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RESEARCH ARTICLE

Branchial ionocyte organization and ion-transport protein expression in juvenile alewives acclimated to freshwater or seawater

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SUMMARY

The alewife (*Alosa pseudoharengus*) is a clupeid that undergoes larval and juvenile development in freshwater preceding marine habitation. The purpose of this study was to investigate osmoregulatory mechanisms in alewives that permit homeostasis in different salinities. To this end, we measured physiological, branchial biochemical and cellular responses in juvenile alewives acclimated to freshwater (0.5 p.p.t.) or seawater (35.0 p.p.t.). Plasma chloride concentration was higher in seawater-acclimated than freshwater-acclimated individuals (141 mmol l^{-1} *vs* 134 mmol l^{-1}), but the hematocrit remained unchanged. In seawater-acclimated individuals, branchial Na^+/K^+-ATPase (NKA) activity was higher by 75%. Western blot analysis indicated that the abundance of the NKA α-subunit and a $Na^+/K^+/2Cl^-$ cotransporter (NKCC1) were greater in seawater-acclimated individuals by 40% and 200%, respectively. NKA and NKCC1 were localized on the basolateral surface and tubular network of ionocytes in both acclimation groups. Immunohistochemical labeling for the cystic fibrosis transmembrane conductance regulator (CFTR) was restricted to the apical crypt of ionocytes in seawater-acclimated individuals, whereas sodium/hydrogen exchanger 3 (NHE3) labeling was present on the apical surface of ionocytes in both acclimation groups. Ionocytes were concentrated on the trailing edge of the gill filament, evenly distributed along the proximal 75% of the filamental axis and reduced distally. Ionocyte size and number on the gill filament were not affected by salinity; however, the number of lamellar ionocytes was significantly lower in seawater-acclimated fish. Confocal *z*-series reconstructions revealed that mature ionocytes in seawater-acclimated alewives occurred in multicellular complexes. These complexes might reduce paracellular Na^+ resistance, hence facilitating Na^+ extrusion in hypo-osmoregulating juvenile alewives after seaward migration.

Key words: *Alosa pseudoharengus*, alewife, ion, Na^+/K^+-ATPase, NKA, NKCC1, CFTR, NHE3.

INTRODUCTION

The alewife (*Alosa pseudoharengus*, Wilson 1811) is an anadromous clupeid that inhabits the coastal waters of eastern North America from Labrador to South Carolina and also exists as landlocked populations, most notably in the Laurentian Great Lakes (Berry, 1964; Scott and Crossman, 1973). The majority of the anadromous alewife life history, as maturing juveniles and adults, is characterized by marine habitation along the continental shelf of the Atlantic coast (Neves, 1981). Marine habitation is punctuated by annual spawning runs, which occur from late March to early July, when adults migrate up coastal rivers to spawn in streams and ponds (Fay et al., 1983). Young alewives develop in freshwater nursery grounds for several months before descending as young-of-the-year (YOY) juveniles to estuarine, and ultimately marine, habitats (Bigelow and Schroeder, 1953; Richkus, 1975).

Anadromy entails ion homeostasis in the face of varied environmental salinities. In freshwater, diffusive ion loss and water gain are counteracted by active ion uptake and retention, and dilute urine production. Conversely, in seawater, diffusive ion gain and water loss are countered by active ion extrusion and increased water consumption. The primary sites of ion flux and water balance in fish are kidney glomeruli and tubules, the intestinal epithelium and the gill epithelium (Evans et al., 2005; Marshall and Grosell, 2006). Within the gill, close proximity between the blood and the aqueous environment facilitates the exchange of ions to maintain blood osmolality and pH. The transfer of ions is driven by highly specialized cells of the branchial epithelium called ionocytes [also known as chloride cells and mitochondrion-rich cells (reviewed in Evans et al., 2005)]. The ionocyte basolateral, or serosal, membrane is contiguous with a convoluted labyrinth of invaginations, called the tubular network, which amplifies the membrane surface area (Karnaky et al., 1976; Hootman and Philpott, 1979). The ionocyte apical membrane contacts the external environment, is isolated from the basolateral membrane by tight junctions and is morphologically distinct in freshwater and seawater (Pisam and Rambourg, 1991). In freshwater, the apical pit has microvillous projections, whereas in seawater the depth of the crypt is pronounced and is frequently shared with accessory cells (Sardet et al., 1979; Laurent and Dunel, 1980; Hootman and Philpott, 1980). Compared with ionocytes, accessory cells are different in that they have a lower abundance of Na^+/K^+-ATPase (referred to hereafter as NKA) and are ultrastructurally distinct; however, they are similar in that they are rich in mitochondria and have a tubular network. The tight junctions between an ionocyte and accessory cell are shallow and might represent a low-resistance pathway for Na^+ extrusion in seawater (Silva et al., 1977; Zadunaisky et al., 1995; Towle, 1990).

In the freshwater gill, Cl^- uptake is thought to be coupled to HCO_3^- efflux through an anion exchanger. There are three predominant models for Na^+ uptake. In the first model, an apical sodium/hydrogen exchanger (NHE3) mediates an electroneutral exchange of cytosolic H^+ for environmental Na^+ and has been implicated in both ion uptake and acid secretion. In the second model, passive Na^+ influx through an apical channel is driven by an electrogenic apical V-type H^+-ATPase. A more recent model for Na^+ import has emerged wherein an apical sodium/chloride cotransporter (NCC) mediates an electroneutral import of Na^+ and Cl^-. Which mechanism predominates appears to be specific to the environment, species and, in some cases, subtype of ionocyte (Evans et al., 2005; Marshall and Grosell, 2006; Wilson, 2007; Evans, 2010; Hwang and Lee, 2007). The subsequent transfer of intracellular Na^+ into the serum is thought to occur through basolateral NKA, which couples the energy of ATP hydrolysis to the exchange of three Na^+ intracellular ions for two K^+ extracellular ions (McCormick, 1995).

In the seawater gill, the basis for ion secretion is attributable, in large part, to the concerted effort of three major ion-transport proteins: NKA, NKCC1 and cystic fibrosis transmembrane conductance regulator (CFTR). The primary driving force for ion secretion in ionocytes is the NKA, which maintains the low intracellular Na^+ required for secondary import of Na^+, K^+ and two Cl^- ions by NKCC1. Elevated intracellular Cl^- is subsequently extruded through an apical anion channel, CFTR. Intracellular Na^+ is cycled back out of the cell by basolateral NKA; Na^+ ions are then secreted down an apical electrical gradient generated by CFTR, into the environment though shallow tight junctions between ionocytes and accessory cells (McCormick, 1995; Evans et al., 2005; Marshall and Grosell, 2006; Hwang and Lee, 2007). An apical crypt in ionocytes, or shared between an ionocyte and accessory cells, might constitute a cellular vessel promoting localized Cl^- and Na^+ extrusion in seawater.

Most of our understanding of how anadromous fishes regulate gill ion-transport proteins has come from work on salmonids, which are thought to originate from a freshwater ancestor (Stearly, 1992). Relatively few studies have been conducted on how other anadromous species, particularly those of primarily marine taxa such as Clupeidae, regulate gill ion-transport proteins. In a preliminary study, alewives had higher brachial NKA activity in seawater when compared with individuals in seawater (McCormick et al., 1997). Notably, studies of changes in osmoregulatory competence and NKA activity in migrating juvenile shad (*Alosa sapidissima*, a congener of alewives) suggest that the physiological preparation for migration might differ between salmonids and anadromous clupeids (Zydlewski and McCormick, 1997a; Zydlewski and McCormick, 1997b; see also McCormick et al., 1997). The purpose of the present study was to investigate changes in alewife physiology and the branchial epithelium after acclimation to freshwater or seawater, and this is the first such examination of multiple major ion-transport proteins in a non-salmonid anadromous fish. We show that alewives acclimate to increasing salinity, as do most euryhaline fishes, by increasing the abundance of NKA, NKCC1 and CFTR in gill ionocytes. Unlike the majority of euryhaline species investigated thus far, we show that NHE3 remains unchanged. A further difference is that accurate quantification of branchial ionocyte abundance required transverse sectioning of the gill filaments, rather than sagittal sectioning. Finally, we demonstrate that mature ionocytes form multicellular arrangements of NKA-rich cells that share an apical crypt in seawater, presumably enhancing Na^+ extrusion.

MATERIALS AND METHODS

Animals and sampling protocols

Anadromous YOY alewives (*Alosa pseudoharengus*, Wilson 1811) were collected before their seaward migration from freshwater (Bride Lake, East Lyme, CT, USA) and transported to the Conte Anadromous Fish Research Center (Turners Falls, MA, USA) on 7 October 2008. Fish were housed for 2 weeks in 1.5 m tanks equipped with a recirculation system; because brackish water improves survival (Stanley and Colby, 1971) (A.K.C. and S.D.M., unpublished observations), they were maintained in 5.0 p.p.t. salinity. Fish were maintained under a simulated natural photoperiod, at a constant temperature of 15±1°C, with particle and charcoal filtration and supplemental aeration. Fish were hand-fed daily to satiation (Zeigler Bros, Gardners, PA, USA). Fish from this source population were used for acclimation experiments.

Forty fish (6–11 g) were divided equally into two test tanks for acclimation experiments. In one tank, salinity was maintained at 5.0 p.p.t. for 1 week and then quickly decreased to 0.5 p.p.t. (148 p.p.m. Na^+, 234 p.p.m. Cl^-, 35.1 p.p.m. Ca^{2+}, 21.1 p.p.m. Mg^{2+}, pH 7.9; Environmental Analysis Lab, University of Massachusetts Amherst, Amherst, MA). In the second tank, salinity was maintained at 26.3 p.p.t. for 1 week and then quickly elevated to 35.1 p.p.t. (pH 8.3), characteristic of seawater. Fish were held in freshwater or seawater for more than 2 weeks (30 October to 14 November). One half of the water volume was exchanged with newly prepared freshwater or seawater after one week. Photoperiod, temperature, filtration and aeration conditions were consistent with those in the rearing tanks. Fish were fed *ad libitum* once daily until one day before sampling, at which point food was withheld.

Fish were anesthetized in 200 mg l^{-1} buffered tricaine methanesulfonate (Argent, Redmond, WA, USA) prior to sampling, and blood was drawn from the caudal vessel with 1 ml heparinized hematocrit tubes. Sampled blood was then either drawn up into a heparinized capillary tube and centrifuged to measure hematocrit (12 fish per group) or transferred into a 1.5 ml tube and centrifuged at 3200 ***g*** for 5 min at 4°C. Plasma was stored at –80°C for measurement of chloride content (12 fish per group) with a digital chloridometer (Labconco, Kansas City, MO, USA). Eight gill arches were removed from each fish, and the tissue was trimmed from the ceratobranchials. Five gill pieces, consisting of four to six filaments each, were excised from the gill tissue. One piece was placed in 100 µl ice-cold SEI buffer (150 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} imidazole, pH 7.3) and stored at –80°C for measurement of NKA activity. The remaining pieces were fixed in 4% paraformaldehyde in 100 mmol l^{-1} phosphate buffer pH 7.4 for 2 h at room temperature. The tissue was rinsed in PBS (1.9 mmol l^{-1} NaH_2PO_4, 8.1 mmol l^{-1} Na_2HPO_4, 138 mmol l^{-1} NaCl, pH 7.4) and equilibrated in 30% sucrose–PBS overnight at 4°C. Tissue was embedded and frozen in Tissue-Tek OCT embedding medium (Sakura Finetek, Torrance, CA, USA) and stored at –80°C for immunohistochemistry. The remainder of the gill tissue was placed in a 1.5 ml tube, flash frozen on an insulated stainless-steel plate equilibrated at –80°C and stored at –80°C for western analysis.

NKA activity

Gill NKA activity (12 fish per group) was determined using the microplate method outlined by McCormick (McCormick, 1993). In this assay, ouabain-sensitive ATPase activity is measured by coupling the production of ADP to NADH using lactic dehydrogenase and pyruvate kinase, in the presence and absence of 0.5 mmol l^{-1} ouabain. Samples (10 µl) were run in duplicate in 96-well microplates at 25°C and read at a wavelength of 340 nm for

10 min on a THERMOmax microplate reader using SOFTmax software (Molecular Devices, Menlo Park, CA, USA). The protein concentration of the homogenate was determined using a BCA (bicinchoninic acid) Protein Assay (Pierce, Rockford, IL, USA).

Western blot analysis

Gill tissue was placed in 10 v/w ice-cold phosphate-buffered RIPA (10 mmol l^{-1} sodium phosphate, 150 mmol l^{-1} NaCl, 1.0% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4) with 1 mmol l^{-1} phenylmethylsulfonyl fluoride and Complete Mini (Roche, Indianapolis, IN, USA) protease inhibitors and mechanically homogenized. The homogenate was vortexed, incubated on ice for 30 min and centrifuged at 13,000 ***g*** for 10 min at 4°C to pellet insoluble material. The pellet was discarded, and the supernatant was subjected to a BCA assay of protein content. Soluble proteins were denatured by heating at 60°C for 15 min in 2× Laemmli reducing sample buffer and diluted to 1 μg μl^{-1} with sample buffer. Protein samples were aliquoted and stored at –80°C. A sample (10 μg) of protein was thawed (five to six fish per group), resolved on a single gel by SDS-PAGE and transferred to an Immobilon PVDF membrane (Millipore, Bedford, MA, USA) in transfer buffer (25 mmol l^{-1} Tris, 192 mmol l^{-1} glycine, 10% methanol, pH 8.3) at 30 V overnight. Following transfer, the membrane was rinsed in distilled water, dried at 37°C and wetted in 100% methanol. The wetted membrane was rinsed in distilled water and placed in blocking solution (PBS, 5% non-fat dry milk, 0.05% triton X-100) for 1 h at room temperature and rinsed three times in PBST (PBS, 0.05% triton X-100). The blocked membrane was incubated with primary antibody anti-NKA (α5; 0.08 μg ml^{-1}) or anti-NKCC (T4; 0.3 μg ml^{-1}), purchased from Developmental Studies Hybridoma Bank (Iowa City, IA, USA), in blocking solution for 1 h and rinsed as before. We made an attempt to identify V-ATPase in alewives using a polyclonal antibody raised against killifish V-ATPase; however, the signal we received was unconvincing. The membrane was then incubated with secondary antibody (1:10,000 horseradish-peroxidase-conjugated goat anti-mouse) in blocking solution for 1 h and rinsed five times in PBST. Chemiluminescence was induced by incubating the blots for 1 min in a 1:1 mixture of enhanced chemiluminescent solution A (ECL A; 396 μmol l^{-1} coumaric acid, 2.5 mmol l^{-1} luminol, 100 mmol l^{-1} Tris, pH 8.5) and ECL B (0.018% H_2O_2, 100 mmol l^{-1} Tris, pH 8.5). Blots were exposed to X-ray film (RPI, Mt Prospect, IL, USA) to record signal. Digital photographs were taken of exposures, and band intensity was measured from a single blot using ImageJ (NIH) with the cumulative 8-bit gray-scale value as a measure of protein abundance.

Immunohistochemistry

Gill tissue sections (25 μm thick) were cut along the transverse or sagittal filamental axis in a cryostat at –24°C. The tissue was placed on Fisherbrand Colorfrost/Plus slides (Fisher Scientific, Hampton, NH, USA), dried and rinsed with PBS. Slides were blocked in 2% normal goat serum in PBS, unless they were intended for labeling with anti-NKCC, in which case they were first subjected to antigen retrieval by heating in 10 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, pH 8.0 at 80°C for 20 min. Slides were incubated with primary antibodies at 4°C overnight. Monoclonal antibodies anti-NKA (α5) and anti-NKCC (T4) were used at of 0.8 μg ml^{-1} and 0.3 μg ml^{-1}, respectively, in antibody dilution buffer (PBS, 0.1% BSA, 2% normal goat serum, 0.02% keyhole limpet hemocyanin, 0.01% NaN_3). CFTR was detected with affinity-purified anti-CFTR polyclonal antibodies that were raised against the C-terminal 20 amino acids of Atlantic salmon (*Salmo salar*) CFTR II. NHE3 was detected with antibodies that were raised against two regions of rainbow trout (*Oncorhynchus mykiss*) NHE3b; these regions are highly conserved across teleost NHE3 sequences but are divergent from corresponding NHE2 sequences. Polyclonal antibodies anti-CFTR and anti-NHE3 were used at dilutions of 1:2000 and 1:1000, respectively, in antibody dilution buffer. After incubation with primary antibodies, slides were rinsed three times with PBST and incubated with Alexa-Fluor-conjugated secondary antibodies (Invitrogen Molecular Probes, Carlsbad, CA, USA) at a dilution of 1:200, and TO-PRO-3 (Invitrogen) nuclear stain at a dilution of 1:1000, in antibody dilution buffer for 2 h at room temperature. The slides were rinsed five times with PBST and cover-slipped in mounting media (50% glycerol, 10 mmol l^{-1} Tris, pH 8.0). Slides were examined with a Zeiss Leica Scanning Microscope 510, and representative confocal images were collected. As a negative control, slides were run excluding the primary antibodies during the overnight incubation step.

Ionocyte enumeration was conducted using anti-NKA as a marker (Witters et al., 1996). Tissue preparation was performed as above with the following exceptions: tissue was sectioned to 5 μm, anti-NKA was used at 0.08 μg ml^{-1} and secondary antibodies were used at 1:1500. Slides were examined with a Nikon inverted fluorescent microscope with a mercury lamp. We evaluated the number, and within-filament distribution, of ionocytes by counting cells in transverse tissue sections rather than sagittal sections because ionocytes are unevenly distributed across the filament in alewives. We assessed the along-filament ionocyte distribution from 14 filaments from sections collected along the filamental axis, from a single seawater-acclimated individual. We assessed the across-filament ionocyte distribution from nine filaments from sections collected approximately halfway along the length of the filamental axis, from a single seawater-acclimated individual. We assessed salinity-dependent differences in ionocyte cell number by recording ionocytes on the filament or lamellae from sections collected approximately halfway along the length of the filamental axis, from fish acclimated to freshwater or seawater (five fish per group). We also recorded ionocyte abundance on lamellae in terms of cell number per lamellar area (μm^2). For ionocyte size (μm^2 cell^{-1}), epifluorescent NKA signal area was measured with MetaMorph 4.1.2 software (Universal Imaging Corporation, Downingtown, PA, USA). A set threshold defined the NKA signal, and the nucleus was included in the total cell area. At least 50 immunoreactive ionocytes from several different tissue sections were analyzed from fish acclimated to freshwater or seawater (five fish per group). Lamellar area and ionocyte size were obtained using MetaMorph software. All exposure levels were consistent across conditions for each antibody.

Statistics

Differences in protein abundance and ATPase activity between freshwater- and seawater-acclimated fish were tested using one-way analysis of variance (ANOVA). Differences in ionocyte number and size were tested by two-way ANOVA, wherein treatments were acclimation salinity and location (filament or lamella). Along-filament and across-filament heterogeneity in ionocyte abundance was tested by one-way ANOVA. Across-filament values were ranked prior to one-way ANOVA. Tukey's HSD tests were subsequently performed to resolve significant differences. A test yielding a P-value of <0.05 was regarded as evidence for rejecting the null hypothesis of no effect. All parameters met test assumptions of normality and equality of variance.

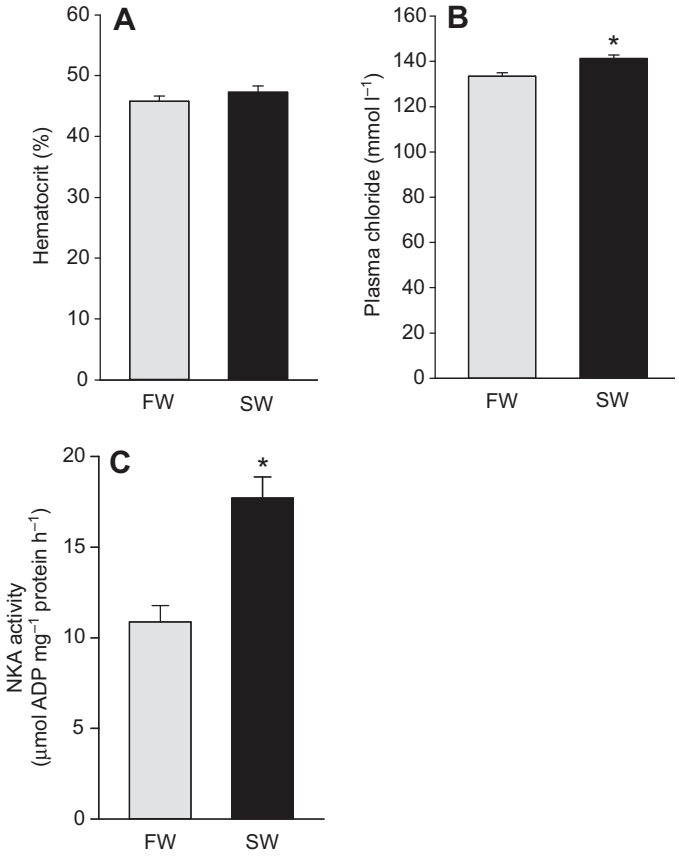


Fig. 1. Indices of osmoregulatory competence, measured in freshwater-acclimated (FW; 0.5‰) and seawater-acclimated (SW; 35‰) alewives. Values are means (+s.e.m.), 12 fish per group. Asterisks indicate significant differences between means (P<0.05; see text for details). (A) Hematocrit; (B) plasma chloride concentration; (C) gill Na^+/K^+-ATPase (NKA) activity.

RESULTS

Two weeks of acclimation affected plasma ion concentration and gill ion-transporter activity (Fig. 1). Acclimation salinity had no effect on hematocrit (Fig. 1). Plasma chloride concentration was 5% higher, and gill NKA activity was 75% higher, in seawater-acclimated fish than freshwater-acclimated fish (Fig. 1). During freshwater and seawater acclimation, there was no mortality, and there were no behavioral changes evident.

We used antibodies immunoreactive with α-subunits of NKA (α5 antibody) and NCC/NKCC1/NKCC2 (T4 antibody) to determine how salinity affected the abundance of these important gill ion-transporters. NKA was detected as a single band of 95 kDa, which was approximately 40% more intense in seawater-acclimated individuals (Fig. 2). T4 was immunoreactive with two prominent bands of approximately 120 and 240 kDa, and occasionally discrete bands of a higher molecular mass; the intensities of these bands were approximately 150% greater in seawater-acclimated individuals (Fig. 2).

Using the α5 antibody as an ionocyte marker, we determined that these cells were heterogeneously distributed over the filament. Across the filament, ionocytes were concentrated on the trailing (afferent blood flow) half (Fig. 3, Fig. 4A). Cells tallied from 14 filaments in a single individual (from seawater) were uniformly distributed along the proximal 75% of the filament length but were sparser towards the tips of the filaments (Fig. 4B).

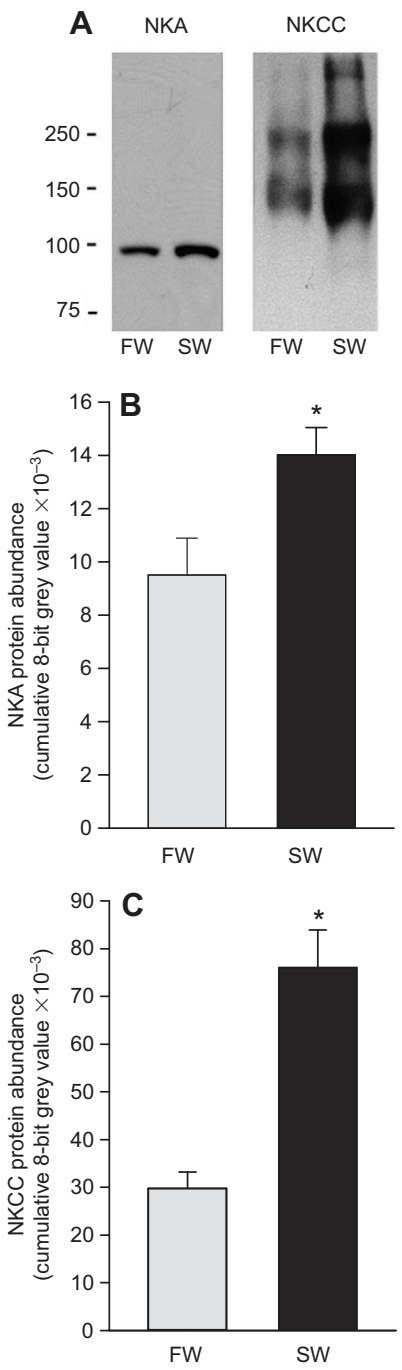


Fig. 2. Abundance of NKA and NKCC in gill tissue of freshwater-acclimated (FW) and seawater-acclimated (SW) alewives. (A) Images of western blots from single individuals chosen to represent band location and intensity. Numbers to the left of the western blot images represent molecular mass (kDa). (B,C) Values are means (+s.e.m.) of protein abundance for NKA (B) and NKCC (C), from five to six fish per group. Asterisks indicate significant differences between means (P<0.05; see text for details).

Acclimation to salinity influenced ionocyte location but not size. The abundance of ionocytes on filaments did not differ between groups. However, there were 80% fewer ionocytes on lamellae in seawater-acclimated fish, assessed in terms of number or number per unit area (Fig. 5A). Acclimation salinity, position on the filament or lamellae and interaction between salinity and position did not

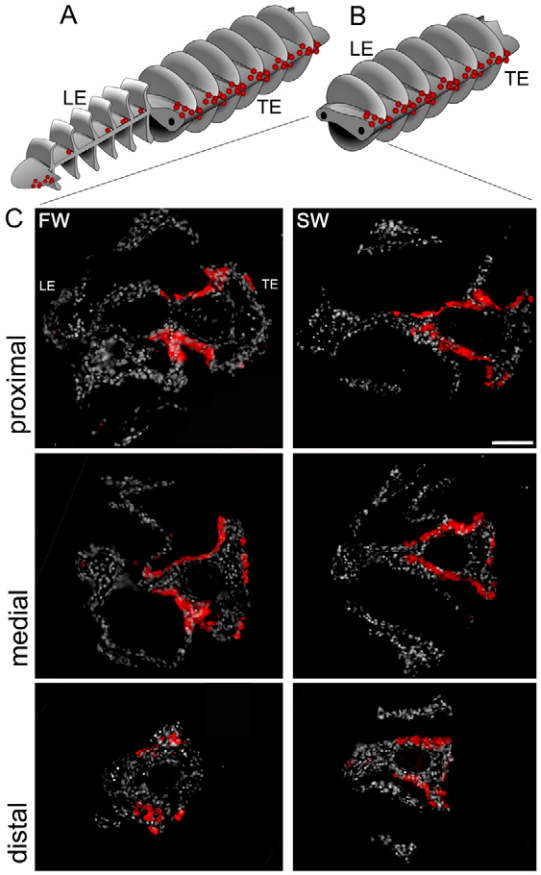


Fig. 3. Gill filament, lamellae and ionocytes in alewives. Diagrams indicate typical locations of ionocytes (red dots) and lamellae projecting above and below a gill filament, illustrating different section orientations used for localizing ionocytes by immunohistochemistry. LE, leading edge; TE, trailing edge. (A) Exposure of across-filament structure with a sagittal section. (B) Exposure of along-filament structure with a transverse section. (C) Distribution of ionocytes across a gill filament in freshwater-acclimated (FW) and seawater-acclimated (SW) alewives. Images are representative 5 μm transverse sections, taken from proximal, medial and distal portions of the filament. LE, leading edge; TE, trailing edge. DAPI staining of cells (gray) indicates the filament and lamellae; fluorescence of antibody (red) indicates the location of ionocytes. In some cases, adjacent filaments were cropped from the image for clarity. A ×25 objective was used; scale bar: 50 μm.

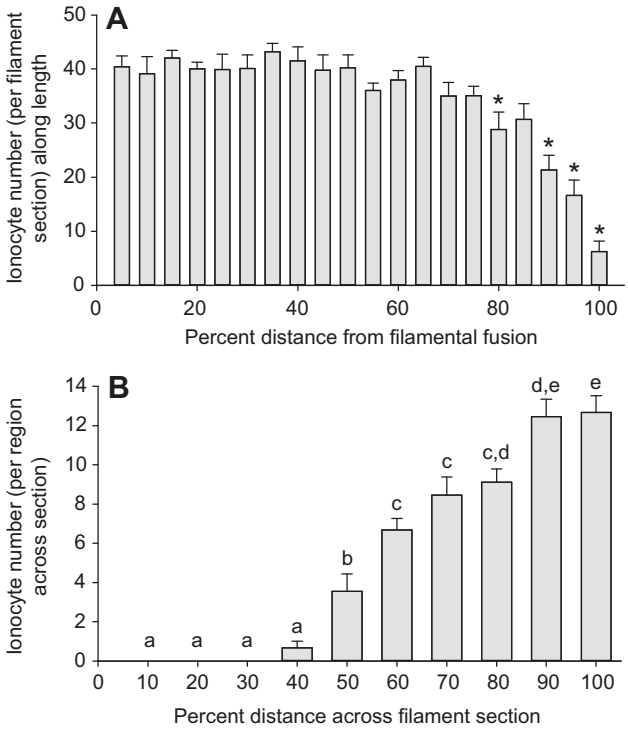


Fig. 4. Along- and across-filament ionocyte distribution in alewife gills. Ionocytes were counted in different sections of the same filament. Bars represent the mean number (+s.e.m.) of ionocytes by location. (A) Along-filament distribution of ionocytes. The ionocyte abundance in transverse sections of 14 filaments of a seawater-acclimated alewife is aggregated into position classes, defined by the proportional distance from the proximal to the distal end of the filament. Asterisks signify significantly different means from that of the origin, or 0–5% filament length (P<0.05; see text for details). (B) Across-filament distribution of ionocytes. Ionocyte abundance in nine sections of a single gill filament from each of five fish is aggregated into position classes, defined by the proportional distance from the leading to the trailing side of the filament. Different letters signify significantly different means (P<0.05; see text for details).

significantly affect ionocyte size, although larger cells tended to be more frequent in seawater (Fig. 5B,C).

The ion-transport proteins we analyzed were localized to different ionocyte membranes. NKA and NKCC1 localized to ionocyte basolateral and tubular network membranes (Fig. 6). CFTR signal was intense in approximately 60% of seawater ionocytes and localized to the circumference of apical crypts (Fig. 6). A low level of CFTR signal occasionally colocalized with the nuclear stain in freshwater and seawater cells (Fig. 6, transverse insets). This signal was not observed in negative controls; however, it is probably nonspecific in light of its unconventional cellular and subcellular localization. We observed NHE3 immunoreactivity in apical crypts of approximately 95% of freshwater and seawater ionocytes, and the intensity was not affected by salinity (Fig. 7). Similarly to CFTR, NHE3 was concentrated at the circumference of seawater ionocyte crypts (Fig. 7, inset).

Confocal microscopy revealed multicellular complexes of ionocytes. We assembled *z*-series confocal stacks from transverse filament sections that spanned an interlamellar and a lamellar surface. These reconstructions presented ionocytes in two orientations – filamental ionocytes were apical-side-up, whereas lamellar ionocytes were viewed *en face*. Ionocytes in freshwater were found on the filament and up the lamellar surface, and they were largely singular. Ionocytes in seawater were combined in dyads and triads with common crypts, filling the junction of filaments and lamellae (Fig. 8).

DISCUSSION

Surprisingly little is known of the biochemical and cellular mechanisms that underlie euryhalinity of alewives and other anadromous clupeids. One early study determined that serum proteins and chlorides were higher in migratory prespawning freshwater alewives compared with postspawning individuals in freshwater (Sindermann and Mairs, 1961). Subsequent studies examined the impacts of salinity on pituitary ultrastructure (Cook et al., 1973; Betchaku and Douglas, 1981) and branchial mucous cell histology (Cook et al., 1979). The present study brings to light important changes in ion-transport protein abundance and ionocyte organization during freshwater and seawater acclimation.

We first established that the alewives had successfully acclimated to freshwater or seawater according to three physiological indices

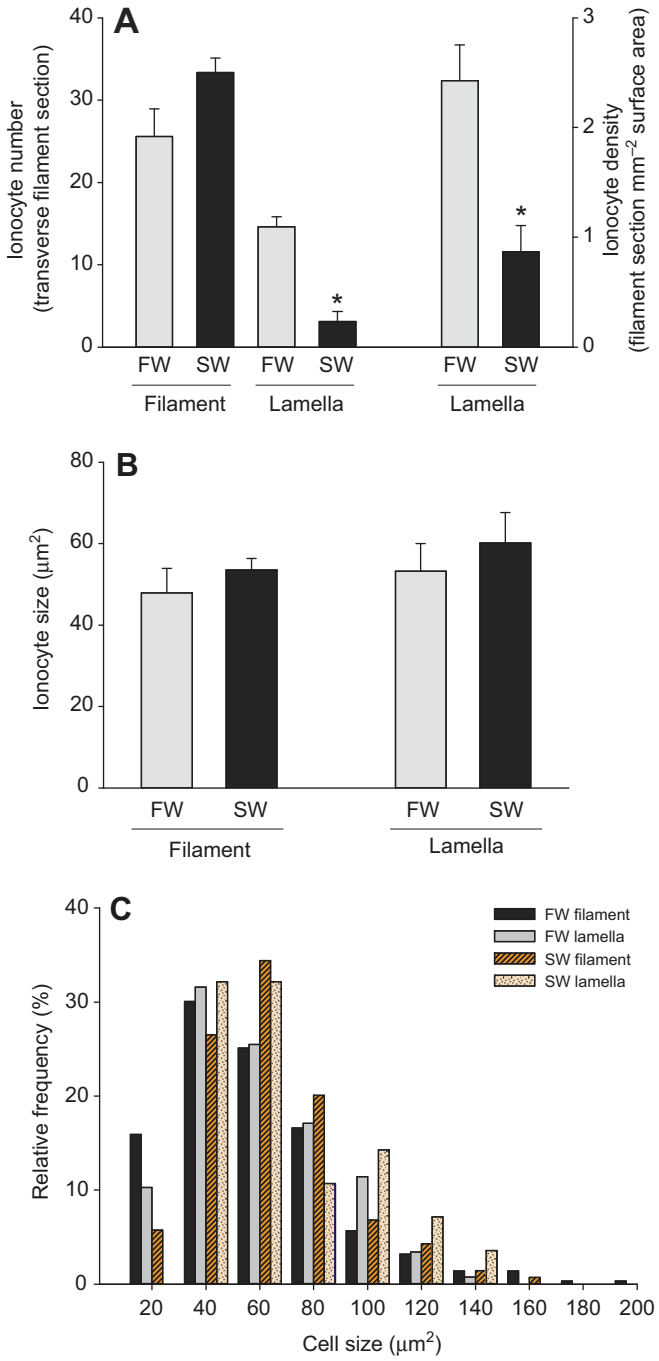


Fig. 5. Ionocyte number, location and size in gills of freshwater-acclimated (FW) and seawater-acclimated (SW) alewives. Bars represent means (+s.e.m.). Asterisks indicate that there is a significant (P<0.05) difference between means. (A) Ionocyte abundance. Seven transverse filament sections were taken from five freshwater-acclimated and five seawater-acclimated individuals. Bars represent ionocyte number on the filament and on lamellae per section (left axis) and ionocyte density on lamellae (right axis). (B) Ionocyte size. (C) Distribution of ionocyte cell size. Areas were measured from 250 ionocytes in total, from five individuals per group.

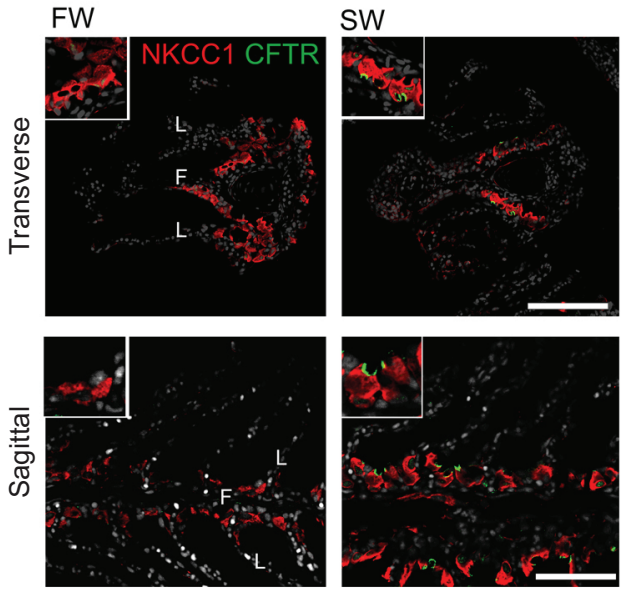
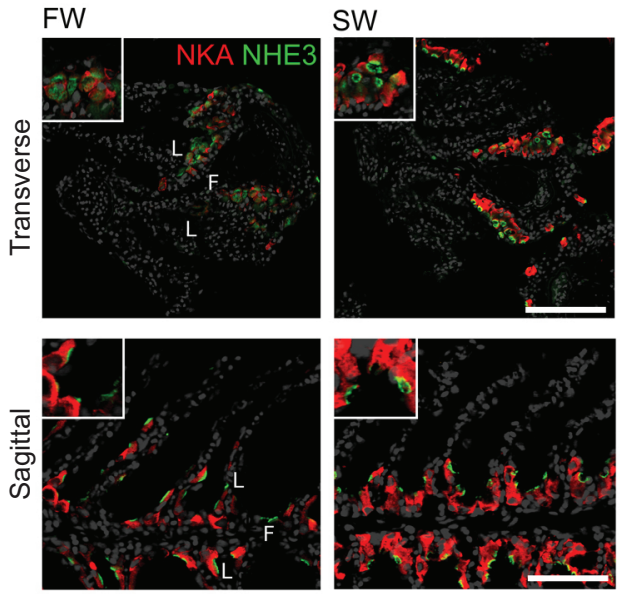


Fig. 6. Immunolocalization of NKCC1 and CFTR in gills of freshwater-acclimated (FW) and seawater-acclimated (SW) alewives. NKCC1 (red) and CFTR (green) signals are illustrated in transverse and sagittal sections of gill filaments. Cell nuclei are stained with TO-PRO-3 (gray). F, filaments; L, lamella. Images are single confocal z-sections, with a ×25 objective. Scale bars: 50 μm. Insets are a ×2 digital magnification.

of hyper/hypo-osmoregulatory competence: survival, maintenance of hematocrit and homeostasis of plasma chloride concentration. There were no mortalities, and there was no significant difference in hematocrit in seawater-acclimated individuals. Plasma chloride levels were only slightly higher in seawater-acclimated individuals than freshwater-acclimated individuals: 141 mmol l^{-1} and 134 mmol l^{-1}, respectively. A similar result was obtained in American shad juveniles collected while on their seaward migration (Zydlewski and McCormick, 1997a).

As is often the case in euryhaline species (McCormick, 1995), gill NKA activity was higher in seawater-acclimated alewives. Activity values of NKA were 75% greater in alewives acclimated to seawater than in freshwater-acclimated alewives. Similar differences were observed in a preliminary investigation of freshwater- and seawater-acclimated alewives (McCormick et al., 1997) and were also reported in shad (Zydlewski and McCormick, 1997a); however, gill NKA activity in freshwater-acclimated alewives is considerably higher than those reported for the American shad (Zydlewski and McCormick, 1997a; Zydlewski and McCormick, 1997b). Importantly, several species of euryhaline fish that are thought to be of marine origin, such as the sea bass (*Dicentrarchus labrax*), thick-lipped mullet (*Chelon labrosus*) and Australian bass (*Macquaria novemaculeata*), have been demonstrated to have increased NKA activity in freshwater (Lasserre, 1971; Langdon, 1987). The gill epithelium might be more ion-permeable in such species, which would facilitate ion export in seawater but would demand higher NKA activity in freshwater to increase Na^+ import. It appears that migratory clupeids do not fall into this marine-origin paradigm.

The abundance of NKA protein was also higher in seawater-acclimated alewives. Western analysis using the α5 antibody revealed a single immunoreactive band in freshwater and seawater of approximately 95 kDa, which is consistent with previous reports for the NKA α-subunit in other fish (Lee et al., 1998). The difference between seawater-acclimated and freshwater-acclimated alewives in branchial NKA enzyme abundance was less pronounced than that of NKA activity (40% *vs* 75%). The explanation for a greater elevation of NKA activity than of NKA expression might lie in the quaternary structure of NKA and in subunit isoforms: shifts



Fig, 7. Immunolocalization of NKA and NHE3 in gills of freshwater-acclimated (FW) and seawater-acclimated (SW) alewives. NKA (red) and NHE3 (green) signals are illustrated in transverse and sagittal gill sections of gill filaments. Cell nuclei are stained with TO-PRO-3 (gray). F, filaments; L, lamella. Images are single confocal *z*-sections, with a ×25 objective. Scale bars, 50 μm. Insets are a ×2 digital magnification.

in isoform composition could yield greater changes in activity than would be indicated by changes in enzyme abundance. NKA functions as a hetero-oligomer that is composed of three subunits (α, β and a FXYD protein). Multiple isoforms of subunits are known (McDonough et al., 1990; Mobasheri et al., 2000; Geering, 2005), and orthologs of each of these isoforms have been identified in fish (Rajarao et al., 2001; Gharbi et al., 2004; Gharbi et al., 2005; Richards et al., 2003; Wang et al., 2008; Tipsmark, 2008). Freshwater isoforms have been reported in several salmonids (Richards et al., 2003; Bystriansky et al., 2006; Bystriansky et al., 2007; Nilsen et al., 2007; Wang et al., 2008; McCormick et al., 2009). Changes in the isoforms that form the holoenzyme and in their phosphorylation state are likely to affect the kinetics of the enzyme pool (Feschenko and Sweadner, 1994; McDonough et al., 1990; Hauck et al., 2009) and should be detected in the NKA activity assay, whereas western analysis yields a measure of total NKA α-subunit abundance rather than isoform abundance, as the α5 antibody epitope is highly conserved among the α isoforms. In any case, our observations that NKA activity and abundance increase upon seawater acclimation are consistent with most reports from euryhaline species, and the potential for freshwater isoforms of NKA in alewives deserves further investigation.

The abundance of T4-immunoreactive bands was two-fold higher in seawater-acclimated alewives. Multiple lines of evidence indicate that this is the result of higher NKCC1 expression, although T4 is known to immunoreact also with two absorptive members of the cation–chloride cotransporter family (NKCC2 and NCC) in a number of species, including Mozambique tilapia, *Oreochromis mossambicus* (Lytle et al., 1995; Wu et al., 2003; Hiroi et al., 2005; Hiroi et al., 2008; Inokuchi et al., 2008). In western blots, T4-immunoreactive bands migrated at 120 and 240 kDa, and occasionally additional bands migrated at a higher molecular mass. This is consistent with patterns previously reported for NKCC1, which typically migrates by SDS-PAGE as multiple poorly resolved bands that probably represent variably glycosylated monomer and higher-order multimers (Pelis et al., 2001; Tipsmark et al., 2002; Moore-Hoon and Turner, 2000). None of these bands was more intense in freshwater-acclimated alewives, as might occur if T4 were cross-reactive to NCC and NKCC2; moreover, NKCC2 expression has not been detected in the gill of any fish. When we de-glycosylated the gill homogenate with PNGase F prior to western analysis, there was a uniform reduction of the molecular mass of all bands (data not shown), as previously reported for NKCC1 in Atlantic salmon (Pelis et al., 2001). The immunohistochemical signal was restricted to the basolateral and tubular network ionocyte membranes. This is also most consistent with NKCC1, as NCC and NKCC2 are usually found on the apical surface of epithelia. We conclude that T4 is specific for NKCC1 in the alewife gill and that NKCC1 promotes ion secretion in hypo-osmoregulating alewives. Acclimation to seawater similarly affects NKCC1 expression in four salmonids [lake trout *Salvelinus namaycush*, brook trout *S. fontinalis*, brown trout *S. trutta* and Atlantic salmon (Hiroi and McCormick, 2007; Pelis et al., 2001; Tipsmark et al., 2004)], the killifish *Fundulus heteroclitus* (Scott et al., 2004), the striped bass *Morone saxatilis* (Tipsmark et al., 2004) and the Hawaiian goby *Stenogobius hawaiiensis* (McCormick et al., 2003). We also detected NKCC1

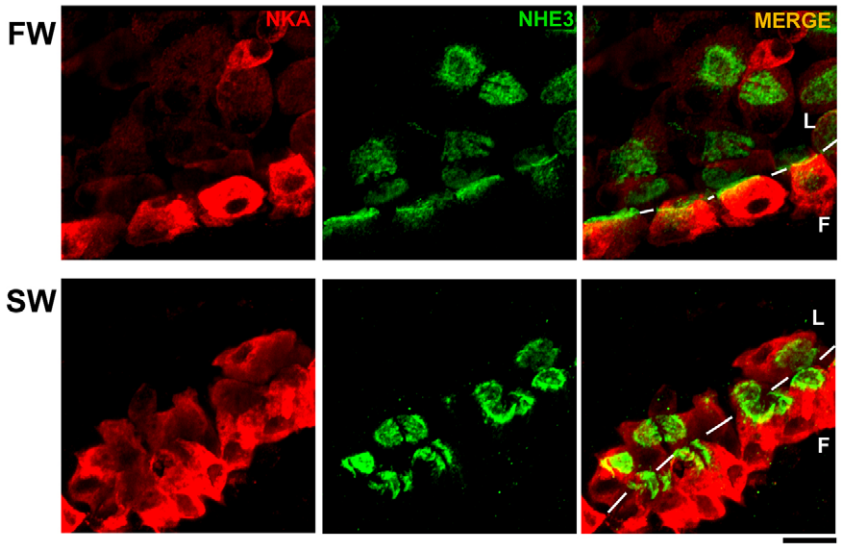


Fig. 8. Ionocyte orientations in gills of freshwater-acclimated (FW) and seawater-acclimated (SW) alewives. NKA (red) and NHE3 (green) signals are illustrated on gill lamellae (L) and filaments (F). Images are *z*-projections, with a ×63 objective and ×3 zoom. The broken white line indicates the junction of lamella and filament. Scale bar, 10 μm.

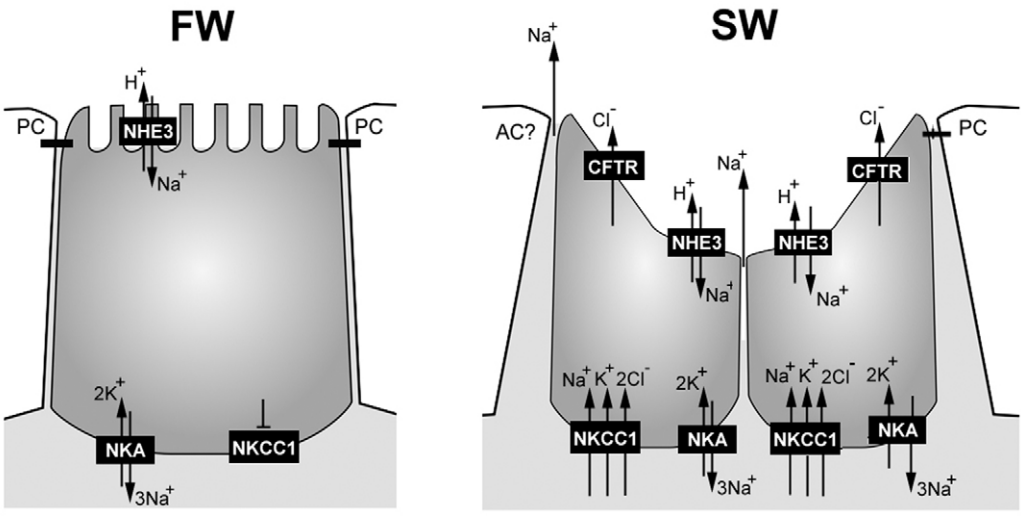


Fig. 9. Summary of ion-transport protein localization in alewife ionocytes and a model for enhanced Na^+ extrusion in seawater. Freshwater (FW) ionocytes express NKA, NHE3 and NKCC1 (possibly latent), whereas seawater (SW) ionocytes form clusters that are rich in NKA and NKCC1, express NHE3 and are frequently CFTR positive. Seawater ionocytes might share shallow Na^+-permeable junctions with each other, as well as with accessory cells (AC), forming NKA-rich complexes with low Na^+ resistance to facilitate Na^+ extrusion in seawater. PC, pavement cell; TJ, tight junction.

in freshwater-acclimated individuals. This might be an inactive nonphosphorylated pool of NKCC1 that could be quickly activated for an acute response upon exposure to elevated salinities (Flemmer et al., 2010). Notably, the proportional increase in NKCC1 abundance upon seawater acclimation was much greater than that of NKA, further suggesting that NKA isoforms have a role in ion absorption. NKCC1 might also be required for regulating ionocyte volume.

The location of CFTR in alewife ionocytes and differences in CFTR abundance with acclimation salinity were consistent with previous findings in other species. We observed CFTR immunoreactivity within the apical crypt of ionocytes, as reported in Hawaiian goby (McCormick et al., 2003), the mudskipper *Periophthalmodon schlosseri* (Wilson et al., 2000), killifish (Marshall et al., 2009), the European eel *Anguilla anguilla* (Wilson et al., 2004) and tilapia (Hiroi et al., 2005). We found the CFTR signal almost exclusively in seawater-acclimated alewives. Similarly, higher acclimation salinity stimulated increased branchial CFTR transcript abundance in killifish (Singer et al., 1998) and Atlantic salmon [CFTR I (Singer et al., 2002; Nilsen et al., 2007)] and CFTR protein expression in killifish (Shaw et al., 2007) and sea bass (Bodinier et al., 2009). We conclude that CFTR functions in Cl^- secretion in hypo-osmoregulating alewives. Although we observed consistent NKA and NKCC1 staining in seawater ionocytes, not all seawater ionocytes had CFTR staining. The absence of CFTR might be an artifact of sectioning wherein the crypt was sheared away or it might be evidence of distinct ionocyte subtypes in seawater (those with and without CFTR). Perhaps CFTR-negative cells represent a freshwater type of ionocyte that persists in seawater to provide osmoregulatory plasticity; this flexibility might be crucial for the transition between river, estuarine and marine environments.

Elevation of branchial NKA, NKCC1 and CFTR in alewives acclimated to seawater is integral to a hypo-osmoregulatory strategy that is not constrained by life history. Catadromous eels, which mature in estuaries, rivers and lakes and return to the ocean to spawn, respond similarly to changes in salinity. In juvenile European eels preparing for upstream migration, the abundance of all three ion-transporters was higher in seawater-acclimated compared with freshwater-acclimated individuals (Wilson et al., 2004). Both transcript and protein levels of these transporters increased in a Japanese eel (*Anguilla japonica*) ionocyte cell culture when transferred to a medium of increased salinity (Tse et al., 2006). Thus, the induction of this cohort of secretory ion-transporters appears to be a pervasive hypo-osmoregulatory strategy in diadromous fish.

In this study, we demonstrate that NHE3 immunoreactivity occurs in the apical region of gill ionocytes, in both freshwater and seawater. This is similar to observations made in rainbow trout (Edwards et al., 1999; Ivanis et al., 2008) and killifish (Claiborne et al., 1999; Edwards et al., 2005), but is in contrast to reports of freshwater upregulation of NHE3 in the gills of the Japanese dace *Tribolodon hakonensis* (Hirata et al., 2003; Hirose et al., 2003), the zebrafish *Danio rerio* (Yan et al., 2007), killifish (Scott et al., 2005) and Mozambique tilapia (Hiroi et al., 2008; Watanabe et al., 2008; Inokuchi et al., 2008; Watanabe et al., 2008; Choi et al., 2011). That we did not observe a greater abundance of NHE3 in freshwater alewives suggests that NHE3 is more important for acid secretion than Na^+ uptake in alewives. The T4 antibody used in the present study has also been shown to cross-react with the apical NCC cotransporter in tilapia (Hiroi et al., 2005). The lack of apical staining in this study might mean that an apical NCC does not exist in the alewife gill, but it is also possible that the T4 antibody simply does not recognize the alewife NCC. Uptake of Na^+ in the alewife gill might be mediated by an apical amiloride-sensitive epithelial Na^+ channel (ENaC) driven by a vacuolar-type proton pump [V-ATPase (Fenwick et al., 1999; Katoh et al., 2003], although ENaC has yet to be identified in the genome of any fish. A molecular or immunohistochemical examination of NCC, ENaC and V-ATPase is warranted and might provide valuable insight into clupeid hyper-osmoregulation.

Ionocytes were heterogeneously distributed along multiple gill filament axes. Along the filament, ionocytes were uniformly abundant for the proximal 75% and were less abundant along the remainder. To our knowledge, this is the first effort to quantify along-filament ionocyte distribution. The along-filament variability in ionocyte abundance might be functionally related to variability in the flow of water, in that water flow and gas exchange might be diminished at the distal filament tips during routine respiratory exchange (Hughes,

1972). We also observed variability in ionocyte abundance across the filament: ionocytes were concentrated on the trailing (afferent) edge. This has previously been reported in the hagfish *Myxine glutinosa* (Bartels, 1998), the sea lamprey *Petromyzon marinus* (Bartels et al., 1996), Atlantic stingray (Piermarini and Evans, 2000), Mozambique tilapia and the Nile tilapia *Oreochromis niloticus* (Cioni et al., 1991; van der Heijden et al., 1997), killifish (Katoh et al., 2001), the medaka *Oryzias latipes* (Sakamoto et al., 2001) and the chum salmon *Oncorhynchus keta* (Uchida et al., 1996).

Ionocyte number, location and size often vary with salinity. We found that the number of filamental ionocytes was unaffected by salinity, but lamellar ionocytes were less abundant in seawater. The reduction of lamellar ionocytes in seawater has also been reported in anadromous salmonids (Uchida et al., 1996; Ura et al., 1996; Hiroi and McCormick, 2007) and American shad (Zydlewski and McCormick, 2001). The shift of osmoregulatory cells to the filament might reflect the adaptive value of increasing lamellar surface area for gas exchange in response to the higher respiratory demands of marine habitation (Evans et al., 2005). We did not observe a more restricted distribution of ionocytes to the leading edge in seawater, as reported in killifish (Katoh et al., 2001).

We used an unconventional approach to sectioning gill filaments for counting ionocytes. Because ionocytes were nearly absent from the leading (efferent) half of the filament, we chose to count ionocytes in transverse sections (Figs 3, 4); ionocyte cell numbers in sagittal sections would vary strongly with section position. In species such as the alewife, where ionocytes are highly skewed towards the trailing edge, transverse sections provide a more complete representation of ionocyte distribution and might be required to quantify treatment responses accurately. In species where ionocytes are more evenly distributed across a filament, and treatment responses are sufficiently robust, sagittal sections are preferred because they view a continuous epithelium covering many lamellae extending from the filament.

The arrangement of ionocytes into multicellular complexes in seawater-acclimated alewives is particularly intriguing. Multicellular complexes consisting of an ionocyte sharing an apical crypt with an accessory cell, which are NKA poor and distinct in their function and ultrastructure, have been widely reported in the literature (Hootman and Philpott, 1980; Laurent and Dunel, 1980). In contrast, multicellular complexes consisting of multiple mature ionocytes that share a crypt have rarely been reported. In seawater-acclimated alewives, multicellular complexes comprised two or more NKA-rich mature ionocytes sharing an extensive apical crypt. Such arrangements have been reported in seawater-acclimated tilapia, but they were rare (Lee et al., 2003; Hwang, 1987; van der Heijden et al., 1997); structurally similar but functionally dissimilar complexes have been reported in freshwater-acclimated killifish (Katoh et al., 2001). Previous ultrastructural analysis of the tight junctions between ionocytes and accessory cells indicated that they are shallow and simple, which might permit paracellular passive efflux of Na^+ in seawater (Sardet et al., 1979; Ernst et al., 1980). If the multicellular complexes we observed share such junctions, they would constitute an NKA-rich unit with low paracellular resistance to Na^+, which would be optimal for Na^+ extrusion in seawater (Fig. 9). Ultrastructural analysis of these cellular units by transmission electron microscopy would provide valuable insight into the precise identity of the cellular constituents and the nature of their junctions.

In the present study, we describe the impacts of freshwater and seawater acclimation on the expression of major ion transporters in the alewife gill. An increase in NKA, NKCC1 and CFTR abundance in gill ionocytes with increasing salinity is in accordance with established models for hypo-osmoregulation. Unlike the majority of reports on the effects of salinity on NHE3 expression in euryhaline fish, we found no differences in freshwater and seawater. All four ion transporters responded to salinity as has been described in salmonids. Such similarity is interesting because salmonids evidently were ancestrally freshwater, whereas clupeids and their sister taxon, the anchovies, are primarily (although not entirely) marine. Our three-dimensional analysis of ionocyte distribution in alewife gill filaments demonstrates that transverse sectioning, rather than more conventional sagittal sectioning, might be more appropriate for ionocyte enumeration in species where these cells are highly skewed towards the trailing edge. Finally, we describe the induction of unique multicellular complexes of mature ionocytes, which share extensive crypts in seawater that might be particularly well suited to Na^+ extrusion.

LIST OF ABBREVIATIONS

CFTR	cystic fibrosis transmembrane conductance regulator
ENaC	epithelial Na^+ channel
NCC	sodium/chloride cotransporter (Na^+/Cl^- cotransporter)
NHE3	sodium/hydrogen exchanger 3 (Na^+/H^+ exchanger)
NKA	Na^+/K^+-ATPase
NKCC1	$Na^+/K^+/2Cl^-$ cotransporter 1
p.p.t.	parts per thousand
YOY	young of the year

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