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Gene expression after freshwater transfer in gills and opercular epithelia of killifish: insight into divergent mechanisms of ion transport

Graham R. Scott[1,*], James B. Claiborne[2], Susan L. Edwards[2,3], Patricia M. Schulte[1] and Chris M. Wood[4]

[1]*Department of Zoology, University of British Columbia, Vancouver BC, Canada V6T 1Z4*, [2]*Department of Biology, Georgia Southern University, Statesboro, GA 30460-8042, USA*, [3]*Department of Physiology and Pharmacology, James Cook University, Cairns, QLD 4879, Australia* and [4]*Department of Biology, McMaster University, Hamilton ON, Canada L8S 4K1*

*Author for correspondence (e-mail: scott@zoology.ubc.ca)

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Summary

We have explored the molecular basis for differences in physiological function between the gills and opercular epithelium of the euryhaline killifish *Fundulus heteroclitus*. These tissues are functionally similar in seawater, but in freshwater the gills actively absorb Na^+ but not Cl^-, whereas the opercular epithelium actively absorbs Cl^- but not Na^+. These differences in freshwater physiology are likely due to differences in absolute levels of gene expression (measured using real-time PCR), as several proteins important for Na^+ transport, namely Na^+,H^+-exchanger 2 (NHE2), carbonic anhydrase 2 (CA2), Na^+,HCO_3^-cotransporter 1, and V-type H^+-ATPase, were expressed at 3- to over 30-fold higher absolute levels in the gills. In gills, transfer from 10% seawater to freshwater increased the activity of Na^+,K^+-ATPase by twofold (from 12 h to 7 days), increased the expression of NHE2 (at 12 h) and CA2 (from 12 h to 7 days), and decreased the expression of NHE3 (from 12 h to 3 days). In opercular epithelium, NHE2 was not expressed; furthermore, Na^+,K^+-ATPase activity was unchanged after transfer to freshwater, CA2 mRNA levels decreased, and NHE3 levels increased. Consistent with their functional similarities in seawater, killifish gills and opercular epithelium expressed Na^+,K^+-ATPase α_{1a}, Na^+,K^+,$2Cl^-$cotransporter 1 (NKCC1), cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel and the signalling protein 14-3-3a at similar absolute levels. Furthermore, NKCC1 and CFTR were suppressed equally in each tissue after freshwater transfer, and 14-3-3a mRNA increased in both. These results provide insight into the mechanisms of ion transport by killifish gills and opercular epithelia, and demonstrate a potential molecular basis for the differences in physiological function between these two organs.

Key words: *Fundulus heteroclitus*, carbonic anhydrase, Na^+,H^+-exchanger, Na^+,HCO_3^-cotransporter, fish.

Introduction

The euryhaline killifish *Fundulus heteroclitus* inhabits brackish water estuaries and salt marshes along the eastern coast of North America. These habitats undergo routine fluctuations in salinity, so killifish in their natural environment must dynamically regulate ion balance. This species is known for its exceptional euryhalinity, as it can tolerate salinities ranging from freshwater to nearly four times seawater (Griffith, 1974). For these reasons, killifish have been used extensively to understand the physiological mechanisms of osmoregulation in aquatic animals, and have revealed many details that are fundamental to our current understanding (Wood and Marshall, 1994). For example, studies on fundulids have been essential for appreciating the role of the gills in modulating ion transport (Motais et al., 1966; Potts and Evans, 1967; Pic, 1978; Marshall, 2003), the importance of mitochondria-rich cells within the gill epithelium (Copeland, 1948; Philpott, 1965), the importance of the intestine for water absorption in seawater (Potts and Evans, 1967), the role of the kidney in water excretion in freshwater (Stanley and Fleming, 1964; Fleming and Stanley, 1965), and various aspects of the hormonal regulation of ion transport (Potts and Evans, 1966; Pickford et al., 1970; Scott et al., 2005).

In addition to many studies on killifish *in vivo*, an early finding of great importance to understanding branchial ion transport was the discovery that skin lining the opercular bone of this species was similar to the gills with regards to many morphological and functional characteristics (Burns and Copeland, 1950; Karnaky et al., 1977). The flat opercular epithelium has since been the subject of numerous studies, many of which have established the mechanisms of ion secretion in seawater teleosts (Wood and Marshall, 1994; Marshall and Bryson, 1998). Although this preparation appears to be an excellent surrogate model for understanding the physiology of gills in seawater fish, recent findings suggest that

the opercular epithelium is not an adequate model for gill physiology in freshwater fish. Briefly, several studies of whole animal ion flux have demonstrated that intact killifish *in vivo* actively absorb Na^+ at high rates, while Cl^- uptake is passive and negligible in freshwater (Patrick et al., 1997; Patrick and Wood, 1999; Wood and Laurent, 2003). In contrast, when the opercular epithelium of freshwater-acclimated killifish is mounted *in vitro* with freshwater on the apical surface, it absorbs Cl^- actively at low rates and Na^+ uptake is passive (Marshall et al., 1997; Burgess et al., 1998). Only 1% of Na^+ and Cl^- transport in the intact animal is accomplished by the opercular epithelium (Degnan and Zadunaisky, 1979), so the patterns of ion flux in killifish *in vivo* are likely representative of what occurs across the gills. Although the molecular mechanisms of Na^+ absorption across killifish gills are poorly understood, apical Na^+,H^+-exchanger (NHE), basolateral Na^+,K^+-ATPase, and possibly V-type H^+-ATPase (V-ATPase) are believed to be important (Patrick et al., 1997; Patrick and Wood, 1999; Katoh et al., 2003; Edwards et al., 2005). Some other genes thought to be important in freshwater fish gills, such as carbonic anhydrase (CA) and Na^+,HCO_3^-cotransporter (NBC), may also be involved (Wood and Pärt, 2000; Marshall, 2002; Perry et al., 2003a,b). The mechanisms of Cl^- absorption across the opercular epithelium, and why these mechanisms are absent from the gills, are largely unknown (see Marshall et al., 1997; Burgess et al., 1998).

While its similarity to the gills has made the opercular epithelium useful for understanding some aspects of fish gill physiology, the functional differences between these tissues in freshwater provide an excellent comparative model to study the molecular basis of physiological function. In particular, differences in gene expression between gills and opercular epithelium could be responsible for their differences in Na^+ and Cl^- transport in freshwater. An important objective of the present study was therefore to compare the expression of several ion transport genes in gills and opercular epithelium of killifish. Because little is known about the role and expression of some of the genes cloned in the present study (e.g. NHE2, NHE3, CA2 and NBC1), an additional objective was to characterize the expression patterns of these genes after abrupt transfer from brackish water (10% seawater) to freshwater. Near-isosmotic brackish water is the preferred salinity for *F. heteroclitus* (Fritz and Garside, 1974), and transfer from brackish water to freshwater may be more environmentally representative of the conditions that killifish naturally encounter in estuaries

Materials and methods

Experimental animals

Adult killifish *Fundulus heteroclitus* L. (3–8 g) were captured from one of three locations for use in this study. All those used in the salinity transfer experiment, as well those used for cloning CA2 and NBC1, were captured from estuaries near Antigonish, Nova Scotia, Canada (i.e. a northern population; see Scott et al., 2004b). Fish used for NHE cloning were caught from estuaries in either Tybee Island, Georgia, USA or from Mount Desert Island, Maine, USA. For the salinity transfer studies, fish were held in static charcoal-filtered fiberglass tanks at a salinity of 10% seawater (3.5 p.p.t.) made up in dechlorinated Hamilton, Ontario, Canada tapwater ([Na^+], 0.82 mmol l^{-1}; [Cl^-], 1.07 mmol l^{-1}; hardness, 120 mg l^{-1} as $CaCO_3$; pH 8.0). Before sampling, fish were maintained 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). All animal care and experimentation was conducted according to McMaster University animal care protocol #02-10-61.

Total RNA extraction, reverse transcription and cloning

Genes of interest were cloned from killifish gills, which were sampled as described below. Total RNA was extracted using either Trizol (Invitrogen, Burlington, ON, Canada) or Tri-Reagent (Sigma-Aldrich, Oakville, ON, Canada) isolation reagents following the manufacturer's instructions. RNA concentrations were determined spectrophotometrically and RNA integrity was verified by agarose gel electrophoresis [~1% (w/v) agarose:Tris-acetate-EDTA]. Extracted RNA samples were stored at –80°C following isolation. First strand cDNA was synthesized by reverse transcribing total RNA using oligo(dT) primer and either RevertAid H Minus M-MuLV reverse transcriptase (MBI Fermentas, Burlington, ON, Canada) or Superscript II RNAse H-reverse transcriptase (Invitrogen), following each manufacturer's protocols.

Several genes of potential importance for ion regulation in fish were cloned using a polymerase chain reaction (PCR)-based approach. Multiple alignments of previously published cDNA sequences were constructed using ClustalW (Thompson et al., 1994) to identify conserved gene regions, from which primers were then designed (Table 1). Specific sequences within CA2, NBC1, NHE2, and NHE3 were amplified from killifish gills using *Taq* polymerase (MBI Fermentas or Invitrogen). Each PCR consisted of 35–40 cycles of 30–60 s at 94°C, 30–60 s at the lower annealing temperature for each respective primer set (see Table 1), and 60 s for every 1000 bp of expected product at 72°C. PCR products were verified by electrophoresis on 1% agarose gels containing ethidium bromide and cloned into pGEM-T Easy (Promega, Nepean, ON, Canada) or pCR2.1 (Invitrogen) vector plasmid. Multiple clones of each fragment were sequenced bidirectionally, and the partial CA2, NBC1 and NHE3 consensus sequences obtained were submitted to the GenBank database (CA2, accession no. AY796057; NBC1, acc. no. AY796058; NHE3, acc. no. AY818825).

The complete cDNA sequence of NHE2 was subsequently obtained by rapid amplification of cDNA ends (RACE). Initial PCR amplification of the 3′ region was achieved using a killifish gene-specific primer (5′-TCT TTG TGG GAC TGT TCT TCG GCT TG-3′) and the Invitrogen GeneRacer 3′

Table 1. *Primers used for cloning*

Gene	Direction	Sequence (5′–3′)	T_m (°C)	Species	Accession no.
CA2	F	(C/T)CG (C/G)CA GTC (C/T)CC (C/T/A)(A/G)T (T/A/G)GA	50	*Danio rerio*	NM_199215.1
	R	CAG (A/T)GG (A/T/G/C)GG (T/A/G)GT GGT C(A/C)G (G/A)G	50	*Mus musculus*	NM_009801.3
				Oncorhynchus mykiss	AY5414870
				Platichthys flesus	AF093622
				Tribolodon hakonensis	AB055617
NBC1	F	GC(G/C) AG(G/A) TGG AT(A/T/C) AAG TTT GA(G/A) GA	52	*Ambystoma tigrinum*	AF001958
	R	GA(G/A/C) CG(C/A) AGG TTG GA(C/T) TT(C/T) TTG	53	*Mus musculus*	AF141934
				Oncorhynchus mykiss	AF434166
				Tribolodon hakonensis	AB055467
NHE2	F	AA(C/T) GA(C/T) G(C/G)I GTI ACI GTI GT		See Towle et al. (1997)	
	R	GG IC(G/T) IAT IGT IAT ICC (C/T)TG			
NHE3	F	GC(C/G/A/T) GT(G/T) CTG GC(C/G/A/T) GTI TT(C/T) GA(G/A) GA		See Hirata et al. (2003)	
	R	GC(C/G/A/T) CC(C/G/A/T) C(G/T)(C/G/A/T) A(G/A)I CCI CC(G/A) TA			

F, forward; R, reverse; T_m, annealing temperature.

primer. Similarly, amplification of the 5′ region used a gene-specific primer (5′-GGT CTC ACT CAC GCT ACT CCA CAT C-3′) and the Invitrogen GeneRacer 5′ primer. For both 3′ and 5′ RACE protocols, the possibility of PCR artifact was reduced by using nested RACE-PCR, which used 1 μl of the original amplification reaction as a template, killifish gene specific primer (3′ RACE: 5′-TCC CCT CTT CGT CTT CCT CTA CTC-3′; 5′ RACE: 5′-GCT CTG ACA CAT TCG CTT CCA C-3′), and the GeneRacer 3′ or 5′ nested primer. Thermal cycling parameters for all reactions included an initial incubation at 95°C (5 min), followed by 35 cycles of 94°C (30 s), 60°C (90 s), and 72°C (180 s), and a final extension at 72°C (7 min). The resulting products were visualized, sub-cloned, and sequenced as previously described, and the complete NHE2 consensus sequence was submitted to GenBank (NHE2, acc. no. AY818824).

In addition to the sequences cloned in this study, several other killifish cDNA sequences were used in this work for real-time PCR analysis of gene expression. These sequences were for the signalling protein 14-3-3a (acc. no. AF302039), cystic fibrosis transmembrane conductance regulator Cl^- channel (CFTR; acc. no. AF000271), elongation factor 1α (EF1α; acc. no. AY430091), Na^+,K^+-ATPase α_{1a} (acc. no. AY057072), Na^+,K^+,2Cl^-cotransporter 1 (NKCC1; acc. no. AY533706), and V-type H^+-ATPase subunit A (V-ATPase; acc. no. AB066243).

Salinity transfer protocol

Fish were acclimated to a salinity of 10% seawater (brackish water) for at least 1 month before salinity transfer. In experiment 1, Na^+,K^+-ATPase activity was measured in the gills and opercular epithelia of fish before transfer, or 12 h, 3 days or 7 days after transfer to freshwater. In experiment 2, mRNA expression in the gills and opercular epithelia was measured 12 h, 3 days or 7 days after transfer either to brackish water (i.e. a sham treatment where the animals were simply transferred between two tanks of their same acclimation salinity) or to freshwater. In all cases, fish were transferred between tanks with a net. Fish were sampled by cephalic blow followed by rapid decapitation, second and third whole gill arches were removed, opercular epithelia were isolated by scraping off the underside of the opercular bone, and both tissues were immediately frozen in liquid nitrogen. Tissues were not perfused before freezing because blood has been previously shown to contribute little to whole-gill expression levels (Perry et al., 2000; Scott et al., 2004a). All tissues were stored at –80°C until analyzed.

Na^+,K^+-ATPase activity assay

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, gills and opercular epithelia 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% sodium 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 analyzed. 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). All samples were run in triplicate (coefficients of variation were ≤10%). Ouabain-sensitive ATPase activity is expressed as μmol ADP mg^{-1} protein h^{-1}.

Real-time PCR analysis of gene expression

Total RNA was extracted and reverse transcribed from killifish gills using the methods described above. Gene expression was assessed using quantitative real-time PCR (qRT-PCR) on an ABI Prism 7000 sequence analysis system (Applied Biosystems, Foster City, CA, USA). Primers for all genes were designed using Primer Express software (version 2.0.0, Applied Biosystems; see Table 2). PCR reactions

Table 2. *Primers used for qRT-PCR*

Gene	Direction	Sequence (5′–3′)
14-3-3a	F	CAA CGA GGA GCG CAA CCT
	R	CGG GCA CCC ACA ACA TTC
CA2	F	AGG GCT GAC GCT GAT TGG
	R	CCT CTG CTG GAA AGC GTT ACC
CFTR	F	AAT CGA GCA GTT CCC AGA CAA G
	R	AGC TGT TTG TGC CCA TTG C
EF1α	F	GGG AAA GGG CTC CTT CAA GT
	R	ACG CTC GGC CTT CAG CTT
Na^+,K^+-ATPase α_{1a}	F	ATT GCT GCC CGC CTG AA
	R	TGA ACG ACG CAA GCT TTG G
NBC1	F	AAA GGC GGA GAA CGT TGG A
	R	CCA TCA AGC TGT GCA AAG ACA
NHE2	F	ACA GCA TCA GGC GCA TTC T
	R	GCT GGC ATC TGC TTG TTG TTA A
NHE3	F	TGT TGA AGT CGT CAG CGA GAA
	R	CAG CTC GTG GAA GAC GTT GA
NKCC1	F	CCC GCA GCC ACT GGT ATT
	R	GCC ATC TGT GGG TCA GCA A
V-ATPase	F	TGA AGT TCA AGG ACC CGG TTA
	R	CTG CGC GTA CTC GCC TTT

F, forward; R, reverse.

contained 1 μl of cDNA, 4 pmoles of each primer and Universal SYBR green master mix (Applied Biosystems) 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 and representative samples were electrophoresed to verify that only a single product was present. Whereas some genes were cloned using killifish from populations in Georgia and Maine, the fish used in the freshwater transfer experiment were from Nova Scotia (described above). All primers used for qRT-PCR were therefore tested in multiple killifish populations, and they were used only if the melt-curve analysis indicated that the same product was amplified in all populations. Control reactions were conducted with no cDNA template or with non-reverse-transcribed RNA to determine the level of background and genomic DNA contamination, respectively. Genomic contamination was always below 1:35 starting cDNA copies for all templates, with the exception of NHE2 in opercular epithelium (NHE2 could not be amplified above background levels in opercular epithelium).

A randomly selected sample was used to develop a standard curve for each primer set, and all results were expressed relative to this arbitrary standard. All samples were run in duplicate (coefficients of variation were ≤10%). Results were then normalized to EF1α. Expression of this gene does not change in killifish gills at any time following salinity transfer when expression is normalized to total RNA concentration (data not shown), demonstrating that EF1α is an appropriate control gene (see also Richards et al., 2003; Scott et al., 2004a). For clarity in graphing, time course mRNA expression data are expressed relative to the 12 h brackish water control samples. In addition, the absolute level of mRNA expression of each gene examined was estimated semi-quantitatively 12 h after transfer using the following formula:

$$\text{Absolute level of mRNA expression} = \text{Efficiency}^{-\text{Ct}}, \quad (1)$$

where efficiency is determined from the slope of the standard curve (and theoretically has a value of 2) and Ct corresponds to the threshold cycle number. These results were subsequently normalized to the estimated absolute expression of EF1α. Due to the nature of analysis using qRT-PCR, these are only used for approximate comparison of expression levels between genes.

Statistical analyses

Data are expressed as means ± S.E.M. All data passed tests of normality and homogeneity of variance. Analysis of variance (ANOVA) was therefore used to ascertain overall differences as a function of time for each salinity, as well as overall differences in absolute expression levels for each tissue. Because measured variables may change due to handling of fish alone (Scott et al., 2004a), the effect of freshwater transfer was assessed whenever possible by comparison with time-matched brackish water controls using Tukey *post-hoc* comparisons. The effect of handling was assessed using Tukey comparisons with 12 h brackish water controls. When time-matched controls were not available (Na^+,K^+-ATPase activity experiment) the effect of freshwater transfer was assessed by comparison with pre-transfer controls. Relative expression levels of genes were compared between tissues using Tukey *post-hoc* comparisons. All statistical analyses were conducted using Sigmastat version 3.0 and a significance level of $P<0.05$ was used throughout.

Results

Na^+,K^+-ATPase activity

Na^+,K^+-ATPase activity in the gills and opercular epithelium of killifish responded differently to freshwater transfer (Fig. 1). In the gills, activity of this enzyme increased twofold above pre-transfer brackish water levels at all times after freshwater transfer. In contrast, activity in the opercular epithelium was unchanged by freshwater transfer, and levels remained consistently 50% below those in freshwater gills.

Absolute gene expression

The estimated absolute expression levels (determined using Formula 1) of several genes in killifish gills were in the following order, from highest to lowest: elongation factor 1α, Na^+,H^+-exchanger 2, 14-3-3a, Na^+,K^+-ATPase α_{1a}, Na^+,HCO_3^-cotransporter 1, carbonic anhydrase 2, $Na^+,K^+,2Cl^-$cotransporter 1, CFTR Cl^- channel, V-type H^+-

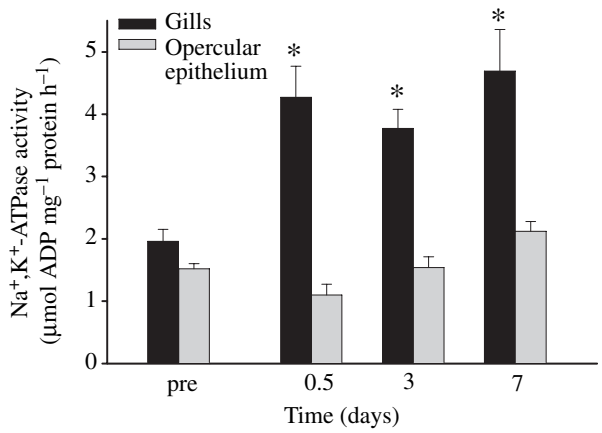

Fig. 1. Na^+,K^+-ATPase activity in killifish gills (black bars) and opercular epithelium (grey bars), before (pre) and after transfer from brackish water (10% seawater) to freshwater (N=8). Values are means ± S.E.M. *Significant difference from pre-transfer brackish water control (P<0.05).

ATPase A and NHE3 (Fig. 2). This pattern of expression was somewhat different in opercular epithelium, however, as the absolute levels differed between tissues for those genes believed to be important for Na^+ uptake from freshwater: levels of expression of NHE2, NBC1, CA2 and V-ATPase were at least 3- to 30-fold higher in the gills, with the greatest differences being observed between tissues for NHE2 and CA2 (NHE2 was not amplified above background levels in opercular epithelium). Numerous other genes were expressed at equivalent levels in each tissue, including many known to be important for ion secretion in seawater, such as Na^+,K^+-ATPase α_{1a}, NKCC1 and CFTR. NHE3 and the signalling protein 14-3-3a were also expressed at equal levels in gills and opercular epithelium.

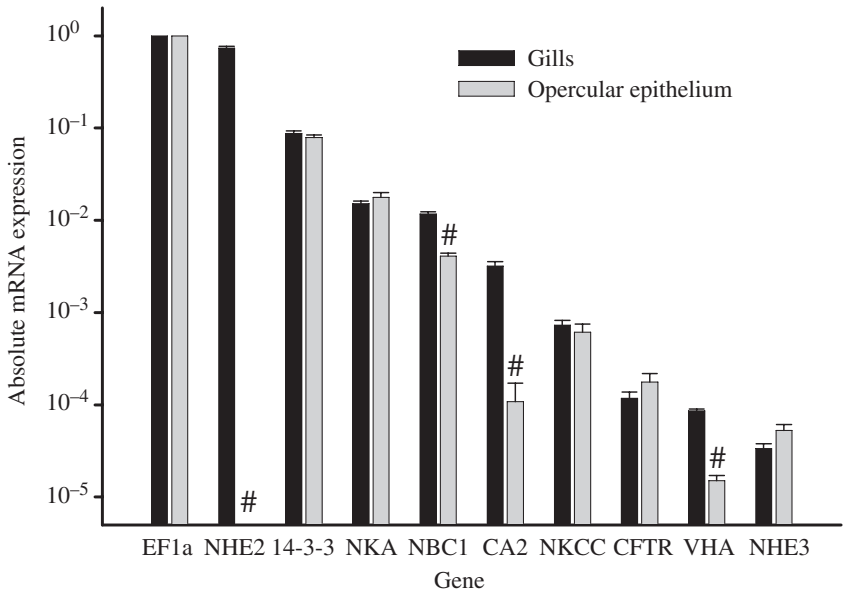

Fig. 2. The estimated absolute expression levels of several genes important for ion transport in the gills (black bars) and opercular epithelium (grey bars) of killifish (N=15–20). Values are determined using Formula 1 and data are normalized to the expression level of elongation factor 1α (EF1α). Na^+,H^+-exchanger 2 (NHE2) was not amplified above background levels in opercular epithelium. NKA, Na^+,K^+-ATPase α_{1a}; VHA, V-type H^+-ATPase A. #Significant difference between tissues (P<0.05).

Table 3. *Gene expression of ion transporters that did not change after freshwater transfer, measured by qRT-PCR*

		Time after transfer		
Gene	Salinity	12 h	3 days	7 days
Gills				
Na^+,K^+-ATPase α_{1a}	BW	1.0±0.1	1.1±0.1	1.1±0.1
	FW	0.9±0.1	1.0±0.3	1.5±0.2
NBC1	BW	1.0±0.1	1.0±0.1	0.8±0.1
	FW	1.0±0.1	1.0±0.1	0.7±0.1
V-ATPase	BW	1.0±0.1	1.1±0.1	1.4±0.1
	FW	1.0±0.1	1.1±0.1	1.2±0.2
Opercular epithelium				
Na^+,K^+-ATPase α_{1a}	BW	1.0±0.2	1.3±0.2	1.0±0.3
	FW	1.1±0.2	2.2±0.6	1.3±0.3
NBC1	BW	1.0±0.1	0.5±0.1†	0.6±0.1†
	FW	0.9±0.1	0.5±0.1†	0.6±0.1†
V-ATPase	BW	1.0±0.4	1.3±0.2	0.9±0.2
	FW	1.0±0.2	0.9±0.2	0.7±0.1

Values are means ± S.E.M. N≥7. †Significant difference from 12 h brackish water control (P<0.05). qRT-PCR expression data are normalized to 12 h brackish water controls.

BW, brackish water; FW, freshwater.

Gene expression after freshwater transfer

Freshwater transfer changed the mRNA expression patterns of some genes in similar ways in gills and opercular epithelium of killifish. Expression of the important seawater ion transporters NKCC1 and CFTR decreased in the gills and opercular epithelium at all times after freshwater transfer, reaching levels as low as 30% and 10% of brackish water controls (Fig. 3). The expression of 14-3-3a, which is an important gene for regulating ion secretion, increased up to 1.5-fold at 12 h and 7 days after freshwater transfer, but was similar to brackish water controls at 3 days (Fig. 3). The mRNA expression of some other genes was unchanged by freshwater transfer in both tissues, including Na^+,K^+-ATPase α_{1a}, NBC1 and V-ATPase (Table 3).

For some other genes, the patterns of expression exhibited in each tissue differed after freshwater transfer. In the gills, mRNA expression of NHE2 and CA2 increased in

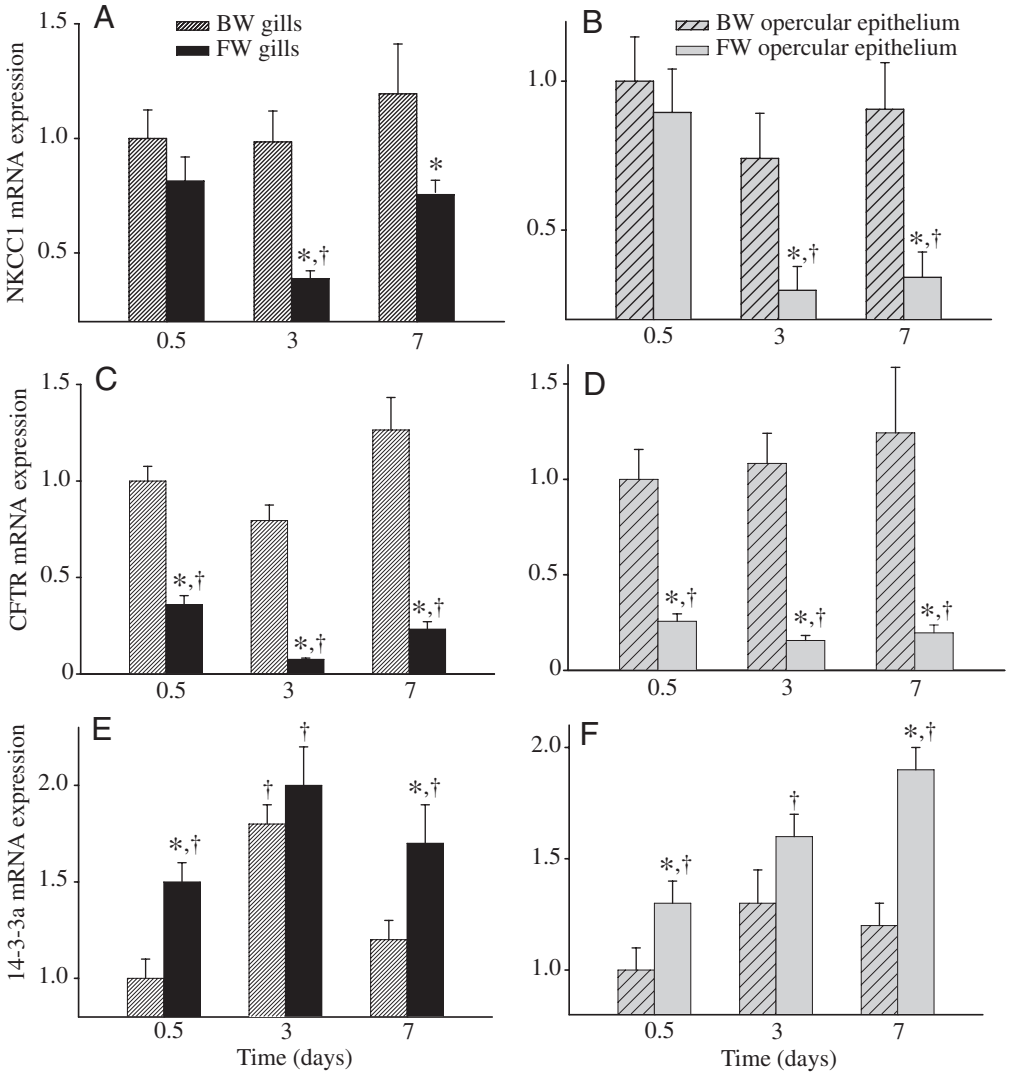

Fig. 3. Expression of genes that have similar patterns of expression between killifish gills and opercular epithelium. Na^+,K^+,2Cl^--cotransporter 1 (NKCC1) (A,B), cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (C,D), and the signalling protein 14-3-3a (E,F) mRNA expression in gills (A,C,E; black/white) and opercular epithelium (B,D,F; grey) after transfer from brackish water (10% seawater) to brackish water (BW, hatched bars) or freshwater (FW, solid bars) (N=7–10). Expression is relative to the expression of EF1α and is normalized to 12 h brackish water controls. Values are means ± S.E.M. *Significant difference from time-matched brackish water control (P<0.05); †significant difference from 12 h brackish water control (P<0.05).

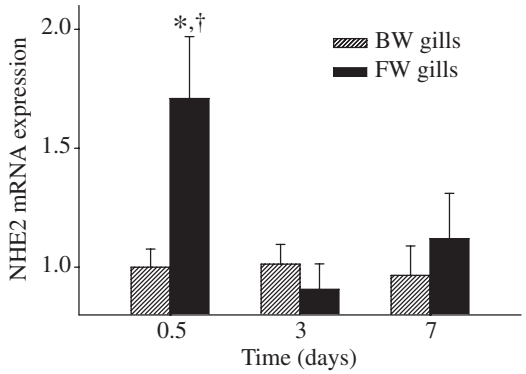

Fig. 4. Na^+,H^+-exchanger 2 (NHE2) mRNA expression in killifish gills after transfer from brackish water (10% seawater) to brackish water (BW, hatched bars) or freshwater (FW, black bars) (N=7–10). NHE2 is not expressed in killifish opercular epithelium. Expression is relative to the expression of EF1α and is normalized to 12 h brackish water controls. Values are means ± S.E.M. *Significant difference from time-matched brackish water control (P<0.05); †significant difference from 12 h brackish water control (P<0.05).

freshwater: NHE2 increased transiently at 12 h (1.7-fold) (Fig. 4), while CA2 increased up to twofold above controls at all times after transfer (Fig. 5). In contrast, NHE2 was not detectable in opercular epithelium and CA2 expression decreased in freshwater (Fig. 5). Curiously, NHE3 expression also differed between tissues, in a manner opposite the patterns exhibited by CA2, as expression of this gene decreased in the gills but increased in the opercular epithelium after freshwater transfer (Fig. 5). In a few cases, changes occurred over time in the brackish water controls in gills (14-3-3a and CA2) and opercular epithelium (NBC1), and this was likely an effect of handling.

Discussion

Killifish routinely encounter fluctuations in environmental salinity in their natural habitat, and can rapidly modulate ion transport rates to maintain ion balance (Marshall, 2003; Wood and Laurent, 2003; Scott et al., 2004b). This modulation is known to occur in many tissues in response to salinity change, including the gills, opercular epithelium, intestine and kidney.

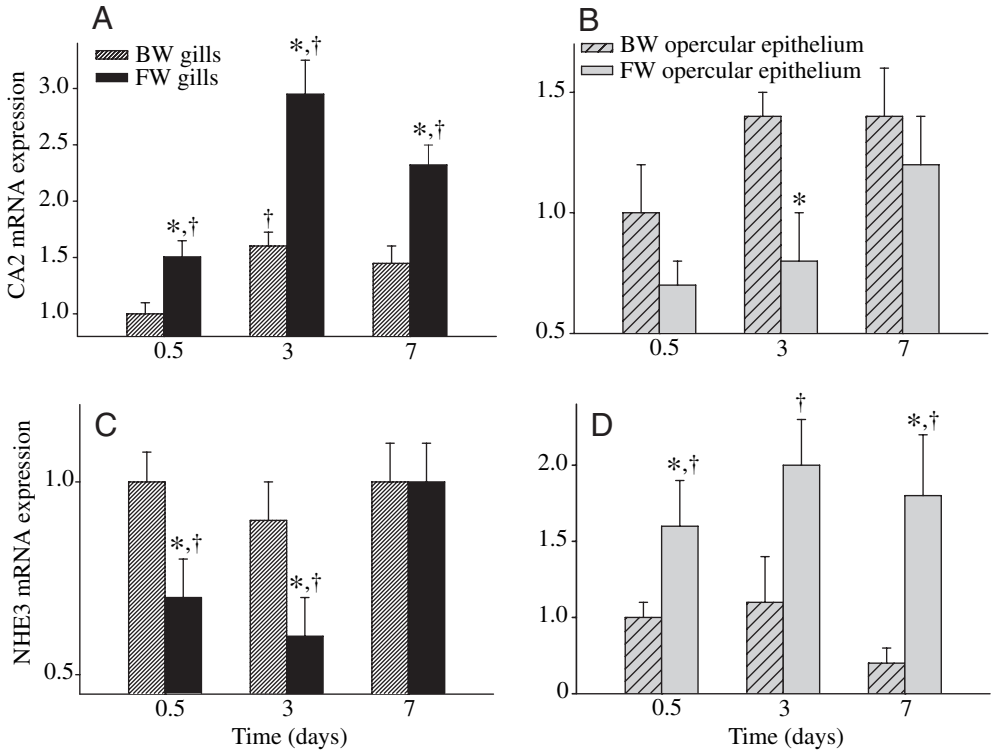

Fig. 5. Expression of genes that have different patterns of expression between gills and opercular epithelium. Carbonic anhydrase 2 (CA2) (A,B) and Na^+,H^+-exchanger 3 (NHE3) (C,D) mRNA expression in killifish gills (A,C; black/white) and opercular epithelium (B,D; grey) after transfer from brackish water (10% seawater) to brackish water (BW, hatched bars) or freshwater (FW, solid bars) (N=7–10). Expression is relative to the expression of EF1α and is normalized to 12 h brackish water controls. Values are means ± S.E.M. *Significant difference from time-matched brackish water control (P<0.05); †significant difference from 12 h brackish water control (P<0.05).

In hyperosmotic environments, both the gills and opercular epithelium secrete Na^+ and Cl^- to counteract passive ion uptake (Karnaky et al., 1977; Marshall, 2003; Wood and Laurent, 2003). In contrast, functional differences exist between the absorptive capacity of these epithelia in freshwater (Marshall et al., 1997; Patrick et al., 1997; Burgess et al., 1998; Patrick and Wood, 1999): the gills actively absorb Na^+ at high rates, but not Cl^-, while the opercular epithelium actively absorbs Cl^- at low rates, but not Na^+. We have demonstrated a probable molecular basis for these differences in function between killifish gills and opercular epithelium. Substantial differences exist between tissues in the absolute mRNA expression levels of certain genes, as well as in the responses of gene expression to freshwater transfer in each tissue. These molecular differences provide evidence for divergent mechanisms of ion transport between these tissues, and demonstrate unique aspects of functional plasticity in the ion transporting epithelia of killifish.

Suppression of ion secretion after freshwater transfer

The opercular epithelium has proved a useful surrogate model for the gills in seawater fish, and previous studies have shown that their mechanisms of ion secretion are likely similar (Wood and Marshall, 1994; Wood and Laurent, 2003). In this regard our study is no exception, as the absolute expression levels of many genes involved in ion secretion (Na^+,K^+-ATPase α_{1a}, NKCC1 and CFTR) were similar between gills and opercular epithelium, as well as the signaling protein 14-3-3a, which is thought to regulate the activity of CFTR (Kohn et al., 2003).

In addition to its use as a model for ion secretion in seawater, the opercular epithelium of killifish has also been useful for understanding how ion secretion is suppressed in hyposmotic environments (Marshall, 2003). Indeed, our results suggest that the gills and opercular epithelium may behave similarly in this regard, as the responses of seawater ion transporters to freshwater transfer in these tissues were similar. Expression of NKCC1 and CFTR decreased after freshwater transfer in both gills and opercular epithelium, and the patterns of these changes were similar in each tissue. These results are also consistent with previous studies of freshwater transfer for NKCC1 and CFTR mRNA levels in the gills of killifish (Scott et al., 2004a,b), as well as NKCC protein abundance in the gills of other species (Tipsmark et al., 2002; Wilson et al., 2004). Furthermore, genes expressed by the opercular epithelium (and possibly the gills as well) also undergo many post-transcriptional alterations after freshwater transfer to suppress ion secretion, including transporter internalization and inactivation (Marshall et al., 1998, 2000, 2002; Hoffmann et al., 2002) and morphological restructuring (Daborn et al., 2001). These post-transcriptional events appear particularly important during short-term salinity change (Marshall, 2003).

The signaling protein 14-3-3a is potentially important for inactivating Cl^- secretion *via* CFTR after freshwater transfer. Previous studies have shown that killifish 14-3-3a promotes ionic homeostasis in *Xenopus* oocytes (Kohn et al., 2003), and mRNA expression of this gene increases in the gills of killifish transferred to freshwater (Kültz et al., 2001). In this study, 14-3-3a expression increased in both gills and opercular epithelium after freshwater transfer, suggesting that some of the intracellular signals responsible for inactivating ion secretion are similar in each tissue. However, the intracellular

signalling pathways that transduce osmotic signals (see Kültz and Avila, 2001; Seale et al., 2003; Marshall et al., 2005) are still unclear, so the direct role of 14-3-3a remains uncertain.

Mechanisms of ion absorption

Although the absolute mRNA expression levels of many genes are similar between gills and opercular epithelium, some other genes appear to be expressed at substantially higher levels in the gills. Interestingly, those genes whose expression differs between tissues, namely Na^+,H^+-exchanger 2, Na^+,HCO_3^-cotransporter 1, carbonic anhydrase 2, and V-type H^+-ATPase A, have all been proposed to be important in the gills of various fish species for Na^+ uptake in freshwater (Claiborne et al., 2002; Katoh et al., 2003; Perry et al., 2003a,b). With few exceptions, most previous studies in fish have found that changes in the mRNA expression of ion transporters occur along with predictable changes in protein abundance (e.g. D'Cotta et al., 2000; Tipsmark et al., 2002; Scott et al., 2004a; Scott et al., 2005). Assuming that the relative levels of transcription measured in this study are correlated with levels of protein expression, it is therefore plausible that the differences in ion transport between gills and opercular epithelium are a result of the observed differences in absolute gene expression: gills express higher levels of NHE2, NBC1, CA2, and V-ATPase and are thus able to actively absorb Na^+.

Based on the molecular and physiological differences between gills and opercular epithelium, we have proposed a preliminary model on which to base future investigation of how Na^+ absorption might be accomplished across the killifish gill epithelium (Fig. 6). In the model, apical Na^+ uptake occurs *via* NHE2 in exchange for H^+ supplied by CA2. Active transport of Na^+ across the basolateral surface is accomplished by Na^+,K^+-ATPase (which creates the electrochemical gradients necessary for transport); therefore, apical Na^+ uptake is not driven by a transepithelial H^+ gradient as in some other fish species (Lin and Randall, 1993; Fenwick et al., 1999). Support for these suggestions come from immunological evidence that CA2 and Na^+,K^+-ATPase localize to mitochondria-rich cells in killifish gills (Flügel et al., 1991; Katoh et al., 2003). Our model assumes a co-localization of NHE2 and CA2, and although CA2 is known to be located at the apical surface (Rahim et al., 1988), the localization of NHE2 in killifish gills has yet to be demonstrated.

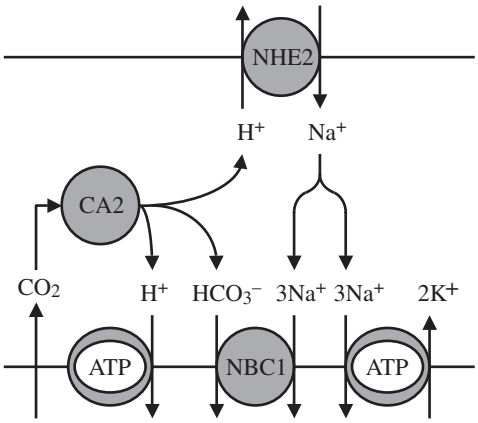

Fig. 6. Preliminary working model of Na^+ absorption by killifish gills. Apical Na^+ uptake occurs *via* NHE2 in exchange for H^+ supplied by CA2. Active transport of Na^+ across the basolateral surface is accomplished by Na^+,K^+-ATPase. Na^+ may also leave the cell in symport with HCO_3^- through NBC1 operating in efflux mode. V-ATPase may pump H^+ across the basolateral surface to help maintain intracellular acid–base balance.

In addition to NHE2, CA2 and Na^+,K^+-ATPase, NBC1 and V-ATPase may be important for Na^+ uptake and/or regulating acid–base balance in freshwater. Na^+ may cross the basolateral surface in symport with HCO_3^- through NBC1, which can operate in both influx ($1Na^+$:$2HCO_3^-$; Wood and Pärt, 2000; Perry et al., 2003a) and efflux ($1Na^+$:$3HCO_3^-$, as shown in Fig. 6; Hirata et al., 2003) modes in freshwater. NBC1 has not yet been localized in killifish chloride cells, but V-ATPase is basolaterally located in killifish gill cells (Katoh et al., 2003), and could therefore export protons across the basolateral surface. Each of these proteins are likely involved in maintaining acid–base balance in freshwater (e.g. Perry et al., 2003a), but may also support Na^+ absorption. For example, the absolute expression of V-ATPase was low in the moderately hard freshwater used in this study, where environmental Na^+ and Cl^- concentrations were similar. However, basolateral localization of this protein in killifish gills increased substantially in an artificial freshwater medium with low Na^+ content (12% of present values) but high Cl^- (130% of present values), an ionic situation that would tend to promote acidosis (Katoh et al., 2003). In this situation, increased basolateral V-ATPase would not be beneficial for regulating acid–base balance (as it would promote internal acidosis), and may instead perform a different function. It has been suggested that basolateral H^+-ATPase facilitates transepithelial Cl^- uptake in the gills of some fish species by extruding protons, and thus driving apical Cl^-,HCO_3^- exchange to maintain intracellular acid–base balance (Piermarini and Evans, 2001; Piermarini et al., 2002). However, killifish gills appear to lack apical Cl^-,HCO_3^- exchange and do not absorb Cl^- in freshwater (Patrick et al., 1997; Patrick and Wood, 1999; Wood and Laurent, 2003; Scott et al., 2004b), so basolateral proton extrusion might instead be involved in Na^+ uptake or other physiological functions that are necessary in dilute environments. The exact roles of V-ATPase and NBC1 in Na^+ uptake, their stoichiometry relative to other transporters, and whether these proteins are expressed in the same cell type as NHE2 and CA2, have yet to be determined.

It is possible that some of the differences in expression between gills and opercular epithelium result from differences in cell type composition within each tissue. The contribution of different cell types (including cells not involved in transport, such as connective tissue) to whole-tissue expression cannot be resolved in this study. However, we hypothesize that the majority of the observed differences seen in this study are due to mRNA expression in the cells responsible for transepithelial

ion transport. Previous studies have demonstrated that ion transporters tend to be expressed at much higher levels in these cells (e.g. Wilson et al., 2000; Piermarini and Evans, 2001), so their contribution to whole-tissue expression is large. For the genes thought to be involved in ion secretion (NKCC1, Na^+,K^+-ATPase α_{1a}, CFTR and 14-3-3a), as well as for NHE3, absolute expression levels in this study were the same in the two tissues. Furthermore, mRNA expression of both NKCC1 and CFTR were reduced after freshwater transfer, and this reduction was of a similar magnitude in both tissues. Therefore, at least for these genes, there is no apparent evidence that differences in cell type composition (either due to non-epithelial cells, or differences in the relative abundance of mitochondria-rich cells or pavement cells) are causing differences in expression. Regardless, the contribution of cell type composition in expression studies using whole-tissue deserves future attention.

The mechanisms through which the opercular epithelium of killifish actively absorbs Cl^-, albeit at a low rate, are presently unclear (Wood and Marshall, 1994; Marshall et al., 1997; Burgess et al., 1998), and remain so because the only differences in expression that we detected between tissues were of genes that likely function in Na^+ absorption. Cl^- uptake is unaffected by mucosal exposure to the anion exchanger inhibitors SITS (4-acetamino-4′-isothiocyanostilbene-2,2′-disulfonic acid) and DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid) (Marshall et al., 1997), suggesting that transport may not occur by apical Cl^-,HCO_3^- exchange. Interestingly, CFTR appears to localize to the basolateral surface of pavement cells in killifish opercular epithelium in freshwater, while it is not expressed in this cell type in seawater (Marshall et al., 2002). This transporter may therefore facilitate Cl^- transport by pavement cells in freshwater. However, CFTR expression decreases substantially after freshwater transfer in opercular epithelium, so if pavement cells transport Cl^- *via* CFTR this may explain the detectable but low rate of Cl^- uptake by the opercular epithelium *in vitro*. Future work should address the role of CFTR and other Cl^- transporters (e.g. SLC26 anion exchangers or Cl^-ATPase; Dópido et al., 2004) in the opercular epithelium of killifish in freshwater.

Activation of Na^+ absorption after freshwater transfer

Killifish rapidly activate Na^+ absorption after freshwater transfer (Wood and Laurent, 2003; Scott et al., 2004a), and this occurs through many coordinated molecular and cellular events. These are likely to include rapid activation of Na^+,K^+-ATPase activity (Towle et al., 1977; Mancera and McCormick, 2000), increased cell proliferation (Katoh and Kaneko, 2003; Scott et al., 2005), cell differentiation and apoptosis (Marshall et al., 1999; Daborn et al., 2001; Katoh et al., 2001) and, in some cases, increased transcription of Na^+,K^+-ATPase (Scott et al., 2004a,b). Expression of both Na^+,H^+-exchanger 2 and carbonic anhydrase 2 mRNA increased as early as 12 h after transfer, so transcriptional regulation of these genes may also play a role in the early stages of freshwater acclimation. Changes in expression after salinity change can occur within a few hours of transfer (Scott et al., 2004a), so NHE2 and CA2 expression may have increased earlier than 12 h into freshwater. Some evidence suggests that changes in whole-gill expression of ion transporters after freshwater transfer may be partly due to alterations in cell proliferation (Scott et al., 2005). If this is indeed the case, differences in cell proliferation and/or regression between gills and opercular epithelium would be expected after freshwater transfer.

The expression of Na^+,H^+-exchanger proteins has been reported in the gills of numerous fish species in freshwater (Wilson et al., 2000; Edwards et al., 2002; Hirata et al., 2003) and their expression is known to be modulated by acid–base disturbances (Claiborne et al., 1999; Edwards et al., 2001). Few studies have examined how these proteins are modulated by freshwater transfer (Claiborne et al., 1999), but it is reasonable to propose that NHE2 upregulation might contribute to Na^+ uptake in killifish. Although the role of carbonic anhydrase in ion absorption is well established (Marshall, 2002; Perry et al., 2003b) and CA2 upregulation is probably also involved in activating Na^+ uptake after freshwater transfer, few previous studies have assessed its mRNA expression patterns after transfer to hyposmotic environments (Henry et al., 2003). The suppression of CA2 expression after transfer in opercular epithelium, a tissue that is unable to actively absorb Na^+, is consistent with a role for this gene in Na^+ uptake.

Previous studies in killifish observed Na^+,K^+-ATPase α_{1a} mRNA expression and activity to increase after freshwater transfer (Scott et al., 2004a,b; Scott et al., 2005). The lack of similarity in expression patterns between this and previous studies was likely due to differences in experimental protocols: killifish were transferred from 10% seawater to freshwater ($[Na^+]$ 0.82 mmol l^{-1}) in this study, while in previous studies fish were transferred from 30% seawater to freshwater with appreciably lower ion levels ($[Na^+]$ 0.17 mmol l^{-1}). The latter case represents a much greater change in ionic gradients after transfer than in the present study, and illustrates an important characteristic of physiological responses to environmental change. That is, the presence and magnitude of the physiological response is dependent on the degree of change, and in many cases not all elements of a physiological system need to be adjusted to maintain homeostasis. It is therefore likely that the post-transcriptional increase in gill Na^+,K^+-ATPase activity in this study (which has also been observed in two other studies; see Towle et al., 1977; Mancera and McCormick, 2000) sufficiently activated Na^+ transport to maintain ion balance.

Unlike the gills, the opercular epithelium did not increase Na^+,K^+-ATPase activity after freshwater transfer. This suggests that while the signalling pathways responsible for eliminating ion secretion after freshwater transfer may be similar between these tissues (e.g. 14-3-3a pathway), those activating ion absorption may be different. Furthermore, increasing Na^+,K^+-ATPase activity likely plays a lesser role in increasing Cl^- uptake in the opercular epithelium after freshwater transfer than for increasing Na^+ uptake in the gills. Because Na^+,K^+-ATPase is important for ion secretion by both gills and opercular epithelium in seawater, however, it is perhaps not surprising that the absolute mRNA expression of this gene is the same in each

tissue. The difference between these tissues may instead be in the post-transcriptional regulation of Na^+,K^+-ATPase activity.

In contrast to the expression of NHE2, which increased after freshwater transfer, the expression of NHE3 decreased in the gills of killifish after freshwater transfer, suggesting that there are freshwater- and seawater-specific isoforms responsible for Na^+,H^+-exchange (see also Edwards et al., 2005). There may be functional differences between these isoforms in kinetics, substrate affinity, regulation or other properties that enabled selection for isoform switching between different osmotic environments. In this regard, isoform switching appears to be an important characteristic of the acclimatory responses of fish to salinity change (Schulte, 2004). In salmonids, for example, Na^+,K^+-ATPase α-isoform switching occurs after transfer from freshwater to seawater (Richards et al., 2003). Curiously, NHE3 expression increased in the opercular epithelium after freshwater transfer, which further demonstrates the divergent mechanisms of ion transport between these tissues in hyposmotic environments.

Taken together, the results of this study demonstrate the potential molecular basis for the differences in function between the gills and opercular epithelium of killifish in freshwater. While these tissues appear to behave similarly in seawater, at both the molecular and physiological levels, and while they also appear to suppress ion secretion in hyposmotic environments using similar mechanisms, their functional and genomic responses to freshwater transfer are markedly different. The gills of killifish actively absorb Na^+ in freshwater while opercular epithelia do not, and this is likely based on substantial differences in the absolute expression of several proteins important for Na^+ transport, namely NHE2, CA2, NBC1 and V-ATPase. Furthermore, the physiological responses of the gills during acclimation to freshwater occur in conjunction with augmented mRNA expression of NHE2 and CA2, events that did not occur in the opercular epithelia. Our results provide insight into the molecular and physiological mechanisms underlying ion transport in killifish gills, and demonstrate the substantial functional plasticity of ion transport in this species.

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