

## A MECHANISM FOR BRANCHIAL ACID EXCRETION IN MARINE FISH: IDENTIFICATION OF MULTIPLE $\text{Na}^+/\text{H}^+$ ANTIporter (NHE) ISOFORMS IN GILLS OF TWO SEAWATER TELEOSTS

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Accepted 18 November 1998; published on WWW 11 January 1999

### Summary

Both  $\text{Na}^+/\text{H}^+$  exchange and the electrogenic extrusion of  $\text{H}^+$  via an  $\text{H}^+$ -ATPase have been postulated to drive acid excretion across the branchial epithelium of fishes. While the  $\text{H}^+$ -ATPase/ $\text{Na}^+$  channel system appears to be the predominant mechanism in some freshwater species, it may play a reduced role in seawater and brackish-water animals, where high external  $\text{Na}^+$  concentrations may thermodynamically favor  $\text{Na}^+/\text{H}^+$  exchange driven by a  $\text{Na}^+/\text{H}^+$  antiporter (NHE). In this study, we used molecular and immunological methods to assess the role of NHE isoforms in the branchial epithelium of the marine long-horned sculpin (*Myoxocephalus octodecimspinosus*) and the euryhaline killifish (*Fundulus heteroclitus*).

Northern blot analysis of RNA probed with the human NHE-1 BamHI fragment suggested the presence of homologous gill NHE mRNA in sculpin. RT-PCR on gill RNA isolated from sculpin recovering from metabolic acidosis provided evidence for two distinct NHE isoforms; one with 76% amino acid homology to mammalian NHE-

2, and another 92% homologous to trout erythrocytic  $\beta$ -NHE. Killifish also have transcripts with 91% homology to  $\beta$ -NHE. Immunological detection using monoclonal antibodies for mammalian NHE-1 revealed a protein antigenically similar to this isoform in the gills of both species. Metabolic acidosis caused an approximately 30-fold decrease in expression of the NHE-1-like protein in sculpin. We speculate that  $\beta$ -NHE in the gills plays the intracellular 'housekeeping' roles described for mammalian NHE-1. During systemic acidosis, apical gill NHE-2 (which is sensitive to external amiloride and low  $[\text{Na}^+]$ ) in parallel with a dramatic suppression of basolateral NHE-1 activity enhances net  $\Delta\text{H}^+$  transfers to the water.

Key words: acid–base, pH balance,  $\text{H}^+$  excretion, salinity, stenohaline, euryhaline, sculpin, *Myoxocephalus octodecimspinosus*, killifish, *Fundulus heteroclitus*, gill.

### Introduction

The role of the fish gill in hydromineral regulation has been studied for nearly 70 years (Smith, 1930; Keys, 1931; for a review, see Evans, 1993). While early work mainly focused on ion- and osmoregulatory requirements, many of the postulated gill transepithelial ion exchanges (e.g.  $\text{Na}^+/\text{NH}_4^+$ ,  $\text{Na}^+/\text{H}^+$  and  $\text{Cl}^-/\text{HCO}_3^-$ ) allow not only ion-regulatory transfers, but acid–base adjustments as well (Cameron, 1976; Claiborne and Heisler, 1986; Heisler, 1993). In the case of freshwater fish, the linkage between acid–base excretion and the uptake of essential  $\text{Na}^+$  and/or  $\text{Cl}^-$  from the dilute medium would serve an adaptive dual acid–base and ion-regulatory role. That several seawater species are also thought to accomplish these transfers (Claiborne and Evans, 1988, 1992; Tang et al., 1989; McDonald et al., 1991; Milligan et al., 1991) is an indication of the importance of acid–base regulation to

the animal. In this case, the excretion of acid–base equivalents for the uptake of  $\text{NaCl}$  would exacerbate the ionic load already faced by the hypo-ionic, seawater fish. In addition, a variety of fish families move between (or are exposed to) waters of varying salinities (Evans, 1984), and several 'stenohaline' species can adjust to water diluted to 20% or less of full-strength sea water (Wu and Woo, 1983). Therefore, ion gradients affecting acid–base exchanges may undergo rapid and/or periodic variations in numerous euryhaline and moderately euryhaline species. While potential sites for acid–base exchanges have been hypothesized to include the skin, urinary system (kidneys/bladder) and intestine (Wilson et al., 1996), the gills appear to account for 90–99% of the acid transfers in most species studied (for a review, see Claiborne, 1998).

To date, the most frequently postulated mechanisms for the apical transfer of  $H^+$  across the gills include  $Na^+/H^+$  or  $Na^+/NH_4^+$  exchange and electrogenic excretion of  $H^+$  (with passive uptake of  $Na^+$  through  $Na^+$  channels). These transfers are net electroneutral exchanges and drive the uptake of  $Na^+$  from the water into the animal. While  $Na^+/NH_4^+$  exchange may occur in seawater species (perhaps *via* the  $Na^+/H^+$  antiporter; Paillard, 1997) when exposed to high external ammonia levels (Claiborne and Evans, 1988), acid excretion is normally accomplished by one or both of the remaining transporters: a  $Na^+/H^+$  antiporter (Tse et al., 1993) or *via* electrogenic excretion of  $H^+$  by an  $H^+$ -ATPase (first suggested by Klungsoyr, 1987; for a review, see Lin and Randall, 1995).

In freshwater species (and perhaps elasmobranchs; Kormanik et al., 1997; Wilson et al., 1997), the  $H^+$ -ATPase/ $Na^+$  channel system appears to be a likely mechanism for acid excretion and salt uptake across the high-resistance, 'tight' gill epithelia (Potts, 1994). In contrast,  $H^+$ -ATPase may play less of a role in seawater-adapted teleosts since the gill epithelium in these fishes is much more permeable than that of freshwater-adapted animals, and thermodynamic considerations should favor  $Na^+/H^+$  exchange at higher external  $[Na^+]$  (Potts, 1994).  $H^+$ -ATPase activity is 50% lower in *Oncorhynchus mykiss* adapted from fresh water to water containing  $100\text{ mmol l}^{-1}$   $Na^+$  and is reduced by 70% when in sea water (Lin and Randall, 1993). Immunofluorescence to antibodies for the V-type  $H^+$ -ATPase is also reduced in seawater fish compared with freshwater fish (Lin et al., 1994). We have recently shown that compensation for acidosis in the marine long-horned sculpin (*M. octodecimspinosus*) is dependent on external  $[Na^+]$ , with 20–30  $\text{mmol l}^{-1}$  required for a positive  $H^+$  flux (Claiborne et al., 1997). The extrusion of  $H^+$  is reversibly inhibited by 75% with amiloride and a  $Na^+/H^+$ -antiporter-specific analog. Thus, indirect evidence points to a role for a  $Na^+/H^+$  antiporter of the NHE family (Bianchini and Pouyssegur, 1994) in acid excretion by seawater fish.

An amiloride-sensitive  $Na^+/H^+$  antiporter has been described in a number of mammalian and non-mammalian systems. This protein appears to function in cell volume regulation, pH balance and  $Na^+$  uptake (Clark and Limbird, 1991). A variety of different isoforms (NHE-1,2,3,4,5; for a review, see Yun et al., 1995) have been identified in mammals. Each of these isoforms may have specialized functions, as well as membrane targeting (apical *versus* basolateral; Noel and Pouyssegur, 1995), and each is activated by a decrease in intracellular pH which drives an increase in  $H^+$  extrusion and  $Na^+$  uptake. The only fish isoform described to date is  $\beta$ -NHE (Borgese et al., 1992), which is most homologous to NHE-1. Other vertebrate NHE-1-like isoforms have been described in *Xenopus laevis* oocytes (Busch, 1997) and turtle colon and small intestine (Harris et al., 1997). The  $\beta$ -NHE isoform has been cloned from trout hemopoietic tissue and detected in erythrocytes (Borgese et al., 1992). This NHE-1-like antiporter is thought to be activated by catecholamines during hypoxia, allowing alkalization of fish erythrocytes and a resulting Bohr shift to increase hemoglobin affinity (Malapert et al., 1997).

In polarized epithelial tissues, NHE-1 is located on the basolateral membrane and is involved with intracellular pH and volume regulation. In contrast, apically located NHE-2 and NHE-3 have only been found in mammals and are thought to function in transepithelial  $Na^+$  reabsorption and proton secretion. NHE-2 is mainly expressed in the gastrointestinal tract and kidney, while the highest levels of NHE-3 are found in the kidney, followed by the intestine and stomach (Yun et al., 1995; Hoogerwerf et al., 1996). In the proximal renal tubule, apical NHE-3 drives the reabsorption of  $Na^+$  and shuttles  $H^+$  to the tubular lumen to allow the net reabsorption of  $HCO_3^-$  (for a review, see Paillard, 1997). Metabolic acidosis has recently been shown to enhance the rate of apical  $Na^+/H^+$  exchange in renal brush border and thick ascending limb due to increases in both mRNA transcription and membrane expression of NHE-3 (Wu et al., 1996; Laghmani et al., 1997). Interestingly, an NHE isoform most homologous to mammalian NHE-3 has been found in green shore crab gill (*Carcinus maenas*) epithelia (Towle et al., 1997), although it is not known whether expression of this mRNA is responsible for the unusual electrogenic stoichiometry of  $Na^+/H^+$  exchange noted in some crustacean tissues.

Thus, one or more isoforms of a homologous  $Na^+/H^+$  antiporter may be present in gill epithelial cells of seawater fish. Indirect evidence for both apical or basolateral  $Na^+/H^+$  exchange in seawater sculpin (*Myoxocephalus octodecimspinosus*) and mummichog (*Fundulus heteroclitus*) has been provided *in vivo* and in opercular epithelium studies (Zadunaisky et al., 1995; Claiborne et al., 1997), and it has been hypothesized that NHE may be responsible for gill acid transfers in these animals (Claiborne, 1998). In the present experiments, we used molecular and immunological techniques to identify and quantify multiple isoforms of NHE in the gills of these two species. We describe the presence of mRNA transcripts for a normally apical  $Na^+/H^+$  antiporter in combination with a down-regulation of putative basolateral NHE expression during acidosis which may enhance net systemic  $H^+$  excretion.

### Materials and methods

*Myoxocephalus octodecimspinosus* (Mitchill) (150–300 g) and *Fundulus heteroclitus* (Linnaeus) (2–8 g) were supplied by commercial fisherman at the Mount Desert Island Biological Laboratory (MDIBL) in Salsbury Cove, Maine, USA, and maintained in tanks with sea water (15–18 °C) pumped from Frenchman Bay. In some cases, animals were transferred directly to dilute media and pre-adapted for 10–14 days (Claiborne et al., 1997; sculpin, sea water diluted to 20%, approximately  $100\text{ mmol l}^{-1}$  NaCl, in an aerated volume of 10l, 15–18 °C; mummichog, aerated, MDIBL tap water in an approximately 38l aquarium, 17–21 °C). Mummichog were fed every other day during this period. Acidosis was induced in some animals by the rapid intraperitoneal injection of  $0.1\text{ mol l}^{-1}$  HCl ( $2\text{ mmol kg}^{-1}$ ) followed by a 5 h recovery period (when measured *in vivo*, net  $H^+$  transfers following

acidosis reach a maximum; Claiborne et al., 1997). Control fish were injected with distilled water. Before tissue removal, animals were killed by MS-222 anesthesia and brain/spinal pithing. In some cases, gill filament samples from opposite arches and other tissues were then rapidly excised and homogenized for isolation of total RNA and/or membrane-bound proteins.

#### Northern blot analysis

To determine whether mRNA transcripts homologous to a human cDNA probe for the NHE-1 Na<sup>+</sup>/H<sup>+</sup> exchanger (Sardet et al., 1989) could be detected in the sculpin, blood and samples of gill, liver, small intestine, kidney, bladder and skeletal muscle tissue were removed from seawater-adapted animals and immediately homogenized and ultracentrifuged according to the method of Sambrook et al. (1989). Following RNA isolation and electrophoresis, total RNA was transferred to Nytran membranes and hybridized with a <sup>32</sup>P-labeled probe of the 1.9 kb BamHI fragment of human NHE-1 cDNA (Sardet et al., 1989; kindly provided by Dr J. Pouyssegur). Filters were then exposed to autoradiographic film for 2–5 days.

#### Reverse transcriptase–polymerase chain reactions (RT-PCR), cDNA subcloning and sequencing

Fresh tissue was excised, frozen in liquid nitrogen and homogenized, or homogenized immediately in TRI reagent and BCP extraction solution (Molecular Research Center, Inc., Cincinnati, OH, USA) and separated *via* a series of solvent extractions. The RT-PCRs were performed using the Perkin-Elmer Gene Amp RNA PCR kit and primers designed to amplify cDNA homologous to published sequences for the NHE antiporter. Initially, degenerate primers designed to include most known isoforms (3F and 4R from Towle et al., 1997) provided evidence of a homologous sequence in sculpin. This sequence in turn allowed us to design degenerate primers (designated SNHE-F and SNHE-R) more specific for the first sculpin sequence and for trout β-NHE (Borgese et al., 1992). The sense primer (SNHE-F) had the sequence 5'-TTCNTNTACAGCTACNTGGC-3' (corresponding to nucleotide positions 967–986 of the trout β-NHE sequence), while the anti-sense primer (SNHE-R) was 5'-GNAGGCCACCGTAGGC-3' (positions 1321–1336 of trout β-NHE). The PCR protocol for sculpin cDNA was denature (95 °C, 1 min), anneal (55 °C, 1 min), elongation (72 °C, 1 min); 40 cycles. Mummichog cDNA was amplified using a combination of primers 3F and SNHE-R using the protocol: denature (95 °C, 1 min), anneal (47 °C, 1 min), elongation (72 °C, 1.5 min); 40 cycles. The PCR products were ligated into Promega pGEM-T Easy or Invitrogen pCR 2.1 vectors and transformed into Promega JM109 or Invitrogen INVαF1 competent cells. Transformants positive for the vector plus insert were selected on LB agar plates containing ampicillin and X-gal (isopropyl β-D-thiogalactopyranoside) and IPTG (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; for pGEM-T Easy Vectors). Positive bacterial colonies were grown

overnight, and plasmids were then isolated using the procedure of Birnboim and Doly (1979). To verify the presence and size of inserts, the isolated plasmids were cut with *Eco*RI in a restriction digest and visualized on a 1% agarose gel containing ethidium bromide. The Sanger method and the SP6 and T7 promoter sequencing primers were then used to sequence the ligated PCR products (Amersham Sequenase 2.0 DNA sequencing kit). The sequences were submitted *via* the WWW (<http://www.ncbi.nlm.nih.gov/>) for NIH Blast comparisons (Altschul et al., 1990) to other known sequences. Alignments were performed using SeqVu 1.1 (Garvin Institute, Sydney, Australia).

#### Protein isolation, gel electrophoresis and western blot analysis

Membrane enrichments were prepared from dissected gill filaments of *Myoxocephalus octodecimspinosus* (first frozen in liquid nitrogen) and *Fundulus heteroclitus* (prepared unfrozen) by disruption with a polytron homogenizer (on ice for 20 s, medium setting) in ice-cold buffer (250 mmol l<sup>-1</sup> sucrose, 1 mmol l<sup>-1</sup> EDTA, 2 μg ml<sup>-1</sup> aprotinin, 2 μg ml<sup>-1</sup> leupeptin, 100 μg ml<sup>-1</sup> PMSF (phenyl methylsulfonyl fluoride) and 30 mmol l<sup>-1</sup> Tris-Cl at pH 7.4). Cell debris was then removed in a low-speed centrifugation (3000 g for 5 min), and membranes were pelleted in a final high-speed centrifugation (50 000 g for 30 min). Pellets were resuspended in a minimal volume of homogenization buffer, and proteins in the suspension were solubilized by the addition of a modified Laemmli sample buffer (Laemmli, 1970; without Bromophenol Blue or β-mercaptoethanol). After determining the total protein concentration (Biorad DC, detergent-compatible assay), 2% β-mercaptoethanol and 0.01% Bromophenol Blue were added to each sample. A 100 μg sample of total protein was separated in a 7% polyacrylamide gel (3 h at 200 V) and transferred to a polyvinylidene difluoride membrane (PVDF, Immobilon-P; Millipore, Bedford, MA, USA) according to the manufacturer's protocol. Immunoblotting procedures were as follows: blocking with Blotto overnight at 4 °C (5% non-fat dry milk in Tris-buffered saline, TBS, pH 7.4); primary antibody incubation with monoclonal antibody 4E9 (culture supernatant diluted 1:5 in Blotto) for 2 h at approximately 25 °C; three washes in TBST (0.1% Tween-20 in TBS pH 7.4); secondary antibody incubation with horseradish-peroxidase-conjugated goat anti-mouse IgG (1:5000 diluted in Blotto; Pierce, Rockford, IL, USA) for 1.5 h at approximately 25 °C. Antibody binding was detected by exposing Kodak X-OMAT-AR scientific imaging film to a chemiluminescent signal (SuperSignal System; Pierce, Rockford, IL, USA) according to the manufacturer's protocol. A digitized image was produced from the resulting negative (Hewlett Packard ScanJet IICx flatbed scanner) and analysed using NIH Image software version 1.61 (National Institutes of Health, USA) on a Power Macintosh 6500/225. Mouse monoclonal antibody 4E9 (Cox et al., 1997) was generously provided by Drs Bliss Forbush and Daniel

Biemesderfer at Yale University School of Medicine. The antibody was produced against a fusion protein representing the cytoplasmic region of porcine NHE-1 (amino acids 514–818).

## Results

### Results from northern blot analysis of RNA from seawater-adapted sculpin

A northern blot of total RNA isolated from sculpin organs (using the 1.9 kb *Bam*HI fragment of human NHE-1 cDNA) is shown in Fig. 1. A transcript of approximately 8.0 kb was evident in sculpin gill, small intestine, kidney, red blood cells and bladder. A possible second transcript at approximately 3.3 kb was observed predominantly in erythrocytes followed by kidney and small intestine. Hybridization of the blot shown in Fig. 1 with a 28S rRNA probe revealed approximately equivalent amounts of RNA loaded in all lanes (not shown). These results suggest that transcripts homologous to NHE-1 are expressed and are clearly detectable in several sculpin tissues including the gill.

### RT-PCR detection of mRNA for NHE isoforms in sculpin and mummichog

Two different sequences were obtained from the PCR products generated by the SNHE-F/SNHE-R degenerate primer pair against mRNA from sculpin (recovering from acidosis). Analysis of nucleotide and amino acid homology suggests that they represent two distinct isoforms of NHE in the gill tissue. RT-PCR on mummichog gill RNA also revealed a sequence homologous to NHE. The first isoform from sculpin (sequence I; Table 1) had the highest BLAST homology score (Altschul et al., 1990) with the NHE-2 isoforms found in rat small intestine (Wang et al., 1993) and human liver (Ghishan et al., 1995). This approximately 360 bp partial sequence proved to be 76 % homologous at the amino acid level and 61 % identical at the nucleotide level to the mammalian sequences. The

second sculpin isoform (sequence II; also approximately 360 bp; Table 2) was 92 % homologous at the amino acid level and had an 84 % nucleotide identity to the trout  $\beta$ -NHE isoform (Borgese et al., 1992). Both sculpin sequences accounted for approximately 15 % of the total translated protein length published for NHE-2 and  $\beta$ -NHE. Importantly, these two partial sequences (again, both generated using the same degenerate primer pair and both of the same length) share only 49 % amino acid identity with each other, suggesting that they are indeed two different isoforms. The approximately 575 bp partial sequence from seawater-adapted mummichog (Table 3) was generated by combining primers 3F (Towle et al., 1997) and SNHE-R. The sequence was 91 % homologous at the amino acid level with 82 % nucleotide identity to the trout  $\beta$ -NHE isoform and accounted for approximately 24 % of the trout isoform length. The mummichog amino acid translation was also 87 % identical to the sculpin  $\beta$ -NHE sequence (Table 2) for the 119-amino-acid shared region.

Using primers SNHE-F and SNHE-R, RT-PCR was performed on RNA isolated from sculpin small intestine, liver, kidney and skeletal muscle. Fig. 2 shows that a PCR product similar to that sequenced from the gill was only obtained from small intestine and kidney. While we do not have sequence information on these PCR products, these data imply that the mRNA for neither  $\beta$ -NHE nor the NHE-2-like isoform is transcribed in liver or skeletal muscle tissue (at least at levels that can be detected by RT-PCR). This predicted tissue distribution is consonant with that of the NHE message revealed by northern blot analysis using the human NHE-1 probe (Fig. 1).

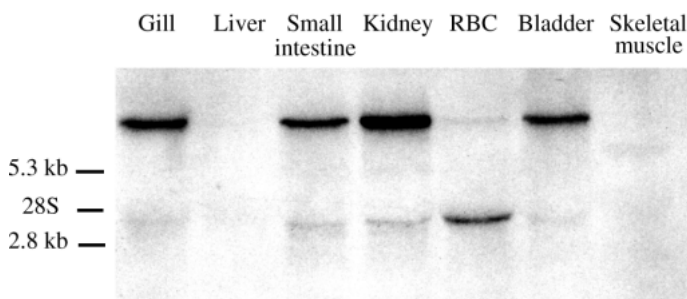


Fig. 1. Northern blot of total RNA isolated from long-horned sculpin (*Myoxocephalus octodecimspinosus*) tissues using the 1.9 kb *Bam*HI fragment of human NHE-1 cDNA. 20 mg of each RNA sample was loaded per lane. Transcripts at approximately 8.0 kb are apparent in gill, small intestine, kidney, red blood cells (RBC) and bladder. The positions of molecular mass given and 28S rRNA are given. The film was exposed for 24 h.

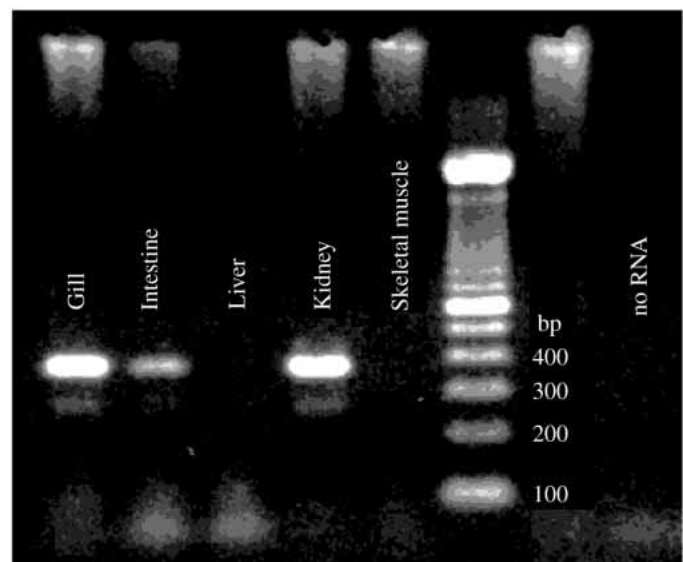


Fig. 2. RT-PCR products obtained from various tissues in seawater-adapted sculpin (*Myoxocephalus octodecimspinosus*). All PCR reactions were carried out using the degenerate primer pair SNHE-F and SNHE-R and identical conditions. The 360 bp band in gills contains both  $\beta$ -NHE and NHE-2-like isoforms. Products are visualized on a 1 % agarose gel stained with ethidium bromide.

Table 1. *Sculpin amino acid sequence I compared with the published rat NHE-2 sequence*

Sculpin I	1	FLYSYXAYLVAELFAISSIMAIIVTCALTMKYYVEENVSQRSC	TIRHVIKMLGSISSETLI	60
		FLYSY +Y+ AE+F +S IMAI CA+TM YVEENVSQ+S TTI++ +KML S+SETLI		
Rat NHE-2	316	FLYSYLSYITAEMFHLGIMAITACAMTMNKYVEENVSQKSYTTIKYFMKMLSSVSETLI		375
Sculpin I	61	FFFLGVVAITTEHEWNNWGYILFTLLFAFVWRGLGVLVLTQIINPFRTIPFNLDQFGLA		119
		F F+GV + HEWNNW ++ FTL F +WR LGV VLTQ+IN FRTIP KDQF +A		
Rat NHE-2	376	FIFMGVSTVGNHEWNNWAFVCFTLAFCLIWRLGTVFVLTQVINWFRTIPLTFKDQFIIA		434

The rat NHE-2 sequence is taken from Wang et al. (1993).

The two sequences are 76 % homologous and 61 % identical.

Homologous amino acids are indicated by matching letters and a + sign. An X indicates a codon with one nucleotide that remains to be confirmed and is presently shown as a gap in the sequence.

BLAST homology smallest sum probability score:  $P=5.4 \times 10^{-47}$  (Gish and States, 1993).

Table 2. *Sculpin amino acid sequence II compared with the published trout  $\beta$ -NHE sequence*

Sculpin II	1	FVYSYXAYLSAEMFHLGIMALIACGATMXPYVEANISHKSHTTIKYFLKMWSXVTETLI		60
		F+YSY AYLS+EMFHLGIMALIACG M PYVEANISHKS+TTIKYFLKMWS V+ETLI		
Trout $\beta$ -NHE	303	FLYSYMAVLSSEMFLGIMALIACGVVMPYVEANISHKSYTTIKYFLKMWSVSETLI		362
Sculpin II	61	FIFLGVATVDGPHWNNWTFVTVTVILCLVSRVIGVVGLTYVINKFRIVKLTTKDQFIVA		119
		FIFLGV+TV GPH WNWTFV TVILCLVSRV+GV+GLT++INKFRIVKLT KDQFIVA		
Trout $\beta$ -NHE	363	FIFLGVSTVAGPHAWNNTFVITTVILCLVSRVLGVIGLTFIINKFRIVKLTTKDQFIVA		421

The  $\beta$ -NHE sequence is taken from Borgese et al. (1992).

The two sequences are 92 % homologous and 84 % identical.

BLAST smallest sum probability score:  $P=3.4 \times 10^{-65}$ .

See Table 1 for details.

Table 3. *Mummichog amino acid sequence compared with the published trout  $\beta$ -NHE sequence*

Mummichog	1	NDAVTVVLYHLFEFSEAGTVTVLDGFLGVISFLVVALGXLLVGAIFYGVAALTSRFTYH		60
		NDAVTVVLY+LFEFES+ GTVTVLD FLGV+ F VV+LG LVGA YGF+AA TSRFT H		
Trout $\beta$ -NHE	234	NDAVTVVLYNLFEFESKVGTVTVLDVFLGVVCFVVSLSGGVLVGAIFYGLAAFTSRFTSH		293
Mummichog	61	IRVIEPLFVVFVYSYMAVLSAEVFLGIMALIACGAVMRPYVEANISHKSHTTIKYFLKM		120
		RVIEPLFVF+YSYMAVLS+E+FLGIMALIACG VMRPYVEANISHKS+TTIKYFLKM		
Trout $\beta$ -NHE	294	TRVIEPLFVFLYSYMAVLSSEMFLGIMALIACGVVMPYVEANISHKSYTTIKYFLKM		353
Mummichog	121	WSSVSETLIFIFLGVATVEGPHQWNVFVMTVILCLVSRVIGVVGLTFVINKFRMVNLT		180
		WSSVSETLIFIFLGV+TV GPH WNW FV+ TVILCLVSRV+GV+GLTF+INKFR+V LT		
Trout $\beta$ -NHE	354	<u>WSSVSETLIFIFLGVSTVAGPHAWNNTFVITTVILCLVSRVLGVIGLTFIINKFRIVKLT</u>		413
Mummichog	181	TKDQFIIA		188
		KDQFI+A		
Trout $\beta$ -NHE	414	<u>KKDQFIVA</u>		421

The two sequences are 91 % homologous and 82 % identical.

BLAST smallest sum probability score:  $P=4.9 \times 10^{-97}$ . See Table 1 for details.

The portion of the sequence comparable to sculpin  $\beta$ -NHE in Table 2 is underlined.

*Immunological detection of NHE-like protein expression in gill tissue*

In a test for expression of NHE in sculpin and mummichog gills, proteins were separated using SDS-PAGE, western-blotted, and then probed with specific antibodies against mammalian NHE-1. A diffuse band centered at approximately 80 kDa was detected in mummichog adapted to both fresh and salt water (Fig. 3), and an approximately 90 kDa protein was observed in sculpin (Fig. 4). The antibody was shown not to cross-react with fibroblast cell cultures expressing the cDNA corresponding to the  $\beta$ -NHE or NHE-2, -3 or -4 isoforms (D. Biemesderfer, personal communication). In addition, control immunoblots of membranes isolated from cell lines transfected with or deficient in NHE-1 (PS127 and PS120, respectively, provided by Dr J. Pouyssegur, University of Nice) exhibited high levels of antibody binding in the positive controls but no specific bands in the negative control (not shown). Blots of proteins isolated from whole blood did not show antibody binding.

To study the effects of acidosis on NHE-1-like protein expression, sculpin were infused with an acid load ( $2 \text{ mmol kg}^{-1}$ ), and 5 h later gill proteins were prepared as described above with equal amounts of total protein separated and immunoblotted (Fig. 4). A diffuse band centered at approximately 90 kDa was detected in both control and acid-loaded sculpin gills. The optical density of the 90 kDa band in acid-loaded fish was 30–40 times lower than in control fish, indicating a large reduction in the abundance of NHE-1-like protein during acidosis. Two additional bands of lower molecular mass were also detected, but these may represent degradative fragments of the functional protein.

### Discussion

We have used several molecular and immunological methods to detect the presence of multiple  $\text{Na}^+/\text{H}^+$  exchanger isoforms in two teleost species. At least three distinct isoforms of NHE (similar to NHE-1, NHE-2 and  $\beta$ -NHE) may be present in the gills of marine sculpin. The isoforms NHE-1 and  $\beta$ -NHE have also been detected in freshwater- and seawater-adapted mummichog.

#### *Northern blot analysis*

The apparent molecular mass of the expressed mRNA transcripts in sculpin differs from that of NHE-1 transcripts in mammalian tissues (7.5–8.0 kb in sculpin *versus* 4.9–5.2 kb in mammals). This may reflect species-specific differences in the coding or noncoding regions of the mRNA. A possible second transcript at approximately 3.3 kb was observed in erythrocytes, and smaller amounts were detected in kidney and small intestine. Smaller molecular mass transcripts have also been reported for northern blots of rat RNA and are thought to represent incomplete transcripts or alternatively spliced products (Orlowski et al., 1992). These bands may also be due to overloading of the lanes and co-migration of the higher molecular mass bands with the 28S ribosomal RNA. It is likely

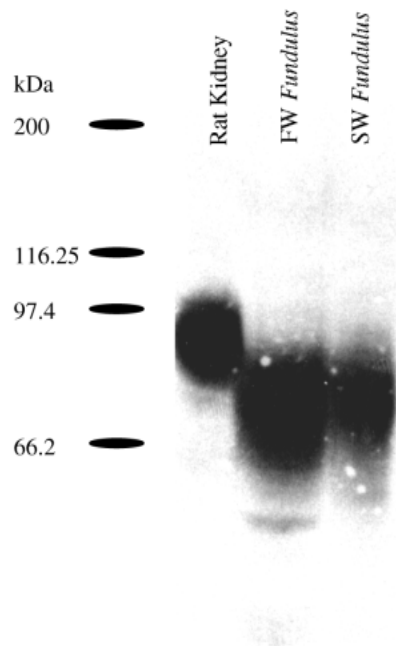


Fig. 3. Immunoblot of gill tissue from mummichog (*Fundulus heteroclitus*) adapted to fresh (FW) and salt (SW) water. Blots were probed with a monoclonal antibody specific for mammalian NHE-1 (4E9). For comparison, rat kidney, transferred and probed under the same conditions, is also shown. Protein loading was not normalized in this experiment. Size markers are in kDa.

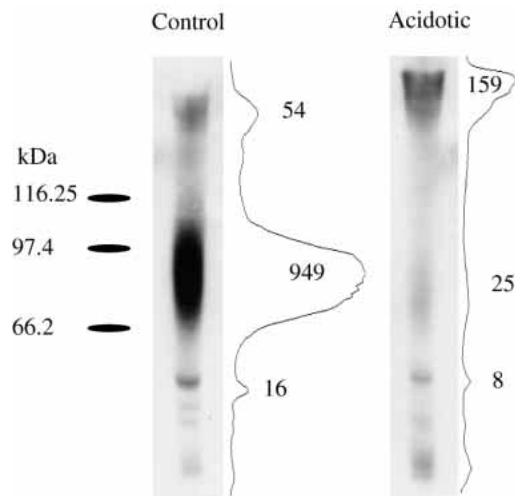


Fig. 4. Immunoblot of gill tissue from control and acid-loaded sculpin (*Myoxocephalus octodecimspinosus*) using NHE-1 monoclonal antibody 4E9. Each lane was loaded with  $100 \mu\text{g}$  of total protein. The uncalibrated optical density of each lane was plotted, and the relative area (arbitrary units) under each peak is given to the right of the respective lane. Size markers are in kDa.

that the large probe (1916bp) used here may also have hybridized to multiple isoforms of the NHE (that we found using RT-PCR; see below) since the probe sequence was designed to include the putative membrane-spanning domain

within the NHE-1 transcript. This domain is well-conserved across isoforms (Noel and Pouyssegur, 1995). These results were also similar to northern blot analysis of flounder (*Pseudopleuronectes americanus*) cDNA using the same probe, which also detected the presence of NHE (Harris et al., 1993).

#### RT-PCR

Analysis of sequences from the RT-PCR products revealed the possibility of two distinct NHE isoforms in the sculpin and one in the mummichog. To our knowledge, this is the first description of an NHE-2-like isoform in transporting epithelia of any non-mammalian species. This is also the first specific identification of the  $\beta$ -NHE isoform in fish branchial tissue. Both isoforms were detected in sculpin recovering from systemic acidosis using the same degenerate primer pair, and both sequences exhibited very high amino acid homology with rat NHE-2 (76%) and trout  $\beta$ -NHE (84%). The degree of amino acid homology between the various mammalian NHE isoforms (as well as  $\beta$ -NHE) ranges from 47 to 74% within the putative membrane-spanning segments of the sequences (the location of the partial sequences described here; Malapert et al., 1997). The NHE-2 isoform is thought to be apically located in the rat, rabbit and human small intestine (for a review, see Yun et al., 1995), so if it plays the same role in the sculpin branchial epithelium, it might be specifically involved in apical H<sup>+</sup> excretion (and Na<sup>+</sup> uptake). Claiborne (1998) originally speculated that NHE-3 (the predominant apical Na<sup>+</sup>/H<sup>+</sup> antiporter in mammalian renal proximal tubule tissue; Paillard, 1997) might be responsible for gill H<sup>+</sup> excretion. The data presented here implicate, albeit indirectly, a different (or additional) apical isoform. The NHE-2 isoform also exhibits a higher amiloride-sensitivity than NHE-3 (Tse et al., 1993), and inhibition of systemic acid excretion by amiloride following acidosis has been noted in this species *in vivo* (Claiborne et al., 1997). In the present study, the NHE-2-like isoform was only detected in animals that had previously been subjected to acidosis, and preliminary data indicate that it may also be present in the gills of control animals (J. B. Claiborne and A. I. Morrison-Shetlar, personal observations). It remains to be seen whether the time course of mRNA transcription is altered during systemic acidosis in these animals.

The  $\beta$ -NHE isoform detected in both mummichog and sculpin gills is highly homologous to the trout erythrocytic  $\beta$ -NHE. While it is possible that the RT-PCR is amplifying  $\beta$ -NHE mRNA from erythrocytes present when the gill tissue samples were collected, neither northern blot analysis (Fig. 1) nor RT-PCR (Fig. 2) detected NHE transcripts in sculpin skeletal muscle or liver homogenates (which contained erythrocytes). To date,  $\beta$ -NHE has only been reported in trout hemopoietic tissue and is thought to be involved with red cell alkalization to increase hemoglobin affinity (Malapert et al., 1997). These authors suggested that  $\beta$ -NHE may also be a basolateral isoform because of its high homology to NHE-1. Interestingly, erythrocytic  $\beta$ -NHE can be stimulated by catecholamines, but is not activated by acidosis until

intracellular pH falls below normal physiological ranges (beginning at a pH of approximately 6.5). If  $\beta$ -NHE plays a functional role in the gill epithelium, it is likely that different kinetic and/or regulatory properties are required. If this is indeed the case and the isoform is basolaterally located,  $\beta$ -NHE could serve the 'housekeeping' functions (e.g. pH homeostasis, volume regulation) often ascribed to NHE-1 in mammalian systems (Yun et al., 1995).

#### Immunological detection of NHE

Monoclonal antibodies against mammalian NHE-1 were used in this study to detect immunologically similar proteins in both sculpin and mummichog gills. Although the apparent molecular mass of 80–90 kDa is smaller than that of mammalian NHE-1 (Rutherford et al., 1997; rat kidney tissue blotted under the same conditions gives a molecular mass of approximately 97 kDa, see Fig. 3), the signal profile was similarly diffuse, indicating that it may also be a heavily glycosylated protein (Sardet et al., 1989). The monoclonal antibody line 4E9 has been shown to be highly specific for the NHE-1 isoform and does not bind to any other isoform (including PS120 cells transfected with  $\beta$ -NHE; D. Biemesderfer, personal communication; Cox et al., 1997; Rutherford et al., 1997). Thus, our results indicate that a protein with antigenic sites similar to those of NHE-1 is present in the gills of these two fish species.

Acidosis induced a 30- to 40-fold decrease in the abundance of NHE-1-like protein in sculpin gill (Fig. 4). NHE-1 has been localized to the basolateral membrane in epithelial tissues such as mammalian kidney and intestine (Biemesderfer et al., 1992; Bookstein et al., 1994), reptilian intestine (Harris et al., 1997) and an amphibian kidney cell line (Coupaye-Gerard et al., 1996). If basolateral targeting is a conserved feature of NHE-1 in the fish gill epithelium, a reduction in serosal Na<sup>+</sup>/H<sup>+</sup> exchange by NHE-1 degradation (concurrent with continued or elevated apical H<sup>+</sup> excretion) would increase the efficiency of systemic net acid excretion. Thus, the large reduction in NHE-1 observed 5 h after acid infusion may represent an adaptive adjustment of NHE-1 expression and/or degradation during metabolic acidosis. These results also agree with the rapid increase observed in acid excretion and eventual over-compensation, over the same time course, that we have noted in acidotic sculpin *in vivo* (Claiborne et al., 1997). While NHE-1 is nearly ubiquitous in mammals, the rat proximal tubule has been shown to exhibit regional differences in NHE-1 expression, and the isoform is not expressed in some cortical segments (Yun et al., 1995). In contrast, basolateral activity and mRNA abundance for NHE-1 in a rabbit kidney cell line were shown to increase during acidosis, perhaps for intracellular pH regulation (Paillard, 1997). While NHE-1 is thought to be mainly regulated by changes in affinity for H<sup>+</sup> (Noel and Pouyssegur, 1995), we believe that our data are the first to show an adaptive decrease in levels of epithelial NHE-1-like protein during acidosis.

Both freshwater- and seawater-adapted mummichog also express the NHE-1-like protein. While the freshwater model for H<sup>+</sup> excretion and Na<sup>+</sup> uptake utilizes a H<sup>+</sup>-ATPase/Na<sup>+</sup>

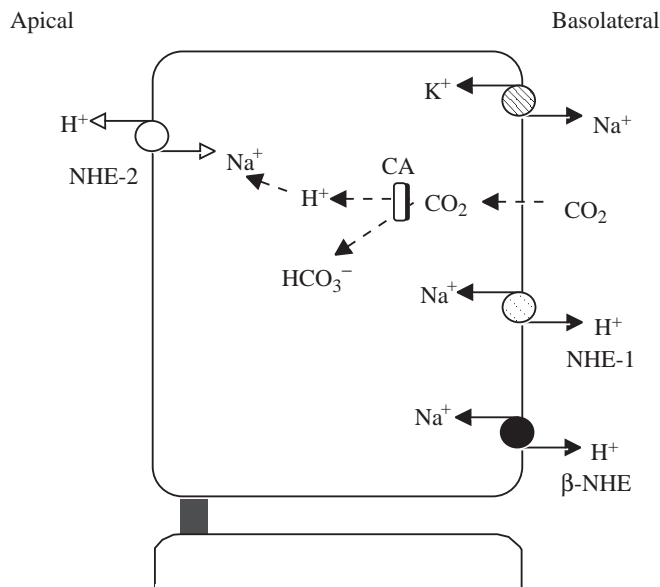


Fig. 5. Speculative working model for  $H^+$  transport via  $Na^+/H^+$  antiporters (NHEs) in the gills of seawater fishes. Net systemic  $H^+$  excretion is accomplished by a decrease in abundance of basolateral NHE-1 and maintenance or up-regulation of apical NHE-2 levels. Basolateral  $\beta$ -NHE may perform pH and volume regulatory 'housekeeping' functions.  $H^+$  and  $HCO_3^-$  are generated within the cell from the hydration of  $CO_2$  catalyzed by carbonic anhydrase (CA). Intracellular  $HCO_3^-$  may be transferred by apical and/or basolateral 'band-3'  $Cl^-/HCO_3^-$  exchange (not shown).

channel system, it is clear that NHE-1 (as well as mRNA for  $\beta$ -NHE detected by RT-PCR; L. A. MacKenzie, preliminary data) is present in the freshwater-adapted animal. Using cultured gill pavement cells from freshwater *Oncorhynchus mykiss*, Pärt and Wood (1996) found evidence for apical, amiloride-sensitive  $Na^+/H^+$  transport and suggested that it was normally a basolateral exchange used for intracellular pH regulation. While thermodynamic considerations do not favor apical  $Na^+/H^+$  exchange for  $Na^+$  uptake in fresh water (Potts, 1994), a basolateral antiporter may still be involved in systemic acid-base regulation (see above). Patrick et al. (1997) have also recently suggested that freshwater-adapted *F. heteroclitus* may not 'fit' the current model for freshwater ion transport and, indeed, active  $Cl^-$  influx may take place across the pavement cell epithelium in rainbow trout (Wood et al., 1998).

#### Working model for seawater acid-base regulation

In summary, the results presented here indicate that at least three distinct isoforms of the  $Na^+/H^+$  antiporter (similar to NHE-1, NHE-2 and  $\beta$ -NHE) may be present in the gills of marine sculpin. The NHE-1 and  $\beta$ -NHE isoforms have also been detected in freshwater- and seawater-adapted mummichog. Immunological detection of NHE-1 is decreased in sculpin following acidosis. These data, in combination with *in vivo* observations (Claiborne et al., 1997), have allowed us to modify our working model (Fig. 5) of seawater gill acid-base

exchange (Claiborne, 1998). We hypothesize that basolateral  $\beta$ -NHE in the gills plays an intracellular 'housekeeping' role analogous to that of NHE-1 in mammals. Basolateral gill NHE-1 activity is down-regulated during acidosis to enhance net  $\Delta H^+$  transfers to the water, while a typically apical isoform (NHE-2; inhibited by external amiloride and low external  $[Na^+]$ ) also drives net systemic acid excretion. While inappropriate for ion regulation in sea water, the  $Na^+$  taken up across the gills may account for only a small portion of total  $Na^+$  influx and may be 'worth' the additional energetic costs to maintain acid-base balance (Evans, 1984). While very speculative, we hope that this working model can serve as a basis for further definition of the molecular regulation, cell (and membrane) location and time course of both mRNA and protein expression during acid-base disturbances in seawater fishes.

The authors wish to thank Drs Dan Biemesderfer, David Evans, Bliss Forbush, Mark Musch, J. Pouyssegur, Bob Shetlar and David Towle for helpful advice and discussions and/or the provision of the antibodies, primers and probes utilized in this study. This work was funded by National Science Foundation RUI-94-19849 and IBN-9808141 to J.B.C., National Institutes of Health DK29786, DK45880, the University of Michigan Gastrointestinal Peptide Center and National Institutes of Environmental Health Science Toxicology grants to D.C.D. and Georgia Southern University Research Grants to J.B.C. A.I.M., C.R.B., L.A.M. and K.P.C.

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