater (Table 2). This appears to occur independent of salinity (seawater, brackish water or freshwater) or whether food is present in the gut, and in seawater is thought to involve Na^+ and Cl^- absorption by the oesophagus or stomach of the fish (Marshall and Grosell, 2005). The apparent $Na^+ + Cl^-$ concentration of fluid absorbed across the intestinal epithelium of killifish appeared to be 298 mmol l^{-1} (Fig. 5) whereas that of the incubating saline was about 276 mmol l^{-1}, suggesting that the transported fluid was slightly hyperosmotic. This value for the apparent $Na^+ + Cl^-$ concentration of absorbed fluid is in accord with previous *in vitro* measurements on this species in seawater (Marshall et al., 2002a) and fresh water (Scott et al., 2006). Our results, therefore, agree with recent suggestions that fluid transported across the fish intestine is hyperosmotic (Grosell, 2006), a phenomenon that is particularly apparent when physiologically realistic fluids (Table 2) are present in the lumen (Grosell and Taylor, 2007).

The mechanisms of intestinal ion and water transport have been the subject of numerous recent studies (e.g. Grosell et al., 2005; Grosell and Genz, 2006; Grosell and Taylor, 2007), and provide an explanation for the observed differences between net Cl^- and Na^+ fluxes (Fig. 4). In addition to cotransport of Na^+ and Cl^-, a significant portion of apical Cl^- transport in the intestinal epithelium occurs in exchange for HCO_3^-. Bicarbonate is partially provided by CO_2 hydration, which also creates a proton that is eliminated across the basolateral membrane. Therefore, luminal movement of HCO_3^- and serosal movement of H^+ (both equivalent to movement of positive charge from lumen to serosa) probably accounted for Cl^- absorption being higher than Na^+ absorption. An increase in Cl^-/HCO_3^- exchange may have also contributed to the apparent positive charge imbalance measured in the intestinal fluids after seawater transfer (Table 2 and Results), in addition to possible increases in unmeasured SO_4^{2-} anion.

Intestinal water and ion absorption *in vitro* decreased after seawater transfer (Fig. 4), in contrast to what occurs in other fish species (Ando, 1975; Aoki et al., 2003). This decrease could occur because intestinal ion absorption from food may be critical to normal ionic homeostasis in freshwater (Marshall and Grosell, 2005), as we have previously suggested for killifish (Scott et al., 2006). By this rationale, the influence of reduced salt demand from the diet on intestinal ion absorption rates exceeds the influence of increased water demand. The decrease in water and ion absorption in killifish after seawater transfer may therefore serve to minimize ion loading from the food when it is not needed; in other words, the ion flux is primary and the water flux follows passively.

Could the decrease in bulk fluid transport measured *in vitro* influence water absorption *in vivo*? This possibility can be assessed by quantitatively comparing the drinking rates to the apparent capacities for water absorption (assuming *in vitro* measurements reflect conditions *in vivo*; see Materials and methods for calculation). The estimated intestinal absorption rate in brackish water would be about 10.9 ml kg^{-1} h^{-1}, which greatly exceeds the measured drinking rate of 1.3 ml kg^{-1} h^{-1}. After 7 days in seawater, the absorptive capacity is reduced to about 5.7 ml kg^{-1} h^{-1}, but still exceeds the measured drinking rate of 2.2 ml kg^{-1} h^{-1}. Because water absorption from the environment by the intestinal tract *in vivo* cannot exceed the drinking rate, intestinal water absorption capacity is likely in excess *in vivo*. This apparent excess capacity would ensure that water balance is not impaired by the decrease in ion absorption, which may be a consequence of a unique strategy for using intestinal salt absorption from food to maintain ionic homeostasis. Greater insight into this issue could be gained by studying water and ion absorption *in vivo*, where intestinal function will be influenced by additional physiological variables (e.g. hormonal/neural control, altered ion composition, etc.).

The decrease in net water and ion absorption occurred without any significant reduction in unidirectional Na^+ influx, unidirectional Na^+ efflux, or intestinal Na^+/K^+-ATPase activity (Table 3). It is unclear if other intestinal ion transporters respond to seawater transfer in killifish, as occurs in other fish species (Grosell et al., 2007). However, the expression of numerous ion transport genes (Na^+/K^+-ATPase α_{1a}, NKCC2, CFTR, carbonic anhydrase II) were unchanged by freshwater transfer in the killifish intestine, despite a threefold increase in net ion absorption (Scott et al., 2006). Regardless, unidirectional fluxes exceeded net fluxes by nearly tenfold, so small undetected changes in unidirectional fluxes may have still contributed to the decline in Na^+ absorption.

Taken together, the results of this study reinforce our understanding of the osmoregulatory plasticity of killifish after transfer from brackish water to seawater. Changes in gene expression in both the gills and opercular epithelium facilitate the large increase in ion secretion that counteracts passive ion loading in seawater. Rapid increases in drinking rate also help replace water that is lost by passive diffusion, facilitated by the excess capacity for water absorption across the intestinal epithelium. As a consequence of their physiological flexibility, killifish can tolerate a broad range of salinities and suffer very little ionic or osmotic imbalance after salinity transfer.

This work was funded by the Natural Sciences and Engineering Research Council of Canada through Discovery Grants to C.M.W. and P.M.S., an Izaak Walton Killam Predoctoral Fellowship to G.R.S., and a Canada Graduate Scholarship to D.W.B. C.M.W. is supported by the Canada Research Chair program. The authors would like to thank W.S. Marshall for kindly supplying killifish, and L. Diao for invaluable technical support.

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