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RESEARCH ARTICLE

Differential regulation of sodium–potassium pump isoforms during smolt development and seawater exposure of Atlantic salmon

Stephen D. McCormick[1,2,*], Amy M. Regish[1], Arne K. Christensen[1,2] and Björn Thrandur Björnsson[3]

[1]USGS, Conte Anadromous Fish Research Center, Turners Falls, MA 01376, USA, [2]Department of Biology, University of Massachusetts, Amherst, MA 01003, USA and [3]Department of Biology and Environmental Sciences, University of Gothenburg, S-405 30 Göteborg, Sweden

*Author for correspondence (mccormick@umext.umass.edu)

SUMMARY

Freshwater and seawater isoforms of the alpha subunit of Na^+/K^+-ATPase (NKA) have previously been identified in gill ionocytes of Atlantic salmon (*Salmo salar*). In the present study we examine the abundance and cellular localization of these isoforms during the parr–smolt transformation, a developmental process that is preparatory for seawater entry. The abundance of NKAα1a was lower in smolts than in parr, remained relatively constant during spring and decreased in summer. NKAα1b increased tenfold in smolts during spring, peaking in late April, coincident with downstream migration and increased salinity tolerance. NKAα1b increased a further twofold after seawater exposure of smolts, whereas NKAα1a decreased by 98%. The abundance of NKAα1b-positive, and NKAα1b and NKAα1a co-labeled ionocytes increased during smolt development, whereas the number of NKAα1a cells decreased. After seawater exposure of smolts, NKAα1b-positive ionocytes increased, NKAα1a-positive cells decreased, and co-labeled cells disappeared. Plasma growth hormone and cortisol increased during spring in smolts, but not in parr, peaking just prior to the highest levels of NKAα1b. The results indicate that the increase in the abundance of NKAα1b during smolt development is directly linked to the increase in salinity tolerance that occurs at this stage, but that significant changes also occur after seawater exposure. Spring increases in circulating levels of growth hormone and cortisol indicate that these hormones may be instrumental in upregulating NKAα1b during smolt development.

Key words: osmoregulation, ion transport, ionocyte, *Salmo salar*.

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INTRODUCTION

Anadromous fish make at least one migration from freshwater to seawater during their life history, and must face the osmoregulatory challenge of a large increase in external salinity. As with all teleost fish, they must maintain a nearly constant internal osmotic pressure, approximately one-third that of seawater, irrespective of whether they are in freshwater or seawater (Evans et al., 2005). In freshwater, fish must counteract the passive loss of ions to the environment by actively transporting them across the gill. In seawater, teleost fish must counteract the passive loss of water and gain of ions by drinking seawater and taking up water and salt across the gut, while excess sodium and chloride are secreted by the gill. Thus the transition from freshwater to seawater calls for the gill to reverse its function from an ion uptake to a salt secretory organ.

Anadromous salmonids have evolved a strategy in which many of the osmoregulatory mechanisms for salt secretion are up-regulated prior to movement into seawater (Hoar, 1988). During transformation from the stream-dwelling parr to the downstream migrating smolt in spring, salmon develop a high degree of salinity tolerance, allowing them to move into seawater with minimal internal osmotic perturbations (McCormick and Saunders, 1987). Previous work has established that the abundance and activity of the branchial sodium–potassium pump (Na^+/K^+-ATPase; NKA) increases during smolt development (Zaugg and Wagner, 1973), and there are changes in the number and characteristics of ionocytes (also known as chloride cells or mitochondrion-rich cells) that are the site of ion transport (Pisam et al., 1988). Although there is a strong correlation between NKA activity and salinity tolerance (McCormick et al., 2009a), it has been suggested that at least some of the increase is due to increased demand for ion uptake capacity as the osmoregulatory function of the gill changes (Primmett et al., 1988).

NKA is one of three major ion transport proteins that are involved in sodium and chloride secretion by the gill when fish are in seawater. NKA moves three sodium ions out of the cell while pumping in two potassium ions, creating an ionic/electric gradient across the cell membrane of the ionocytes, with the inside of the cell highly negative and low in sodium. The sodium gradient is then used by a $Na^+/K^+/2Cl^-$ co-transporter (NKCC1) to bring chloride into the cell. Chloride then leaves the cells on a favorable electrochemical gradient through an apical chloride channel, a homolog of the cystic fibrosis transmembrane conductance regulator (CFTR) (Marshall, 2002). In addition to providing the electrochemical gradient for chloride secretion, NKA also pumps sodium into the paracellular space where sodium leaves through leaky tight junctions on a favorable electrochemical gradient. In seawater-adapted salmon, these three ion transport proteins have been localized specifically to ionocytes (McCormick et al., 2003; Hiroi et al., 2005).

NKA is composed of two essential subunits, α and β. The α-subunit is the main catalytic unit containing the binding sites for ATP, Na^+, K^+ and ouabain (a specific inhibitor of the enzyme). The

β-subunit is a glycosylated polypeptide that promotes folding and positioning of the protein into the basolateral plasma membrane. A third subunit termed γ, also known as FXYD, is not necessary for the catalytic function of NKA, but apparently acts to adapt the kinetic properties of sodium and potassium transport for the functions of different cell types (Garty and Karlish, 2006). Vertebrates express at least four isoforms of the α-subunit that vary in their kinetic properties for Na^+, K^+ and ATP binding, sensitivity to ouabain and calcium, and their regulation by intracellular second messengers (Blanco and Mercer, 1998). These unique properties along with their cell-specific distribution suggest that the NKA isoforms are distinct in their regulation and physiological function.

Transcription studies indicate that there are salinity-dependent isoforms of the α-subunit expressed in the gills of salmonids and tilapia (Richards et al., 2003; Nilsen et al., 2007; Tipsmark et al., 2011). In a recent study on Atlantic salmon parr, we used specific antibodies to characterize two NKAα1 isoform proteins, NKAα1a and NKAα1b, which are the major isoforms expressed in freshwater and seawater, respectively (McCormick et al., 2009b). It was demonstrated that these isoforms are normally present in distinct ionocytes that are presumably involved in ion uptake and salt secretion, respectively. Recent studies on gill NKAα1a and NKAα1b mRNA levels indicate that there is differential transcription of these isoforms during smolt development (Nilsen et al., 2007). In the present study, we have examined changes in NKAα1a and NKAα1b protein abundance and the characteristics of ionocytes expressing these isoforms during smolt development. In particular, we wished to determine how each of the isoforms contributes to the increases in NKA abundance that occur during smolt development. We also examined changes in NKA isoform abundance and localization in response to seawater exposure at the peak of smolt development, to determine responses that would occur during normal smolt migration into the ocean.

MATERIALS AND METHODS

Animals and experimental protocols

In September of 2008, juvenile Atlantic salmon (*Salmo salar* L.) were obtained from the Kensington State Hatchery (Kensington, CT, USA) and brought to the Conte Anadromous Fish Research Center (Turners Falls, MA, USA). Until the initiation of the experiment, fish were maintained in 1.7 m diameter tanks supplied with ambient river water at a flow rate of 4 l min^{-1} and provided with continuous aeration. They were maintained under natural photoperiod conditions and fed to satiation using automatic feeders (Zeigler Bros, Gardners, PA, USA). At the start of the experiment (January 2009), fish were separated by size into parr [fork length (L_f) ≤ 10.0 cm] and pre-smolt (L_f≥12.1 cm) groups based on a previously established winter threshold for smolt development in this strain of Atlantic salmon (McCormick et al., 2007). Each group was maintained in duplicate tanks throughout the experiment under natural photoperiod and identical temperature regimes. At the time of peak smolt development (6 May 2009), 25 parr and 25 smolts were transferred to seawater (35 p.p.t.) recirculation tanks with particle and charcoal filtration, and maintained at 14°C with continuous aeration. Fish in seawater were hand-fed *ad libitum* once daily. All rearing and experiments were carried in accordance with US Geological Survey institutional guidelines and an approved IACUC review.

Food was withheld for 24 h prior to sampling of fish, which occurred between 10:00 and 12:00 h Eastern Standard Time. Fish were anesthetized with MS-222 (100 mg l^{-1}, pH 7.0) and blood was drawn from the caudal vessels into a 1 ml ammonium heparinized syringe, spun at 3200 ***g*** for 5 min at 4°C; plasma was then aliquoted and stored at −80°C. Gill arches were removed, and gill filaments trimmed from the ceratobranchials and placed in a 1.5 ml microcentrifuge tube and frozen immediately at −80°C for western blots. Four to six gill filaments were placed in 100 µl of ice-cold SEI buffer (150 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} imidazole, pH 7.3) and frozen at −80°C within 30 min for measurement of NKA activity. Similarly sized gill samples were placed in 4% paraformaldehyde in 10 mmol l^{-1} phosphate-buffered saline (PBS) at room temperature for 2 h. Fixed gill tissue was then rinsed in PBS, placed in PBS with 30% (w/v) sucrose overnight at 4°C, and then frozen in Tissue-Tek OCT embedding medium (Sakura Finetek, Torrance, CA, USA).

Antibodies

Rabbit anti-NKAα1a and rabbit anti-NKAα1b were developed as previously outlined (McCormick et al., 2009b). Chicken anti-NKAα1a IgY antibodies (GeneTel Laboratories LLC, Madison, WI, USA) were raised against an NKAα1a-specific sequence peptide and affinity-purified using NKAα1a-specific peptide conjugated to Sulfolink Coupling Gel (Pierce, Rockford, IL, USA). The NKAα1a-specific antibody fraction was enriched by immunodepletion against NKAα1b-specific peptide conjugated to Optimized Affinity Resin (OAR, 21st Century Biochemicals, Marlborough, MA, USA). Goat anti-rabbit-HRP conjugate (KPL, Gaithersburg, MD, USA) was used as a secondary antibody for western blotting. Goat anti-rabbit or anti-chicken antibodies conjugated to Alexa fluorophores (Invitrogen Molecular Probes, Carlsbad, CA, USA) were used as a secondary antibody for immunohistochemistry.

Western blots

Ion transporter abundance was quantified by western immunoblotting as previously outlined (Pelis et al., 2001). Gill tissue was homogenized in 10 volumes of 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) containing 30% sucrose (w/v), 2 mmol l^{-1} EDTA, 1 mmol l^{-1} phenylmethylsulfonyl flouride (PMSF), and Complete Mini protease inhibitor tablets (Roche, Indianapolis, IN, USA). The tissue homogenate was centrifuged at 5000 ***g*** for 10 min at 4°C. The supernatant was then centrifuged at 20,000 ***g*** for 10 min at 4°C. The resulting supernatant was centrifuged at 48,000 ***g*** for 2 h at 4°C. The final pellet was resuspended in homogenization buffer plus 0.1% Triton X-100 (LabChem, Pittsburg, PA, USA). Protein concentration was determined with a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Samples were then placed in an equal volume of 2× Laemmli buffer, heated for 15 min at 60°C and stored at –80°C. Samples were thawed and proteins were resolved by SDS-PAGE using a 6.75% gel. Ten or five micrograms of protein were loaded per lane for the detection of NKAα1a or NKAα1b, respectively. Two lanes were reserved on each gel for Precision Plus relative molecular weight markers (Bio-Rad Laboratories, Hercules, CA, USA) and a standard consistent tissue preparation reference to control for blot-to-blot variation. Following electrophoresis, proteins were transferred to Immobilon PVDF transfer membrane (Millipore, Bedford, MA, USA) at 30 V overnight in 25 mmol l^{-1} Tris and 192 mmol l^{-1} glycine buffer; pH 8.3. PVDF membranes were blocked with 5% non-fat dry milk in PBST (PBS with 0.05% Triton X-100) for 1 h at room temperature, rinsed in PBST, and exposed to primary antibody in blocking buffer for 1 h at room temperature. After rinsing in PBST, blots were exposed to goat anti-rabbit-HRP diluted 1:10,000 in blocking buffer for 1 h at room temperature. After rinsing in PBST, blots were incubated for 1 min in a 1:1 mixture of enhanced

chemiluminescent (ECL) solution A (396 μmol l^{-1} coumaric acid, 2.5 μmol 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), then exposed to X-ray film (RPI, Mount Prospect, IL, USA). Digital photographs were taken of individual gels and band staining intensity measured using ImageJ (National Institutes of Health, Bethesda, MD, USA), and protein abundance expressed as a cumulative 8-bit grayscale value. Gray values were standardized to the internal reference lanes for each isoform.

Immunohistochemistry

Sections (5 μm thick) were cut in a cryostat at –24°C, parallel to the long axis of primary filaments and perpendicular to the attachment of secondary lamellae. The tissue was placed on Fisherbrand Colorfrost Plus slides (Fisher Scientific, Hampton, NH, USA), dried, rinsed with PBS, and then incubated in 8% normal goat serum in PBS for 30 min at room temperature. Slides were exposed to primary antibody (chicken anti-NKAα1a, 1.9 μg ml^{-1}; rabbit anti-NKAα1b, 1 μg ml^{-1}) in antibody dilution buffer (0.01% NaN_3, 0.05% Triton X-100, 8% normal goat serum and 0.02% keyhole limpet hemocyanin in PBS) and incubated overnight at 4°C. The slides were rinsed several times with PBS and exposed to fluorescently labeled secondary antibody for 2 h at room temperature. After incubation, the slides were rinsed several times with PBS, covered by a coverslip and examined with a Nikon Diaphot-TMD inverted fluorescence microscope with a mercury lamp. From each fish, immunoreactive ionocytes on the primary filament and secondary lamellae (tallied separately) were counted from sagittal sections of gill filament (700 μm of primary filament/sagittal section) and expressed per millimeter of primary filament. Mean numbers of ionocytes for each group were obtained using the means calculated from each fish. Cell area (μm^2 per cell) was obtained from immunoreactive ionocytes using MetaMorph 4.1.2 (Universal Imaging Corporation, Downingtown, PA, USA). A set threshold defined the NKAα1a, NKAα1b and co-localized signal, and the nucleus was included in the total cell area. When present, at least 50 immunoreactive ionocytes from several different tissue sections were analyzed from each of at least five fish per date. Fifty cells were not available in some cases (e.g. lamellar NKAα1a-positive ionocytes in seawater) so all available cells were counted and used for analysis.

Gill and plasma analyses

NKA activity in gill homogenates was determined using a temperature-regulated microplate method (McCormick, 1993). In this assay, ouabain-sensitive ATPase activity was 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). Protein concentration of the homogenate was determined using a BCA protein assay (Pierce). Plasma chloride was analyzed by the silver titration method using a Buchler-Cotlove digital chloridometer (LABCONCO, Kansas City, MO, USA) and external standards. Plasma growth hormone (GH) levels were measured by a radioimmunoassay validated for Atlantic salmon (Björnsson et al., 1994). Typical measuring range was 0.1 to 50 ng ml^{-1} with ED_{50}=2.2 ng ml^{-1} and an average intra-assay and inter-assay variation of 5.4% (N=9) and 3.9% (N=9), respectively. Plasma cortisol levels were measured by a validated direct competitive enzyme immunoassay as previously outlined (Carey and McCormick, 1998). Sensitivity, as defined by the dose–response curve, was 1–400 ng ml^{-1}. The lower detection limit was 0.3 ng ml^{-1}. Using a pooled plasma sample, the average intra-assay variation was 7.2% (N=6) and the average inter-assay variation was 11.8% (N=6).

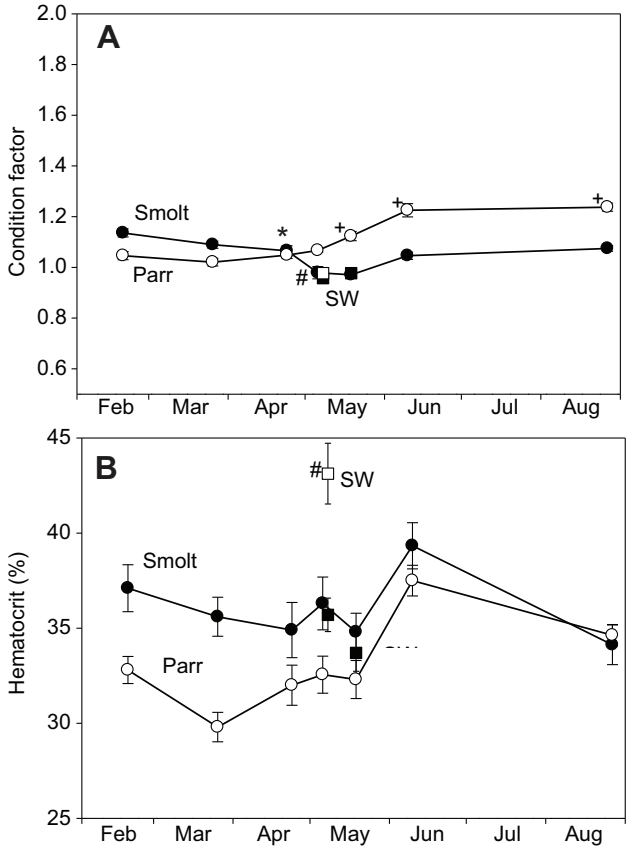

Fig. 1. Condition factor (A) and hematocrit (B) of Atlantic salmon *Salmo salar* parr (open symbols) and smolts (filled symbols) during freshwater rearing (circles) and seawater (SW) exposure (squares). Values are means ± s.e.m. of 10 fish per group. *Significant change from initial February sampling date [one-way ANOVA, Student–Newman–Keuls (SNK) *post hoc* test, P<0.05]. #Significant change after seawater transfer; fish were transferred to seawater on 6 May and sampled 2 and 14 days after transfer.

Statistics

Condition factor was calculated as 100(weight/length^3). Differences between parr and smolts over time in freshwater were compared using two-way ANOVA followed by Student–Newman–Keuls (SNK) *post hoc* comparisons. Due to the low plasma volumes of parr, several parameters were not measured on every date, and thus comparison of parr and smolts occurred only for time points when there was data for both parr and smolts. Changes after seawater exposure were analyzed by one-way ANOVA followed by SNK *post hoc* comparisons.

RESULTS

Condition factor of smolts decreased progressively from February to May, and then increased in June and August (Fig. 1A). Condition factor of parr was lower than smolts in winter, then increased in late spring to become higher than smolts in May and thereafter.

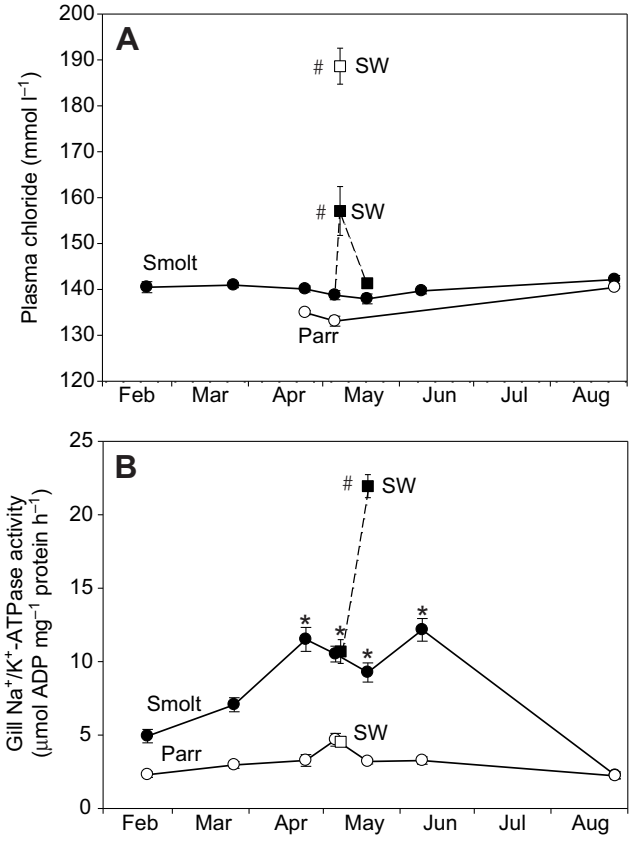

Fig. 2. Plasma chloride (A) and gill NKA activity (B) of Atlantic salmon parr (open symbols) and smolts (filled symbols) during freshwater rearing (circles) and seawater (SW) exposure (squares). Values are means ± s.e.m. of 10 fish per group. *Significant change from initial February sampling date (one-way ANOVA, SNK *post hoc* test, $P<0.05$). #Significant change after seawater transfer; fish were transferred to seawater on 6 May and sampled 2 and 14 days after transfer.

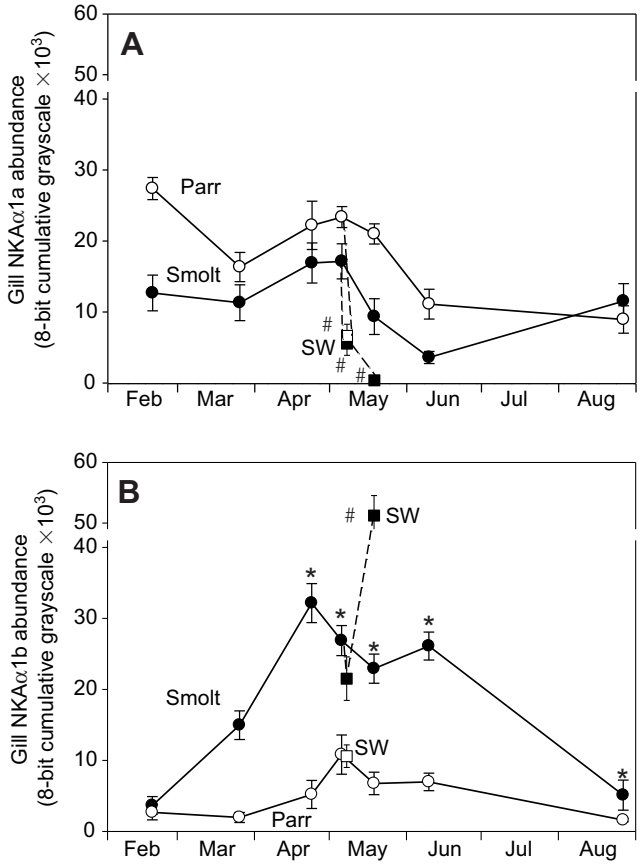

Fig. 3. Abundance of gill NKAα1a (A) and NKAα1b (B) of Atlantic salmon parr (open symbols) and smolts (filled symbols) during freshwater rearing (circles) and seawater (SW) exposure (squares). Values are means ± s.e.m. of six fish per group. *Significant change from initial February sampling date (one-way ANOVA, SNK *post hoc* test, $P<0.05$). #Significant change after seawater transfer; fish were transferred to seawater on 6 May and sampled 2 and 14 days after transfer.

There was a significant effect of developmental stage ($P<0.0001$) and time ($P<0.0001$), and a significant interaction (two-way ANOVA, $P<0.0001$). Seawater exposure for 2 days caused a significant drop in condition factor of parr ($P=0.0002$), probably due to dehydration. Seawater exposure did not significantly affect condition factor of smolts.

Hematocrit of smolts remained fairly constant from February to May, increased in June and then decreased in August (Fig. 1B). Hematocrit of parr was initially lower than smolts, but followed the same general pattern as smolts. There was a significant effect of developmental stage ($P<0.0001$) and time ($P<0.0001$), but no significant interaction (two-way ANOVA, $P=0.13$). Seawater exposure did not affect hematocrit in smolts, but resulted in a significant elevation in parr after 2 days ($P=0.01$).

Plasma chloride levels remained stable in smolts from February to August (Fig. 2A). Plasma chloride levels of parr were lower than those of smolts in spring, but increased in August to levels that were similar to those of smolts. There was a significant effect of developmental stage ($P<0.0001$) and time ($P<0.0001$), and a significant interaction (two-way ANOVA, $P=0.037$). There was a significant increase in plasma chloride after 2 days in seawater in parr, and these levels were substantially higher than in smolts (188 and 158 mmol l^{-1}, respectively), indicative of higher salinity tolerance of smolts. All parr died within 4 days of seawater exposure. There was no mortality of smolts in seawater and after 14 days, plasma chloride levels were similar to those in freshwater fish.

Gill NKA activity was higher in smolts than in parr in February, indicating that some increase had already occurred in smolts in early winter (Fig. 2B). Gill NKA activity of smolts increased progressively through April, remained high through June and then decreased to low levels in August. Gill NKA activity of parr increased slightly in spring but were one-third those of smolts at the peak of smolt development in mid-April and early May. There was a significant effect of developmental stage ($P<0.0001$) and time ($P<0.0001$), and a significant interaction (two-way ANOVA, $P<0.0001$). There was no change in gill NKA activity of parr or smolts after 2 days of seawater exposure, but a twofold increase in smolts after 14 days in seawater ($P<0.0001$).

Gill NKAα1a abundance was higher in parr than in smolts throughout the spring, and both groups showed a similar pattern of relatively constant levels in winter and spring followed by lower levels in summer (Fig. 3A). In smolts, there were no significant differences between dates from February to May, but levels in June and August were significantly lower than peak levels in April and May (SNK test, $P<0.05$). There was a significant effect of developmental stage ($P<0.0001$) and time ($P<0.0001$), but no

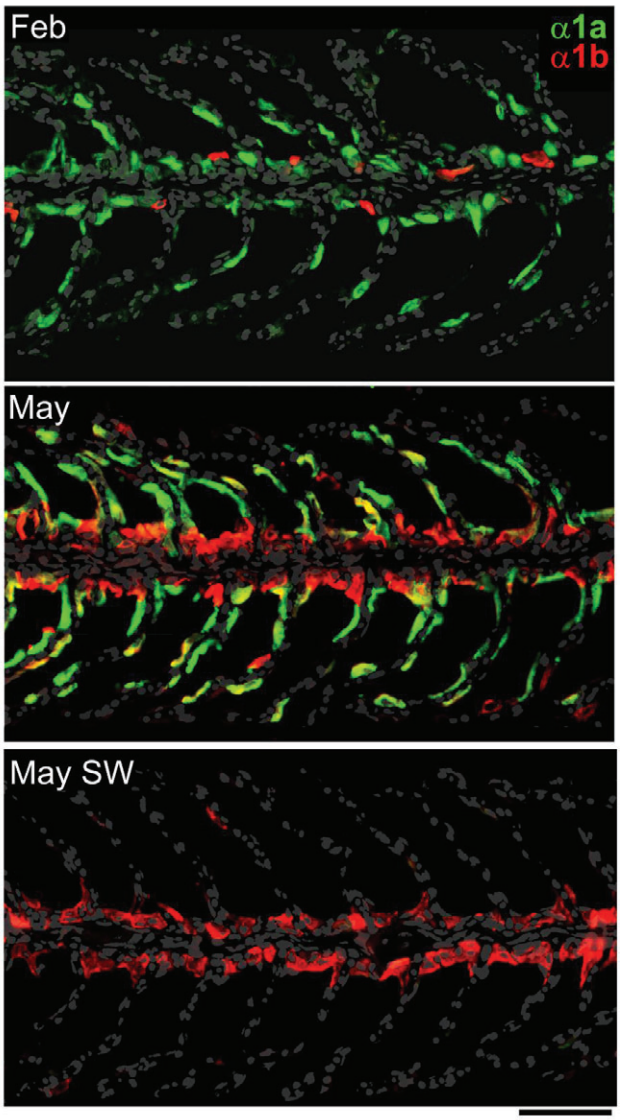

Fig. 4. Localization of NKAα1a (green), NKAα1b (red), and co-labeled NKAα1a and NKAα1b (yellow–orange) immunoreactive ionocytes in gill tissue of Atlantic salmon pre-smolts (19 Feb), freshwater smolts (6 May), and 2 week seawater-adapted smolts (SW, 19 May). Scale bar, 50 μm.

significant interaction (two-way ANOVA, P=0.09). The largest changes occurred after seawater exposure, when levels of both parr and smolt NKAα1a decreased by 75% after 2 days in seawater, and were nearly undetectable in smolts after 14 days in seawater (P<0.0001).

Gill NKAα1b abundance was similar in parr and smolts at the first sampling in February (Fig 3B). Gill NKAα1b abundance increased progressively in smolts during spring, reaching values that were 10-fold higher in late April than in February. Values decreased dramatically between June and August and were nearly as low in August as in February. Parr had a similar pattern of increase in spring and decrease in summer, but the levels were usually less than 25% of those seen in smolts. There was a significant effect of developmental stage (P<0.0001) and time (P<0.0001), and a significant interaction (two-way ANOVA, P=0.0002). There was no significant change in gill NKAα1b abundance after 2 days in seawater in parr or smolts, but after 14 days in seawater gill NKAα1b of smolts increased twofold compared with freshwater levels (P=0.0002).

In pre-smolts sampled in February, NKAα1a filamental ionocytes were the primary cell type, with only limited numbers of NKAα1b ionocytes and very few co-labeled cells (Figs 4, 5). As smolt development proceeded there were significantly increased numbers of NKAα1b and co-labeled filamental ionocytes and decreased NKAα1a ionocytes. In August the numbers of co-labeled filamental ionocytes decreased and the NKAα1a filamental ionocytes increased. A similar pattern was seen in lamellar ionocytes, with the exception that there were very few NKAα1b cells, and NKAα1a ionocytes numbers remained stable. In smolts between February and early May the total number of filamental ionocytes increased by 20% and was not significantly different, whereas lamellar ionocytes increased significantly by 60% (Fig. 5).

During smolt development in freshwater there was a moderate but significant increase in the size of NKAα1a ionocytes on both the filament and lamellae (Fig. 5C,D). The size of co-labeled cells also increased in the spring, whereas the size of NKAα1b did not change substantially and were smaller than other ionocytes.

After 14 days in seawater, the number of NKAα1b ionocytes increased, whereas the number of NKAα1a and co-labeled ionocytes decreased in both the filament and lamellae (Fig. 6). The total numbers of filamental ionocytes remained constant, whereas almost all lamellar ionocytes disappeared in seawater. The size of NKAα1a ionocytes of smolts decreased significantly in both filament and lamellae after 2 and 14 days (Fig. 4), and the few remaining cells appeared to be located beneath a covering of pavement cells (authors' personal observation). The size of NKAα1b ionocytes was not significantly different after 2 days in seawater but had nearly doubled after 14 days (Fig. 6C,D). In seawater, many NKAα1b ionocytes had a connection both to the external environment and to the blood vessels of the filament.

Plasma growth hormone levels were slightly higher in smolts compared with parr in February (Fig. 7A). Plasma growth hormone increased slightly in smolts between February and April, increased steeply in May, and then subsequently declined in June and August. There was no significant change in parr over time. There was a significant effect of developmental stage (P=0.0015) and time (P=0.0009), but no significant interaction (two-way ANOVA, P=0.08). Although mean plasma growth hormone levels doubled after 2 days in seawater, these levels were not significantly different from initial (May) freshwater levels (P=0.26), and had returned to freshwater levels after 14 days in seawater.

Plasma cortisol levels in smolts increased steadily between February and early May, then decreased steadily from mid-May to August (Fig. 7B). The levels in parr did not change over the period of sampling and were much lower than in smolts in April and May. There was a significant effect of developmental stage (P=0.0007) and time (P<0.0001) and a significant interaction (two-way ANOVA, P=0.00012). Plasma cortisol of parr increased significantly after 2 days in seawater (P<0.0001), whereas the levels in smolts were not affected after 2 days in seawater and were actually lower than initial freshwater levels after 14 days.

DISCUSSION

There is a developmental increase in salinity tolerance during the parr–smolt transformation of salmonids that allows smolts to withstand movement into seawater with minimal ionic and osmotic perturbations (Hoar, 1988), and this is associated with increased growth and swimming performance in seawater and increased marine survival (McCormick et al., 1998). The present study

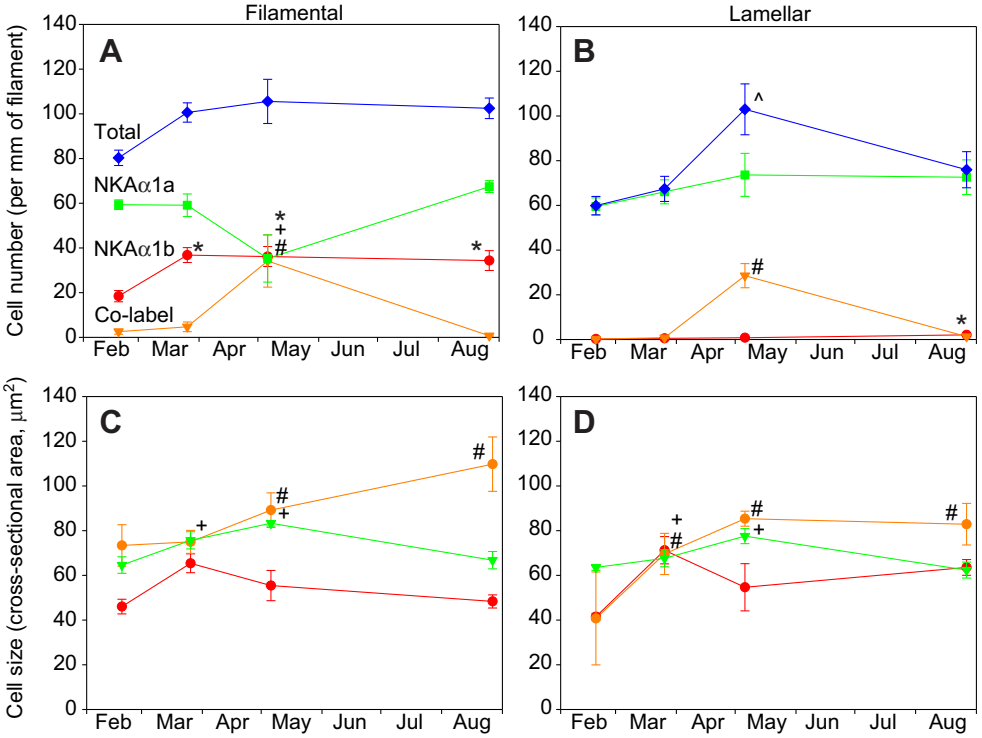

Fig. 5. Filamental cell number (A) and size (C), and lamellar cell number (B) and size (D) of NKAα1a, NKAα1b, co-labeled NKA, and total count of immunoreactive NKA gill ionocytes in Atlantic salmon freshwater smolts over time. Values are means ± s.e.m. of five to six fish per group. Significant change from initial February sampling date is indicated by: *NKAα1b, +NKAα1a, #co-labeled NKA and ^total count of immunoreactive NKA cells (one-way ANOVA, $P<0.05$).

elucidates the functional basis for a key mechanistic switch in the gill, from a freshwater- to a seawater-adapting osmoregulatory organ during the parr–smolt transformation. By using specific antibodies, we have shown that the abundance of gill NKAα1b increases 10-fold during smolt development, peaking in late April and early May when salinity tolerance also peaks. In contrast, the 'freshwater' NKAα1a isoform remains relatively constant throughout. Thus it is suggested that increased levels of the NKAα1b isoform in the gill are likely to be causal to the increased salinity tolerance of smolts.

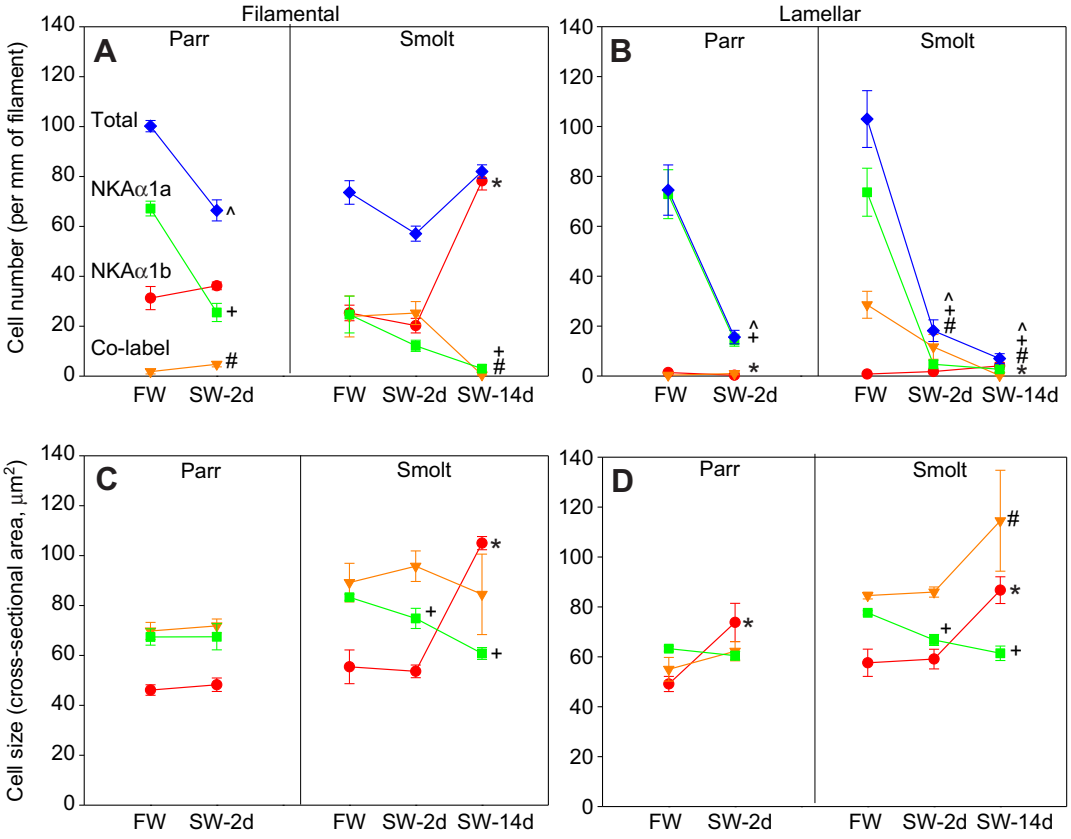

Fig. 6. Filamental cell number (A) and size (C), and lamellar cell number (B) and size (D) of NKAα1a, NKAα1b, co-labeled NKA, and total count of immunoreactive NKA gill ionocytes in Atlantic salmon parr and smolts at peak smolt development before and after seawater acclimation for 2 days (SW-2d; smolt and parr) and 14 days (SW-14d; smolts only). Values are means ± s.e.m. of five to six fish per group. Significant change from the 6 May sampling date is indicated by: *NKAα1b, +NKAα1a, #co-labeled NKA and ^total count of immunoreactive NKA cells (one-way ANOVA, $P<0.05$).

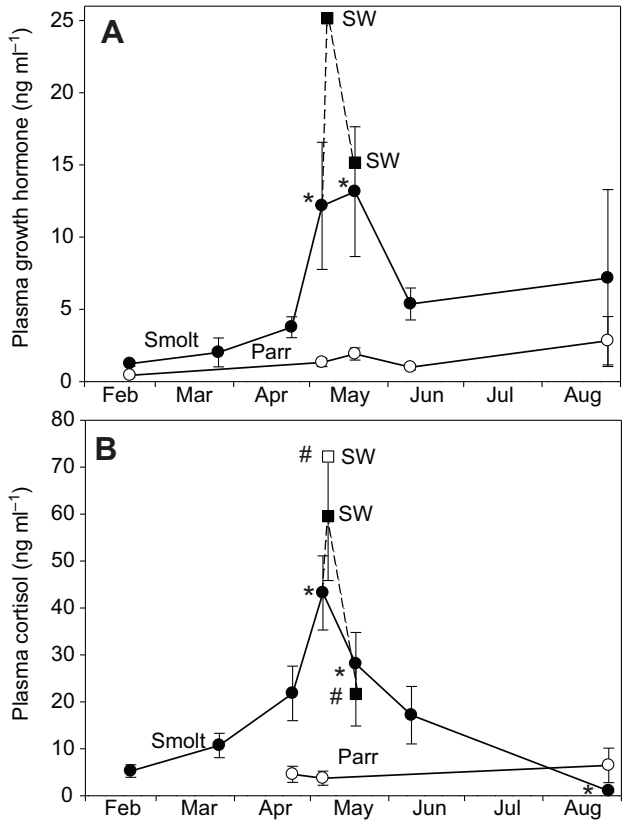

Fig. 7. Plasma growth hormone (A) and cortisol (B) of Atlantic salmon parr (open symbols) and smolts (filled symbols) during freshwater rearing (circles) and seawater (SW) exposure (squares). Values are means ± s.e.m. of eight to 10 fish per group. *Significant change from initial February sampling date (one-way ANOVA). #Significant change after seawater transfer; fish were transferred to seawater on 6 May and sampled 2 and 14 days after transfer.

While a previous study has shown that the abundance of the NKAα1a and NKAα1b isoforms in the gills of Atlantic salmon parr is tightly correlated with environmental salinity, with the NKAα1a being the predominant isoform in freshwater and NKAα1b more abundant after seawater acclimation (McCormick et al., 2009b), the present study is the first to profile the abundance of gill NKAα1a and NKAα1b isoforms during smolt development. Increased gill NKAα1b is consistent with molecular studies demonstrating that transcription of this isoform increases during smolt development of Atlantic salmon (Nilsen et al., 2007). These studies also found that gill NKAα1a mRNA decreases steadily from February to June, whereas in the present study NKAα1a protein levels are relatively stable from February to May with only a minor decrease in the late spring. It is not clear whether these differences are due to source fish, experimental differences, or a consequence of distinct regulatory pathways at the mRNA and protein level.

It is well known that gill NKA activity increases during smolt development and is correlated with increased salinity tolerance (Zaugg and McLain, 1970). As gill NKA activity also increases in salmonids after exposure to seawater, the increase during smolt development has usually been interpreted as being causal to the increase in seawater tolerance (Hoar, 1988). Gill NKA is also thought to be involved in ion uptake in fish, and it is possible that some of the increase in NKA activity observed during smolt development is due to an increased demand for ion uptake to compensate for changes in the gill associated with development of salt secretory capacity (Primmett et al., 1988). The present study suggests that changes in gill NKA activity are primarily due to the up-regulation of NKAα1b, as the pattern of change in gill NKA activity and NKAα1b abundance is very similar, whereas there is almost no change in the abundance of gill NKAα1a during smolt development in freshwater (Fig. 2B, Fig. 3B). It should be pointed out that the magnitude of change during smolt development is much greater for NKAα1b than gill NKA activity (10- *vs* 2.5-fold). In addition, the nearly complete disappearance of gill NKAα1a after seawater exposure is not reflected in gill NKA activity. Our results thus indicate that at least with regard to changes in NKA isoform abundance, the primary smolt-related changes are associated with up-regulation of the seawater isoform and salinity tolerance, with little to no evidence for compensatory changes in ion uptake capacity.

It is interesting to note that in addition to the large increases of gill NKAα1b abundance in smolts, there were also moderate increases in the abundance of gill NKAα1b in parr (Fig. 3B). Although all of the parr died within 4 days of exposure to seawater, in past studies we have observed that the time to death after seawater exposure increases in parr during spring (S.D.M., A.M.R. and A.K.C., unpublished results), providing some evidence of a moderate, endogenous change in salinity tolerance in parr. This is consistent with the idea that seasonal changes in salinity tolerance may be an ancestral state in salmonids and that parr–smolt transformation has evolved as accentuation and intensification of seasonal changes that occur in parr (Dickhoff and Sullivan, 1987). This observation also suggests that the greater magnitude of increases in gill NKAα1b observed in smolts are likely to be critical to the greater salinity tolerance of smolts compared with parr.

After 2 weeks of seawater exposure at the peak of smolt development, the abundance of NKAα1b increased another twofold above freshwater levels. This increase occurred gradually, as there were no detectable changes after 2 days in seawater. In contrast, the abundance of gill NKAα1a decreased within 2 days of seawater exposure and is only a fraction of its initial levels after 14 days in seawater. This is consistent with previous research on the influence of salinity on protein abundance (McCormick et al., 2009) and gene transcription (Nilsen et al., 2007). This increase probably results in a greater overall capacity for salt secretion in the gill, representing the steady state needed for all activities in seawater.

Gill ionocytes of smolts in February primarily contain NKAα1a, with a few small NKAα1b cells also present. During smolt development, the number of NKAα1b cells and co-labeled cells increases, with a slight decrease in the number of NKAα1a ionocytes. Many of these cells do not appear to have exposure to the surface, but are covered by pavement cells, and overall there is only a modest increase in the total number of ionocytes. After seawater exposure, NKAα1b becomes the dominant ionocyte, with a small number of NKAα1a cells that are below the tissue surface. Thus we propose a model (Fig. 8) in which NKAα1b and co-labeled cells of freshwater smolts have no external exposure and are inactive prior to exposure to seawater, then are rapidly activated upon exposure to seawater. We propose that most NKAα1a cells are deactivated, and either transform to NKAα1b cells (especially if they are on the filament) or undergo apoptosis, and that any remaining NKAα1a cells are below pavement cells and inactive. More detailed work on surface exposure of each cell type will be necessary to confirm this model. An important part of this model is that a large pool of NKAα1b is already present in cells and ready to be activated. Parr have poorer seawater tolerance because they

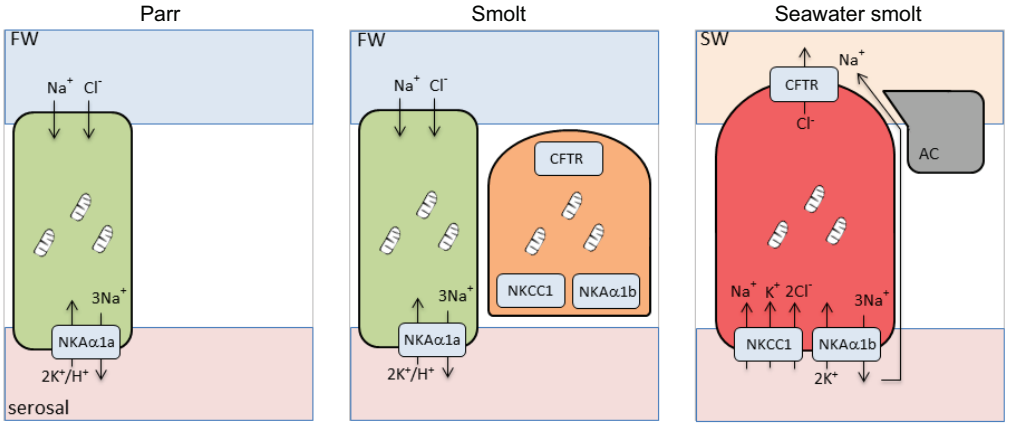

Fig. 8. Model for differential expression of NKAα1a and NKAα1b during smolt development. In freshwater (FW) parr and smolts, the gill epithelium is rich in ionocytes that express the freshwater NKA isoform, NKAα1a. In freshwater smolts, an additional class of ionocytes represented as an orange cell are present; these cells express NKAα1b or both NKAα1a and NKAα1b, as well as other ion transporters (CFTR and NKCC1) that are important for salt secretion. These NKAα1b-expressing ionocytes in freshwater smolts are likely to be inactive in freshwater but rapidly initiate salt secretion after exposure to seawater. AC, accessory cell.

have very few NKAα1b and associated cells, and cannot induce them rapidly enough to counteract the passive gain of ions and loss of water.

In general, there is a strong correlation between the abundance of NKA isoforms and their respective ionocytes. However, there are some differences in their absolute quantitation during smolt development and after seawater exposure. In particular, we observed a 10-fold increase in NKAα1b abundance, whereas the number of NKAα1b and co-labeled cells increased relatively modestly. This is probably due to the semi-quantitative nature of immunocytochemistry, which can uncover the number of cells, but not the quantity of NKA pumps within each cell. Previous work indicates that the density of ionocyte NKA pumps is higher in seawater- than in freshwater-adapted fish (Karnaky et al., 1976). It seems likely that the amount of NKAα1b in each ionocyte increases progressively during smolt development, and indicates that the quantitation of cell numbers underestimates the amount of NKAα1b that is available for rapid activation after seawater exposure.

It should also be noted that while we did not find significant changes in the abundance of gill NKAα1a during smolt development, there was a significant decrease in the number of filamental NKAα1a ionocytes in spring. If NKAα1b and co-labelled ionocytes are less functional for ion uptake, then this reduction in NKAα1a ionocytes may result in reduced ion uptake capacity of smolts. Such an impact would explain the reduced ion influx observed in smolts (Primmett et al., 1988) and the greater ion perturbations following handling stress in smolts relative to parr (Carey and McCormick, 1998). Superior ion uptake capacity in parr compared with smolts is also supported by the greater abundance of NKAα1a in the former (Fig. 2).

It is of interest to note that there were physiological differences between parr and smolt even in early winter, when hematocrit, gill NKA activity and gill NKAα1a abundance were lower in parr than in smolt, whereas gill NKAα1a was greater in parr. As is typical of smolts, condition factor decreased in spring, whereas condition factor of parr increased. There were parallel changes in gill NKA activity and NKAα1b in parr and smolt in spring, though the magnitude of change was substantially greater in smolts. Smolts had substantially higher salinity tolerance, as indicated by the lower plasma chloride after 2 days in seawater, 100% survival and no change in either hematocrit or condition factor. In contrast, all parr died within 4 days of seawater exposure, had high levels of plasma chloride and hematocrit and reduced condition factor, all of which are characteristic of high salt/dehydration stress. The basis for this difference is likely to be the high initial levels of gill NKAα1b, and the inability of parr to induce this enzyme and other ion transporters quickly enough to counteract the passive influx of ions.

Previous studies have indicated that the distribution of ionocytes differs between fish in freshwater and seawater, with the most common pattern being the occurrence of ionocytes in both filament and lamellae in freshwater, but primarily in the filament in seawater (Evans et al., 2005). We examined the distribution of NKAα1a and NKAα1b in both filament and lamellae to examine whether smolt development or seawater exposure would alter this pattern in Atlantic salmon. We found that the total abundance of ionocytes did not change in the filament during smolt development in freshwater but increased slightly in the lamellae. Cells staining only for NKAα1b only were found only in the filament, whereas co-labeled cells were found in both filament and lamellae. These results are consistent with observations of *in situ* hybridization studies that found NKAα1b primarily in the filament, whereas NKAα1a was expressed in both filament and lamellae (Madsen et al., 2009). New ionocytes appear to arise from the basal membrane of the filament (Evans et al., 2005), and the observation that NKAα1b-only cells are found exclusively on the filament provides indirect support that these cells arise from stem cells, whereas co-labeled cells may be transforming from NKAα1a to NKAα1b cells. The appearance of NKAα1b during smolt development was therefore primarily, though not exclusively, in the filament, and after seawater exposure almost all ionocytes were NKAα1b and filamental.

We found that there were significant increases in plasma growth hormone and cortisol that preceded the peak in gill NKAα1b of smolts. In contrast, plasma GH and cortisol did not increase in parr. These findings are consistent with previous studies comparing parr and smolts, and suggest that the capacity of GH and cortisol to respond to increased day length may be the major factor underlying smolt development (McCormick et al., 2007). This is further supported by the ability of exogenous GH and cortisol to up-regulate

transcription of gill ion transporters involved in salt secretion (e.g. NKAα1b, NKCC1, CFTRI) and salinity tolerance (McCormick et al., 2008; Tipsmark and Madsen, 2009). Thus it is likely that the increase in gill NKAα1b and salinity tolerance seen in smolts in the present study is due to increases in plasma GH and cortisol that occur during smolt development.

Previous studies indicate that cortisol can increase the transcription of gill NKAα1a and NKAα1b in Atlantic salmon (McCormick et al., 2008; Tipsmark and Madsen, 2009). In the present study, there is no increase in gill NKAα1a during smolt development in spite of large increases in plasma cortisol levels. One possible explanation is that the increase in growth hormone that occurs during smolt development may promote gill NKAα1b at the expense of gill NKAα1a. Alternatively, divergence in the promoter sequences of the NKA isoform genes might have given rise to NKAα1a-specific silencing elements that are not present in the regulatory regions of the NKAα1b gene. Promoter sequence analysis upon publishing of the Atlantic salmon genome should yield some insight into this possibility. In either event, the effects of cortisol appear to be dynamic and dependent on the induction, or extent, of smolt development.

Increases in plasma cortisol after seawater exposure have been widely reported in teleosts (Mommsen et al., 1999). We observed significant increases in plasma cortisol and chloride in parr after exposure to seawater for 2 days, and all parr had died by 4 days. In contrast, smolts exhibited only moderate increases in plasma cortisol and chloride after seawater exposure, and no mortalities. In coho salmon (*Oncorhynchus kisutch*) the levels of plasma cortisol after exposure to seawater for 24 h were lower at the peak of smolt development in late April than before or after when salinity tolerance was poorer (Young et al., 1995). These results suggest that the increase in plasma cortisol is not solely driven by exposure to seawater, but relative to the levels of plasma ions in each individual.

The focus of the present study has been changes in the major isoforms of the catalytic subunit of NKA that are present in gill ionocytes. Previous research has established that salt secretion operates primarily through the action of NKA, NKCC and CFTR (Evans et al., 2005). Increased transcription of NKCC and CFTR in the gill has been observed during smolt development (Nilsen et al., 2007), and the abundance of gill NKCC and NKCC-rich ionocytes has also been found (Pelis et al., 2001). It therefore seems likely that increased abundance of NKAα1b and NKAα1b-ionocytes during smolt development is part of an ionocyte differentiation process that also includes changes in NKCC and CFTR, a possibility that is currently under investigation.

Protein isoforms can arise from duplicated genes, alternative splicing of a single gene or allelic diversity. NKAα1a and NKAα1b are the products of two distinct genes (Richards et al., 2003). Exchanging key residues that differ between NKAα1b and NKAα1a into porcine NKA, Jorgensen (Jorgensen, 2008) found that the affinity for K^+ was reduced, and suggested that H^+ may substitute for K^+ during ion translocation. If these changes are a reflection of NKAα1a function, this could reduce the need for K^+ recycling in freshwater, thereby reducing energetic costs. The need for an electrogenic sodium–potassium pump to make the inside of the ionocyte highly negative in order to drive Cl^- excretion would probably preclude such a development for fish in seawater.

Genome duplication events have occurred several times during vertebrate evolution, including a genome duplication that occurred between 25 and 100 million years ago within the ancestral salmonids. As a result, existent salmonids can be considered pseudo-tetraploid, with duplicate copies of most genes (Davidson et al., 2010). Such gene duplication may allow for diversification of enzyme function without compromising existing capabilities (Nei, 1969; Lynch, 2007). Duplication of NKA alpha subunit genes may have relaxed the selective pressure on some paralogs such that they could acquire novel functions and accumulate distinct regulatory elements. These concepts are reflected in our results, which indicate that salmon have evolved two functionally distinct, differentially regulated, branchial sodium–potassium pumps. The demonstration that the abundance of these sodium–potassium pumps are differentially regulated by development, salinity and hormones suggests that diversity of isoform function and regulation may represent a key evolutionary mechanism that has allowed for the migratory life history strategy of anadromous salmonids. Differential regulation of key enzyme isoforms associated with life history transitions has not been widely reported and may be a more common feature than currently appreciated.

LIST OF SYMBOLS AND ABBREVIATIONS

CFTR	cystic fibrosis transmembrane regulator
GH	growth hormone
NKA	Na^+/K^+-ATPase
NKCC	$Na^+/K^+/Cl^-$ co-transporter

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