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Short-Term Low-Salinity Tolerance by the Longhorn Sculpin, *Myoxocephalus octodecimspinosus*

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ABSTRACT The bottom-dwelling, longhorn sculpin, *Myoxocephalus octodecimspinosus*, is traditionally viewed as a stenohaline marine fish, but fishermen have described finding this sculpin in estuaries during high tide. Little is known about the salinity tolerance of the longhorn sculpin; thus, the purposes of these experiments were to explore the effects of low environmental salinity on ion transporter expression and distribution in the longhorn sculpin gill. Longhorn sculpin were acclimated to either 100% seawater (SW, sham), 20% SW, or 10% SW for 24 or 72 hr. Plasma osmolality, sodium, potassium, and chloride concentrations were not different between the 20 and 100% treatments; however, they were 20–25% lower with exposure to 10% SW at 24 and 72 hr. In the teleost gill, regulation of Na^+, K^+-ATPase (NKA), Na^+–K^+–$2Cl^-$ cotransporter (NKCC1), and the chloride channel, cystic fibrosis transmembrane conductance regulator (CFTR) are necessary for ion homeostasis. We immunolocalized these proteins to the mitochondrion-rich cell of the gill and determined that acclimation to low salinity does not affect their localization. Also, there was not a downregulation of gill NKA, NKCC1, and CFTR mRNA or protein during acclimation to low salinities. Collectively, these results suggest that down to 20% SW longhorn sculpin are capable of completely regulating ion levels over a 72-hr period, whereas 10% SW exposure results in a significant loss of ions and no change in ion transporter density or localization in the gill. We conclude that longhorn sculpin can tolerate low-salinity environments for days but, because they cannot regulate ion transporter density, they are unable to tolerate low salinity for longer periods or enter freshwater (FW). The genus *Myoxocephalus* has three FW species, making this group an excellent model to test evolutionary and physiological mechanisms that allow teleosts to invade new low salinities successfully. *J. Exp. Zool. 311A:45–56, 2009.*

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Sculpin (Scorpaeniformes: Cottidae) are a speciose (~400 species; Nelson, 2006) group of teleost fishes that display a great variety in habitat use including: rivers, demersal-freshwater (FW) lakes, inshore coastal marine areas (including brackish waters), and demersal-marine areas. The genus *Myoxocephalus* consists of 17 species, of which 9 are demersal-marine fishes, 5 enter brackish water, and 3 spend a portion of their life in FW (Froese and Pauly, 2000). The longhorn sculpin, *Myoxocephalus octodecimspinosus*,[1] is distributed in coastal waters from Virginia to Newfoundland, Canada (Bigelow and Schroeder, 2002). Although they are primarily distributed in marine waters, they have been found entering estuaries during high tides, but never in FW (Bigelow and Schroeder, 2002), suggesting that they have some level of low-salinity tolerance. The only laboratory study to subject longhorn sculpin to low-salinity challenges was conducted by Claiborne et al. ('94). They tested the effects of 4, 8, and 20% seawater (SW) acclimations and acid loads on longhorn sculpin and determined that in 4 and 8% SW they lose Cl^- to a lethal level by 48 and 60 hr, respectively. In 20% SW, there is an initial

[1]Also spelled as *octodecemspinosus*, but we will use the spelling of Bigelow and Schroeder (2002).

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decrease in plasma Cl^-, but by 72 hr this value returned to control values; however, they did not determine what effect low environmental salinity had on gill ion transporter expression.

In teleost fishes, ion balance is regulated by specialized epithelial cells in the gill called mitochondrion-rich cells (MRCs) (also termed as chloride cells). As the name implies, they contain a high density of mitochondria as well as ion transporters and channels necessary for ion movement. These include the basolateral membrane proteins: Na^+, K^+-ATPase (NKA) and Na^+–K^+–$2Cl^-$ cotransporter (NKCC1) and the apical membrane chloride channel, cystic fibrosis transmembrane conductance regulator (CFTR) (recently reviewed by Evans et al., 2005). Studies in euryhaline fishes, such as the killifish (*Fundulus heteroclitus*), or anadromous fishes such as the rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), or Atlantic salmon (*S. salar*) have explored the effects of changes in environmental salinity on gill ion transporter density (e.g. Mancera and McCormick, 2000; Seidelin et al., 2000; Pelis et al., 2001; Marshall et al., 2002; Singer et al., 2002; Scott et al., 2004; Hiroi and McCormick, 2007). In general, when these fishes move from FW/brackish water to marine waters, there is a rapid increase in plasma $[Na^+]$, $[Cl^-]$, and total osmolality (e.g. Seidelin et al., 2000; Scott et al., 2004), and to help maintain ion homeostasis, euryhaline fishes upregulate gill NKA, NKCC1, CFTR, and other transporters and channels. The opposite occurs when going from marine waters to brackish/fresh; there is a loss of ions and, generally, these ion transporters are downregulated. In addition to turning off SW osmoregulatory proteins, FW ion transporters are upregulated during FW acclimation. These include Na^+–H^+ exchangers (NHEs), V–H^+-ATPase, and Cl^-/HCO_3^- exchangers, and these are also necessary for proper acid–base regulation (e.g. Evans et al., 2005). Recently, NKA, NHE-2, and V–H^+-ATPase were immunolocalized to MRCs in the longhorn sculpin gill (Catches et al., 2006); however, the effects of salinity on the expression of these ion transporters in the longhorn sculpin are yet to be elucidated.

The purpose of this study was to explore the effects of low environmental salinity on SW osmoregulatory ion transporter expression and distribution in the longhorn sculpin gill to further our understanding of why they are not found in FW. We hypothesize that longhorn sculpin can only tolerate low-salinity environments for short periods (days) because they are unable to properly regulate these ion transporters necessary for ion homeostasis.

METHODS

The following experiments were conducted in August of 2006 and 2007, and all methods were approved by Institutional Animal Care and Use Committee at the University of Florida and the Mount Desert Island Biological Laboratory (MDIBL, Salisbury Cove, ME). Longhorn sculpin, *M. octodecimspinosus* (mass = 247.7 ± 23 g), were collected by local fishermen in Frenchman Bay, ME. Longhorn sculpin were transported to MDIBL and maintained in 6 ft circular tanks with free-flowing SW from Frenchman Bay, under a natural summer photoperiod, and fed squid every other day. All animals were fasted 48 hr prior to and during experimentation.

Low-salinity acclimation

In a preliminary experiment, we maintained two longhorn sculpin in 10% SW for 6 days before seeing visual signs of stress (sluggish behavior, color change, lack of righting) (data not shown). Thus, we terminated the full experiment after 3 days (72 hr), when there were no obvious signs of stress. Longhorn sculpin were randomly assigned to one of the four treatments: 24-hr sham (100% SW), 24-hr 10% SW, 72-hr sham (100% SW), or 72-h 10% SW. Each sculpin was placed in five gallons of the appropriate, aerated solution in a ten-gallon bucket. The buckets were maintained in a trough with free-flowing SW (15°C) for temperature control. For experiments that lasted longer than 24 hr, 50% of the water was replaced in each bucket daily. Owing to the confined space, only eight fishes were run in each experiment (three shams, five treatments); thus, the experiment was run four times (10% SW and 20% SW for 24 or 72 hr). Twenty percent SW was made by mixing two parts SW with eight parts of dechlorinated FW (by bubbling air into a bucket of FW for 24 hr). The salinity was confirmed by measuring the osmolality with a Wescor vapor pressure osmometer 5520 (Logan, UT). Within the 72-hr treatments, 50% of the water was replaced daily to ensure that ammonia/nitrates/nitrites were zero. A similar experiment was repeated using 20% SW and 100% SW (sham) as the treatments. After 24 or 72 hr, fishes were anesthetized in either100% SW, 20% SW, or 10% SW (depending on the assigned treatment) with $0.379\,g\,L^{-1}$

benezocaine (initially dissolved in absolute ethanol, final concentration of ethanol was 0.1%) (Sigma, St. Louis, MO). Blood (0.5–1.0 mL) was taken from the bulbus arteriosus and then the fish was pithed. Half of the filaments of the second gill arch were cut off and snap frozen for RNA extraction and the other half were fixed in 4% paraformaldehyde in 10 mM phosphate buffered saline (PBS; pH = 7.3) for immunohistochemical analyses. The rest of the filaments from the seven gill arches were cut off from the arch into a dish of 10 mM PBS, mixed, divided into two tubes, and snap frozen for protein analyses.

Plasma chemistry

Blood samples were immediately spun at 1,000*g* for 5 min at 4°C and the plasma was aliquoted and frozen (−20°C) until analyzed. Total plasma osmolality was measured using a Wescor vapor pressure osmometer. Plasma sodium and potassium were measured using an IL943 automatic flame photometer (Instrumentation Laboratory, Lexington, MA) and chloride by the Labconco digital chloridometer (Kansas City, MO). All samples were measured in triplicate.

Molecular techniques

RNA was extracted using TRI reagent (Sigma) as previously described in Hyndman and Evans (2007). RNA pellets were reconstituted in 10 μL of diethyl pyrocarbonate treated water, and the concentration of RNA measured using a Nanodrop ND-1000 spectrophotometer (Fisher Scientific, Wilmington, DE). Total RNA (5 μg) was reverse transcribed using a First Strand cDNA Synthesis Kit With Superscript III (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

Degenerate primers were designed using CODEHOP (Rose et al., 2003) to amplify *NKA*, *NKCC1*, and *CFTR* (Table 1). The polymerase used was 0.625 U of Ex Taq, hot start, DNA polymerase (Takara Bio, Madison, WI) and the reactions were run in a Px2 thermocycler (Thermo Fisher Scientific, Waltham, MA). The polymerase chain reaction (PCR) parameters were: 94°C for 2 min, 40 cycles of 94°C for 30 sec, 45–60°C (gradient) for 30 sec, 72°C for 30 sec, and a final 72°C for 5 min. These products were then singly nested with a primer listed in Table 1, and the PCR was run using the PCR product from the first PCR. With all transcripts, there was a bright single band, and these transcripts were ligated into pCR®4-TOPO vectors and transformed into TOP10 chemically competent cells using a TOPO TA Cloning® Kit for sequencing (Invitrogen). Cells were grown on agar plates with Kanamycin (500 ×) antibiotic and positive colonies were grown in LB broth (20 g L^{-1}) overnight at 37°C while shaking. Plasmids were extracted from the cells using a miniprep kit (Roche Applied Science, Indianapolis, IN) and sequenced at the Marine DNA Sequencing Center at MDIBL. These partial sequences have been deposited into GenBank (accession numbers): *NKA* ♯EU391598, *CFTR* ♯EU391599, and *NKCC1* ♯EU391600.

TABLE 1. Primers used in sequencing and quantitative real-time PCR of longhorn sculpin Na^+, K^+-ATPase (NKA), Na^+, K^+, $2Cl^-$ cotransporter (NKCC1), and the cystic fibrosis transmembrane conductance regulator (CFTR)

Primer	5′ to 3′ Orientation
CFTRF1*	AAA TGT AAC TGC CTC CTG GGA YGA RGG
CFTRF2*	TCC CCT CAG ACC TCT TGG ATH ATG CC
CFTRR1*	CCT CGG CTT CCA GCT GTT TNA RYT GYT G
NKAF1*	GGA TGA ACT AAA GAA GGA AGT AGA TAT GGA YGA YCA YAA
NKAR1*	CCA GAC AAT TCT TTT TCG CCA TNC KYT T
NKAR2*	CAC GCC GGT GAT TAT GTG TAT RAA RTG NTC
NKCC1F1*	CCC CCT CTC AGT CTC GGT TYC ARG TNG A
NKCC1F2*	CAT CAT TAT GAT ACG CAC ACG AAY CAN TAY TA
NKCC1R1*	GGA TGT ACC CTC GTA GAG GCT CRT TRT TYT T
qCFTRF1	TTC GAC CTC ATT CAG CTC ACA
qCFTRR1	TGG CGG CGA TGA AGA TGT A
qNKAF1	ACG AAC CGG CCA ACG ATA A
qNKAR1	TTG GTA GTA GGA GAA GCA GCC A
qNKCC1F1	GGA TTT GTA CGA GGA GGT GGA G
qNKCCR1	GCA AAG GCA AAG ATC AGA CCA A

The asterisks denote degenerate primers and q represents quantitative real-time PCR primers. PCR, polymerase chain reaction.

Quantitative real-time PCR

To determine the effects of dilute environments on longhorn sculpin gill *CFTR*, *NKA*, and *NKCC1*, mRNA levels, quantitative real-time PCR (qRT-PCR) was performed. Nondegenerate primers were designed to amplify a product between 50 and 100 bp (Table 1). L8 was used as an internal control gene as previously described (Choe et al., 2005, 2006). Each sample was run in duplicate using 2 μL of 1/10 diluted original oligo-dt cDNA, 7.4 pmol of primers, and SYBR® Green Master Mix (Applied Biosystems, Foster City, CA) in a total volume of 25 μL. The cycling parameters used were: an initial denaturing step of 95°C for 10 min, 40 cycles of: 95°C for 35 sec, 60°C for 30 sec, and 72°C for 30 sec, followed by a melting curve analysis to ensure that only one product was amplified. Random samples were also sequenced following qRT-PCR confirming amplification of the target of interest. To determine the degree of possible genomic contamination, qRT-PCR was run using RNA samples that were not reverse transcribed, and we determined that there was no genomic contamination. All qRT-PCRs were run on a MyiQ quantitative thermocycler (Biorad, Hercules, CA).

Each primer pair's efficiency was determined by performing a ten-fold dilution curve using plasmid cDNA. Efficiency (E) for each primer pair was calculated using the equation $E = -1 + 10^{(-1/\text{slope})}$ where ''slope'' was the slope of the dilution curve. Each cycle threshold (CT) value was subtracted from a randomly chosen control sample resulting in a ΔCT and was analyzed using the Pfaffl equation (Pfaffl, 2001): ratio $= E^{\Delta\text{CT target}}/E^{\Delta\text{CT L8}}$. Each Pfaffl ratio was then standardized to the average sham Pfaffl ratio.

Immunohistochemistry

A portion of the second gill arch from the longhorn sculpin (see above) was fixed in 4% paraformaldehyde in 10 mM PBS for 24 hr, dehydrated in an increasing concentration of ethanol series, cleared in Citrisolv (Fisher Scientific, Pittsburgh, PA), and embedded in paraffin wax. The tissue blocks were cut at 7 μm, placed on superfrost plus slides (Fisher Scientific), and heated at 37°C for 30 min. The slides were analyzed following the methods of Piermarini et al. (2002) and Hyndman et al. (2006). In short, five slides/animal for each treatment were rehydrated, blocked with 3% H_2O_2 in water for 30 min, and washed in 10 mM PBS. Next, there was a 20-min protein block with Biogenex's protein block (BPB; Biogenex, San Ramon, CA), followed by 10 mM PBS washes. Finally, the slides were incubated in primary antibody (see below) overnight at 4°C. The primary antibody was washed off with 10 mM PBS, and the immunoreactivity was visualized using Biogenex's Super Sensitive™ Link-Label IHC Detection System. The chromagens used in this study were 3, 3′-diaminobenzidine tetrahydrochloride (brown color; Biogenex), Vector SG (blue color; Vector Laboratories, Burlingame, CA), and Vector VIP (purple color; Vector Laboratories). Following this, some sections were double-labeled with a second primary antibody following the same procedures.

Western and dot blotting

Western blots were made following the methods of Hyndman and Evans (2007). Gills were homogenized in 2 mL of ice-cold homogenization buffer (250 mM sucrose, 30 mM Tris, 1 mM EDTA, 0.5% of Sigma's protease inhibitor cocktail, and 100 μg mL^{-1} phenylmethylsulfonyl fluoride; pH 7.8). The homogenates were centrifuged at 12,000g for 10 min at 4°C and the supernatant decanted. Protein content of the supernatant was measured using Pierce's BCA protein assay kit (Rockford, IL). A portion of the supernatant was diluted with an equal portion of Laemmli sample buffer with 0.01% bromophenol blue and 2% β-mercaptoethanol (Laemmli, '70) and heated at 65°C for 10 min. Twenty-five micrograms of protein was separated using SDS-PAGE (10% Tris–HCl gels, Biorad) for 2 hr at 100 V and then transferred to an immunoblot polyvinylidene difluoride membrane according to the manufacturer's protocol (Biorad). Next, the membrane was placed in blotto, 5% nonfat dry milk in 10 mM Tris buffered saline (TBS: 25 mmol L^{-1} Tris, 150 mmol L^{-1} NaCl; pH 7.4) for 1 hr at room temperature (~25°C), shaking, and then placed in primary antibody and incubated at room temperature, overnight. Next the membrane was washed in three changes of 10 mM TBS with 1% Tween 20 (TBST) and incubated in 1/3,000 alkaline phosphatase-conjugated, goat anti-mouse IGg secondary (Biorad) diluted in blotto for 1 hr at room temperature while shaking. Again, the membrane was washed in three changes of 10 mM TBST. The membrane was developed using a chemiluminescent signal (Biorad) following the manufacturer's instructions and developed on ECL hyperfilm

(Amersham, Piscataway, NJ). All films were digitized using a flat bed scanner.

Dot blots were used to accurately quantify ion transporter protein level differences among our treatments, and the methods of Joyner-Matos et al. (2006) were used. In short, gills were homogenized and centrifuged as described above. The supernatant was heated at 65°C for 15 min, then diluted to 2.5 μg μL^{-1} in 10 mM TBS, and continued heating at 65°C until blotted. Another randomly picked control sample was diluted (in 10 mM TBS) out in a series of two-fold dilutions to make an eight-point dilution curve. Proteins were blotted in 1 μL dots (thus 2.5 μg of protein), in triplicate onto dry nitrocellulose membrane (Millipore, Billerica, MA), and left to air dry for 10–20 min. Next, the membrane was placed in blotto and followed the above-mentioned western incubation protocol. The developed filmed was digitized using a flat bed scanner, and dot density determined using Biorad's Quantity One software. All values were standardized to the dilution curve and made relative to protein content (relative units mg protein^{-1}).

Antibodies

Monoclonal, anti-chicken NKA (α5, 1/1,000) was developed by Dr. D. Fambrough and monoclonal, anti-human NKCC1 (T4, 1/500) was developed by Drs. Lytle and B. Forbush III and were obtained from the Developmental Studies Hybridoma Bank, which was developed under the auspices of the National Institute of Child Health and Human Development of the University of Iowa, Department of Biological Sciences, Iowa City, IA. Monoclonal anti-human CFTR (1/500) (R&D Systems, Minneapolis, MN) was made against the C-terminal of human CFTR and is ~61% identical to teleost CFTR (Singer et al., '98; Katoh and Kaneko, 2003). All antibodies were diluted in BPB.

Gill NKA activity

NKA activity was measured using an NADH-linked, spectrophotometric microassay, similar to the one developed by McCormick ('93). Briefly, gills were homogenized on ice in 125 μL of 5 × SEID buffer (250 mM sucrose, 10 mM Na_2EDTA, 50 mM imidazole, 0.05% deoxycholic acid; pH = 7.3) and 1 mL of 1 × SEID (diluted in SEI: 250 mM sucrose, 10 mM Na_2EDTA, 50 mM imidazole). Samples were centrifuged at 3,000*g* for 30 sec at 4°C to remove any large particulates. The protein content of the supernatant was determined using Pierce's BCA protein assay. In a 96-well microplate, 10 μL of the supernatants was added to 200 μL of reaction mixture (80 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 50 mM imidazole, 3 mM ATP, 2 mM phosphoenol pyruvate, 0.2 mM NADH, 3.1 U mL^{-1} lactic dehydrogenase, 3.84 U mL^{-1} pyruvate kinase) either with or without 1 mM ouabain. All samples were run in triplicate. The plate was read every 10 sec, for a total of 20 min, at 25°C on a Biorad benchmark plus microplate reader (340 nm). An ADP standard curve was also run for every lot of reaction mixture to determine the extinction coefficient for the ADP-dependent conversion of NADH to NAD^+ (used in our final calculation of NKA activity).

The difference in the slopes (the rate of [NADH] reduction) between the nonouabain and ouabain wells was calculated for each sample. These values were standardized to the ADP standard curve and normalized to total protein content per sample (μmol ADP mg protein^{-1} hr^{-1}) (McCormick, '93).

Statistics

Plasma chemistry and gill NKA activity data are displayed as mean ± SEM. All other data were made relative to the mean sham value for each time and are displayed as relative mean ± SEM. With the plasma, qRT-PCR, and dot blot data, statistical differences among the treatments were assessed by two-factor analyses of variance (for salinity and time), followed by Bonferroni's post hoc test to determine differences compared with sham treatments (100% SW). The 10 and 20% SW experiments were not run concurrently (see above); however, the shams within each time point (24 or 72 hr) did not differ significantly; thus, they were combined in the 100% sham mean that is reported ($n = 6$). Statistical significance was set at $\alpha = 0.05$. All statistics were run using SPSS (v.15, Chicago, IL).

RESULTS

Plasma chemistry and gill NKA activity

Total plasma osmolality, sodium, potassium, and chloride concentrations did not differ between the 24- and 72-hr 100% SW (sham, $n = 6$) treatments ($n = 5$, Table 2). These parameters were not different between the sham and 20% SW at 24 or 72 hr; however, plasma osmolality and sodium were 14% lower after 24-hr acclimation to 10% SW ($P < 0.001$) and 22% lower after 72-hr compared with sham ($P < 0.001$). Plasma potassium

TABLE 2. Plasma parameters and gill Na^+, K^+-ATPase for longhorn sculpin acclimated to 100% SW (n = 6), 20% SW (n = 5), or 10% SW (n = 5) for 24 or 72 hr

	24 hr			72 hr		
	100% SW	20% SW	10% SW	100% SW	20% SW	10% SW
Plasma osmolality ($mOsm\,kg^{-1}$)	340.0 (3.2)	331.6 (7.1)	295.2 (8.5)*	329.5 (7.4)	317.3 (10.1)	259.1 (4.7)*
Plasma [Na^+] ($mOsm\,L^{-1}$)	178.2 (6.38)	162.7 (5.4)*	152.8 (4.2)*	174.5 (3.5)	171.7 (4.1)	134.1 (2.7)*
Plasma [K^+] ($mOSm\,L^{-1}$)	4.2 (0.2)	3.6 (0.4)	3.1 (0.4)*	4.1 (0.4)	3.4 (0.1)	3.1 (0.2)*
Plasma [Cl^-] ($mEquiv\,L^{-1}$)	166.1 (2.9)	158.0 (4.8)	133.7 (3.8)*	160 (5.8)	159.3 (5.0)	118.0 (4.2)*
Gill Na^+, K^+-ATPase activity ($\mu mol\,ADP\,mg\,protein^{-1}\,hr^{-1}$)	8.0 (0.66)	8.2 (0.7)	8.3 (0.5)	9.3 (1.51)	8.12 (0.7)	8.5 (1.0)

Means (SEM) are recorded. Asterisks represent significant differences from 100% SW within 24- or 72-hr treatments. SW, seawater.

decreased by 24% with 24-hr acclimation to 10% SW ($P = 0.009$) and this decrease was maintained at 72 hr. Chloride also decreased by 20 and 27% with 24- and 72-hr acclimation to 10% SW, respectively ($P < 0.001$). Longhorn sculpin acclimated to 10 or 20% SW for 24 or 72 hr did not have a significant change in gill NKA activity compared with sham values (Table 2).

Immunolocalization of CFTR, NKA, and NKCC1

Longhorn sculpin gills from all of the treatments were immunopositive for CFTR, NKA, and NKCC1 (Fig. 1). Epithelial cells in the interlamellar region were immunopositive for all three transporters. CFTR was found on the apical membrane, as indicated by a small, brown dot near to the edge of the epithelial cells (Fig. 1A, D, G). NKA and NKCC1 were immunolocalized throughout the cell, representing staining of the large, infolded basolateral membrane of MRCs (Karnaky et al., '76) (Fig. 1B, C, E, F, H). Longhorn sculpin that were acclimated to 20% (data not shown) or 10% SW for 24 hr (Fig. 1D–F) had similar immunostaining patterns as the shams (Fig. 1A–C). These immunoreactive patterns were also found with longhorn sculpin acclimated to 20% (data not shown) or 10% SW for 72 hr. Gills from longhorn sculpin that were acclimated to 10% SW for 72 hr were single-labeled for CFTR (Fig. 1G) and then the adjacent section (7 μm further) was double-labeled with CFTR and NKA (Fig. 1H). As shown in Figure 1G and H, CFTR and NKA were immunolocalized to the same epithelial cells, with CFTR staining the apical membrane and NKA found on the basolateral membrane. Likewise, this double labeling was repeated using CFTR and NKCC1, and again CFTR and NKCC colocalized to the same epithelial cell (data not shown).

Westerns and dot blots

The CFTR, NKA, and NKCC1 antibodies used in our western blot experiments yielded bands of the expected molecular weights (Fig. 1I). As seen in Figure 1I, a single CFTR band of ~140 kDa and a single NKA band of ~120 kDa were found in the sham sculpin gill. With the anti-NKCC1 antibody, we found two bands of ~200 and ~130 kDa in the longhorn sculpin gill. Because we found only single (CFTR, NKA) or double bands (NKCC1) with our western blots, and these findings mirror those from other teleosts (Tipsmark et al., 2002; Hiroi and McCormick, 2007), we quantified protein differences with dot blots (total protein, not proteins separated by molecular weight).

The sham treatments for 24 and 72 hr were not statistically different from each other for CFTR, NKA, or NKCC protein levels. We did not find any significant changes in gill CFTR or NKA protein level with acclimation to 10 or 20% SW for 24 or 72 hr compared with their respective sham treatments (Fig. 2A, C). There was a significant 2.5-fold increase in NKCC1 protein level with 24-hr acclimation to 20% SW ($P < 0.001$) (Fig. 2E); however, there were no statistically significant changes in NKCC1 protein level within the 72-hr treatment.

Quantitative real-time PCR

Gill *L8* mRNA levels did not change during acclimation to 10 or 20% SW for 24 ($P = 0.552$) or 72 hr ($P = 0.651$, Fig. 3). Sham treatments for 24 and 72 hr were not significantly different for

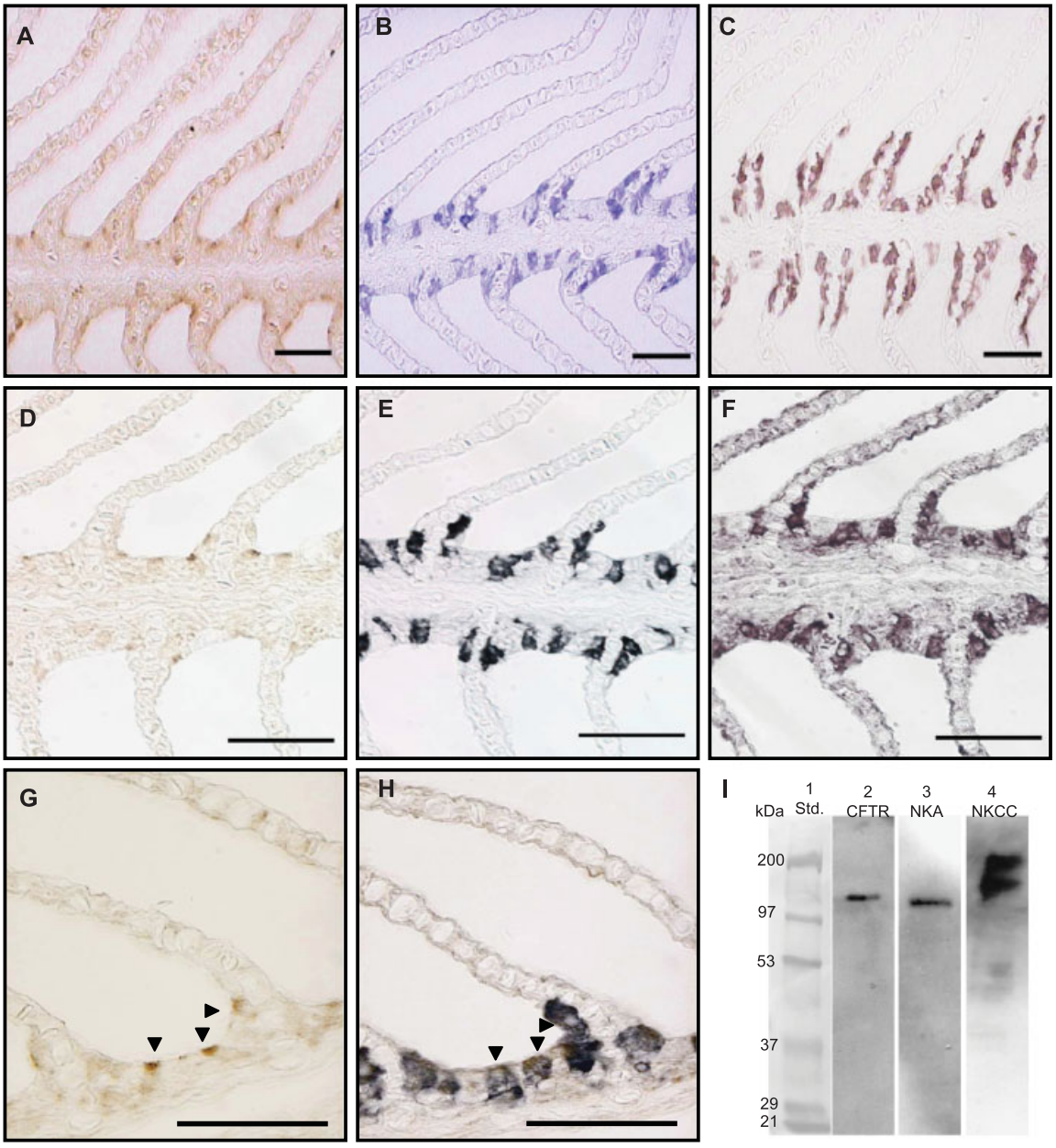

Fig. 1. Representative light micrographs of the immunolocalization of the cystic fibrosis transmembrane conductance regulator (CFTR; **A**, **D**, **G**), Na^+, K^+-ATPase (NKA; **B** and **E**), and Na^+, K^+, $2Cl^-$ cotransporter (NKCC1; **C** and **F**). The gills are from longhorn sculpin acclimated to: 100% SW for 72 hr (**A–C**), 10% SW for 24 hr (**D–F**), and 10% SW for 72 hr (**G** and **H**). CFTR was immunolocalized to the apical membrane, and NKA and NKCC1 to the basolateral membrane of epithelial cells of the interlamellar region. (**H**) is the next serial section of gill (7 μm deeper) from (**G**) and is double-labeled with anti-CFTR, indicated by the arrows (brown, apical membrane), and anti-NKA (blue, basolateral membrane), and shows that CFTR and NKA are expressed in the same epithelial cells. (**I**) Western blots of longhorn sculpin gills acclimated to 100% SW. The first lane is the molecular mass ladder (std), second lane is a blot incubated with anti-CFTR, third lane is a blot incubated in anti-NKA, and fourth lane is a blot incubated with anti-NKCC. SW, seawater. Scale bar = 50μm (micrometer).

CFTR, *NKA*, or *NKCC1*. Gill *NKCC1* and *CFTR* mRNA levels did not significantly change with acclimation to 10 or 20% SW for 24 or 72 hr (Fig. 2B, F). Longhorn sculpin gill *NKA* mRNA levels were not different from sham with 24-hr acclimation to 20% SW (Fig. 2D); however, they did increase 2.6-fold after 24-hr acclimation to 10% SW ($P = 0.001$). After 72 hr, gill *NKA* mRNA levels were 2.2-fold higher in the 10% SW treatment compared with sham ($P = 0.004$) (Fig. 2D).

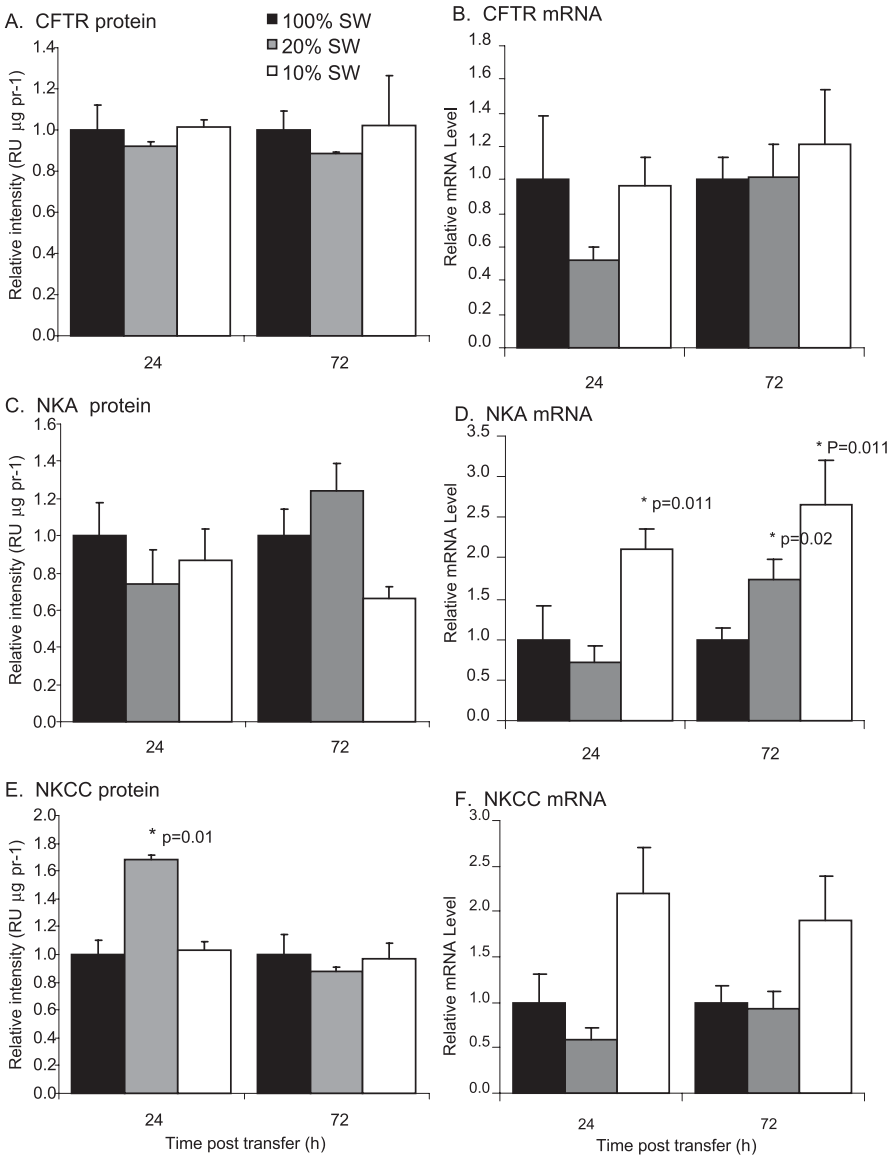

Fig. 2. Longhorn sculpin gill CFTR, NKA, and NKCC1 expression levels following acclimation to 100% SW ($n = 6$), 20% SW ($n = 5$), or 10% SW ($n = 5$) for 24 and 72 hr. (**A**, **C**, **E**) are protein levels determined from immunoblots (dot blots). (**B**, **D**, **F**) mRNA levels determined by quantitative real-time PCR. mRNA values are normalized to *L8* mRNA levels. All values are relative to the mean sham (100% SW) level at 24 or 72 hr and are mean ± SEM. Asterisks represent statistically significant differences compared with the sham value at 24 or 72 hr (P values are listed on the graph). CFTR, cystic fibrosis transmembrane conductance regulator; NKA, Na^+, K^+-ATPase; NKCC1, Na^+, K^+, $2Cl^-$ cotransporter; PCR, polymerase chain reaction; SW, seawater.

DISCUSSION

This study is the first to examine the effects of low salinity on SW osmoregulatory ion transporters, from a marine teleost, in order to determine why they are incapable of inhabiting FW environments. In the wild, longhorn sculpin have been found in estuaries during high tides suggesting that they have some low-salinity tolerance (Bigelow and Schroeder, 2002). We determined that acclimation to 20% SW for 24 or 72 hr did not elicit any significant changes in plasma osmolality or ion concentration (Table 2), but acclimation to 10% SW resulted in a significant loss of ions. This suggests that down to 20% SW, longhorn sculpin can regulate plasma ion concentrations. Claiborne et al. ('94) determined that longhorn sculpin could not survive past 60 hr in 8% SW or 48 hr in 4% SW;

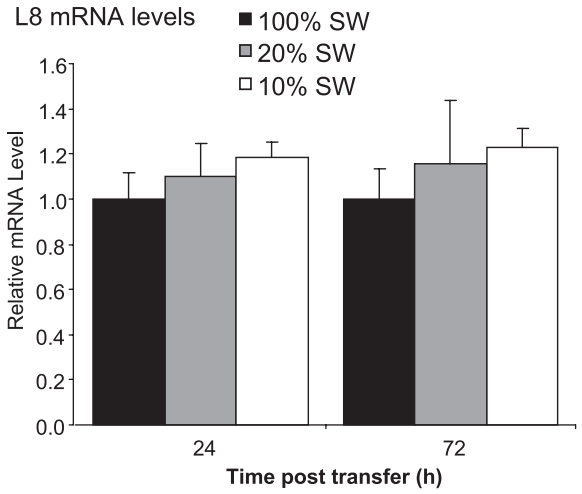

Fig. 3. Longhorn sculpin gill *L8* mRNA expression levels following acclimation to 100% SW ($n = 6$), 20% SW ($n = 5$), or 10% SW ($n = 5$) for 24 and 72 hr. Mean ± SEM. SW, seawater.

thus, we conclude that longhorn sculpin can tolerate salinities down to 8–10% SW for days, but salinities below this level are lethal within a few days. We propose that longhorn sculpin are missing the mechanism that allows euryhaline (or anadromous/catadromous) fishes to survive in fresh and marine environments—proper regulation of gill ion transporter densities. Longhorn sculpin do express CFTR, NKA, and NKCC1 in epithelial cells that match the morphology of the MRCs (e.g. Katoh and Kaneko, 2003; Hiroi and McCormick, 2007). Recently, Catches et al. (2006) immunolocalized NKA in the basolateral membrane of the MRC of the SW longhorn sculpin gill. We determined that CFTR and NKCC1 are also expressed on the MRC as was determined for other teleosts (Katoh and Kaneko, 2003; e.g. Pelis et al., 2001; Hiroi and McCormick, 2007). Presented here, acclimation to 20% did not affect gill CFTR or NKA protein levels or immunolocalization of these proteins in the longhorn sculpin gill (Figs. 1 and 2). There was a significant increase in NKCC1 protein level after 24-hr acclimation to 20% SW, but the NKCC1 protein level was not significantly different from sham at 72 hr. The increase in NKCC1 protein level while the longhorn sculpin were acclimating to a hypoosmotic environment is puzzling, because it is well documented that NKCC1 is stimulated by cell shrinking (as it occurs during acclimation to marine environments) and is involved in volume regulation in teleosts (see a recent review in Hoffmann et al., 2007). Interestingly, we did not find a significant increase in *NKCC1* mRNA at 24 hr compared with sham. Unfortunately, the time lag between de novo mRNA production and de novo protein production is not known for these transporters; therefore, it is plausible that there was an increase in *NKCC1* mRNA hours before our 24-hr sampling resulting in more NKCC1 protein at 24 hr. It may also be that there are posttranscriptional modifications occurring, resulting in a change of protein without a change in mRNA for NKCC1. In any event, these longhorn sculpin were able to maintain a plasma osmolality of $\sim$330 mmol kg^{-1} (this is within the normal range for euryhaline and stenohaline marine species; see Evans et al., 2005) during these experiments, without any obvious changes in CFTR and NKA ion transporter density, NKA activity, or localization of all three proteins in the gill.

Unlike acclimation to 20% SW, longhorn sculpin acclimated to 10% SW suffered a significant loss of ions. Euryhaline, catadromous, or anadromous teleosts that experience changes in environmental salinity regulate gill ion transporters to maintain proper ion balance. For example, the euryhaline killifish (*F. heteroclitus*) downregulates CFTR and NKCC1 mRNA and protein levels within a day or two of entering FW (Katoh and Kaneko, 2003; Choe et al., 2006) resulting in a conservation of ions. Killifishes that were transferred from low (0.1 ppt) to high salinity (35 ppt) increased NKA activity 3- and 72-hr posttransfer (Mancera and McCormick, 2000) to help excrete excess ions. Unlike the killifish, the longhorn sculpin did not downregulate CFTR, NKCC1, or NKA during acclimation to 10% SW, and subsequently suffered a significant loss of ions at 24 and 72 hr (Table 2). *NKA* mRNA levels were higher than shams at 24 and 72 hr in the 10% SW treatments, but there was no obvious change in NKA protein level or immunolocalization. Collectively, this could again suggest differences in time lag between de novo production of mRNA and protein. We did not find an increase in NKA activity level either (Table 2); therefore, this observed increase in NKA mRNA is intriguing. An alternative hypothesis is that there is high NKA protein turnover; therefore, to maintain constant NKA protein levels an increase in *NKA* mRNA would be required. This hypothesis has been proposed to explain high increases in *carbamoyl phosphate synthetase III* (*CPSase III*) mRNA in the Gulf toadfish (*Opsanus beta*) to maintain a constant CPSase III activity level during ureagenesis in this fish (Kong et al.,

2000). This may also be occurring with the NKA. Unlike euryhaline fishes such as the killifish, the longhorn sculpin does not downregulate gill SW osmoregulatory ion transporter densities in dilute environments. Recently, a study determined that landlocked, FW populations of Atlantic salmon (*S. salar*) were not capable of upregulating NKA, NKCC1, and CFTR during smoltification, as was observed in anadromous salmon (Nilsen et al., 2007). Likewise, landlocked Arctic char (*Salvelinus alpinus*) were incapable of maintaining ion homeostasis during hyperosmotic stress and were incapable of upregulating NKA (specifically NKAα1b subunit) (Bystriansky et al., 2007). This is likely because these landlocked fishes have completed many generations in only FW and have adapted to an FW existence (Bystriansky et al., 2007). To the best of our knowledge, we are the first to determine physiologically why a marine fish is incapable of surviving in low-salinity environments.

Although the gill plays an integral role in regulating salt homeostasis, we cannot disregard the role of the kidney in osmoregulation. In FW, teleost kidneys function to reabsorb salts and excrete excess water (see a recent review in Beyenbach, 2004). Thus, it is plausible that another restriction of marine teleosts from FW environments is owing to limitations in kidney function. This hypothesis warrants further investigation. In addition, future experiments should determine the effect of low salinity on FW osmoregulatory proteins, such as the Cl^-/HCO_3^-, V–H^+-ATPase, or NHEs. Catches et al. (2006) determined that NHE-2 is expressed in the apical membrane of MRCs and V–H^+-ATPase in the basolateral membrane of the SW longhorn sculpin gill. This suggests that the machinery involved in ion transport uptake may be present in the sculpin; however, whether it is expressed properly in the MRC to drive Na^+ absorption and/or upregulated during low-salinity exposure remains to be determined.

The exclusion of longhorn sculpin from FW is intriguing. There are records of them entering estuaries during high tide (Bigelow and Schroeder, 2002), and the salinity of this environment during this time is likely higher because of the tide. This is probably less of an osmotic challenge than entering during low tide or entering near the FW source, and we have determined that down to 20% SW there is no obvious detriment to the fish. Because longhorn sculpin are incapable of properly regulating ion transporter densities in the gill below 20% SW, they suffer a net loss of ions eventually to a level that is lethal for the fish. There are ''FW'' *Myoxocephalus*: *M. polyacanthocephalus*, *M. sinensis*, and *M. thompsonii* (Froese and Pauly, 2000). *M. polyacanthocephalus* is amphidromous, spending a portion of its lifecycle in FW, and is distributed in the North Pacific; *M. sinensis* is a demersal-FW species found in China; and *M. thompsonii* is also a demersal, FW species distributed from the St. Lawrence River to the Arctic (Froese and Pauly, 2000). Within the sculpin, there are three other genera that have FW species: *Cottus*, *Trachidermus*, and *Messocottus*. The genus *Cottus* is a group of 57 species of FW sculpin (Froese and Pauly, 2000) that diverged from the marine sculpin approximately 2–5 MYA (Yokoyama and Goto, 2005). It seems plausible that the marine ancestor(s) to the FW sculpin had the ability to regulate gill ion transporter density and or activity, and this lead to their invasion of FW habitats. To help elucidate this question, a complete sculpin phylogeny, mapping habitat use to the different species, would be helpful in understanding the evolution of this group of fishes. A portion of the *Myoxocephalus* phylogeny has been completed using 7/17 species, and it depicts two distinct groups of the *Myoxocephalus*: Arctic–Atlantic and Pacific groups (Kontula and Vainola, 2003). Both groups contain ''FW'' representatives, suggesting that there were independent invasions of FW by *Myoxocephalus*.

In conclusion, the *Myoxocephalus* is an interesting group to test mechanistic questions to help understand habitat invasion and use. The longhorn sculpin can tolerate short-term exposure to low-salinity water ($<10\%$ SW) for days but not much longer, because they cannot regulate ion transporter density or activity, resulting in a significant loss of ions (eventually to a lethal level). The diverse habitat use of the species of *Myoxocephalus* makes it an excellent model to complete comparative studies to explore the relationship between environmental salinity and gill ion transporter density, furthering our fundamental knowledge of the mechanisms and evolution of salinity tolerance.

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