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$Na^+/K^+/2Cl^-$ cotransporter and CFTR gill expression after seawater transfer in smolts (0^+) of different Atlantic salmon (*Salmo salar*) families

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Abstract

Smoltification involves morphological and physiological changes in the gills that prepare anadromous salmonids to osmoregulate efficiently in seawater. In a previous study, we found that different families of Atlantic salmon (*Salmo salar*) smolts vary in their ability to osmoregulate when abruptly transferred to cold seawater and that these differences are correlated with gill Na^+/K^+ ATPase activity. Here we extend these findings to test whether other key transport proteins, namely $Na^+/K^+/2Cl^-$ contransporter (NKCC) and the Cl^- channel or cystic fibrosis transmembrane conductance regulator (CFTR), play a significant role in osmoregulatory differences between families. To facilitate molecular analysis of NKCC, we first isolated a gill cDNA containing the complete coding region (1147 aa) of an isoform previously reported as a partial sequence. Phylogenetic analysis showed that this isoform is most closely related to isoforms of the NKCC1a subfamily found in European eel and Mozambique tilapia. In a second step, we quantified NKCC protein abundance as well as mRNA expression levels for NKCC1a and two CFTR isoforms (CFTRI and CFTRII) in 0+ smolts from three families prior to and following seawater transfer. The family with the lowest salinity tolerance also showed significant increases in gill NKCC1a mRNA after seawater transfer. Taken together with our previous study, these data indicate that family differences in expression of transport proteins are in part related to salinity tolerance, although the best indicator of osmoregulatory performance between families may be gill Na^+/K^+ ATPase activity and CFTR I mRNA levels, rather than Na^+/K^+ ATPase and NKCC1a mRNA levels or NKCC protein abundance.

Keywords: Smoltification; Osmoregulation; Chloride cell; Salinity tolerance; *Salmo salar*; NKCC

1. Introduction

Smoltification in anadromous species such as Atlantic salmon (*Salmo salar*) involves a series of complex physiological, morphological and behavioural changes that prepare the fish for seaward migration. One of the critical aspects of this transformation is the ability

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of the gills to secrete NaCl against a large inward gradient (water-to-blood). This task falls to the gill chloride or mitochondrial rich (MR) cells that increase in number and size during both smoltification (Langdon and Thorpe 1985; Richman et al., 1987; Uchida et al., 1997) and seawater transfer (eg. Foskett et al., 1981; Shikano and Fujio 1998; Pelis et al., 2001; McCormick et al., 2003; for reviews see Marshall 2002; Evans et al., 2005). In salmonids, chloride cell proliferation and seawater acclimation is regulated by cortisol along with growth hormone/insulin-like growth factor I (Pelis and McCormick 2001; for a review, see McCormick 2001). Three key transport proteins co-localized to gill MR cells are associated with hyposmotic regulation in seawater: Na^+/K^+ ATPase, $Na^+/K^+/Cl^-$ cotransporter (NKCC), and a chloride channel, homologous to the cystic fibrosis transmembrane conductance regulator (CFTR) type anion channel. Marshall (2002) suggested that all three transporters can be up-regulated in concert in response to seawater acclimation.

Gill Na^+/K^+ ATPase activity is up-regulated both during smoltification and seawater transfer in salmonids, as well as other euryhaline fishes (eg. Madsen et al., 1995; Pirhonen and Forsman 1998; Lysfjord and Staurnes 1998; D'Cotta et al., 2000; Mancera and McCormick 2002; Sangiao-Alvarellos et al., 2005; Bystriansky et al., 2006), but this is not universal (McCormick et al., 2003). Of the many isoforms of Na^+/K^+ ATPase expressed in salmonids (Semple et al., 2002; Gharbi et al., 2005), gill α1a mRNA levels decrease in response to seawater exposure, whereas α1b mRNA levels increase in both rainbow trout (Richards et al., 2003) and Atlantic salmon (Mackie et al., 2005). There is less information on NKCC and CFTR responses to seawater because, unlike Na^+/K^+ ATPase, these proteins do not lend themselves to simple assay procedures.

There are two major subclasses of NKCC isoforms identified in vertebrates (see Hebert et al., 2004 for a recent review). NKCC1, also known as SLC12A2, is the secretory form of the protein. It is found on the basolateral membrane of epithelial cells and ubiquitously expressed in a wide range of tissue types. NKCC2 (SLC12A1), in contrast, is recognized as an absorptive isoform specific to the kidney whose localization is restricted to the apical membrane of thick ascending limb (TAL) cells. In mammals, NKCC1 and NKCC2 are each encoded by a single gene (Russel, 2000). In the European eel, however, NKCC1 is known to be expressed as two distinct genes, with NKCC1a found in most tissues and NKCC1b primarily expressed in the brain (Cutler and Cramb 2001, 2002). More recently, duplicate eel isoforms have also been reported for NKCC2 (accession no. AJ564602, AJ564603) and two NKCC1 homologs have been isolated from Mozambique tilapia (Hiroi et al., 2005). Gill NKCC1a mRNA levels were significantly higher in European yellow eels (Cutler and Cramb, 2002) and seabass (Lorin-Nebel et al., 2006) in seawater versus freshwater acclimated-fish. In Atlantic salmon, gill NKCC protein abundance was found to increase during smolting and in response to seawater in Atlantic salmon (Pelis et al., 2001; Tipsmark et al., 2002).

Following entry of chloride into MR cells via NKCC, chloride secretion occurs on a downhill electrical gradient via an apical chloride channel homologous to CFTR. Gill CFTR mRNA levels and protein abundance increase after transfer to seawater in killifish (Singer et al., 1998; Scott et al., 2004b). Atlantic salmon express two isoforms of CFTR (CFTRI and II) in gill tissue (Chen et al., 2001), which appear to be regulated independently. Following transfer of Atlantic salmon from freshwater to seawater, gill CFTRI mRNA levels increased progressively over 2 weeks, whereas CFTRII mRNA levels were elevated only at 24 h (Singer et al., 2002). Furthermore, cortisol implantation in Atlantic salmon stimulated CFTRI mRNA levels in the gills but not CFTRII mRNA levels (Singer et al., 2003). Thus, gill Na^+/K^+ ATPase, NKCC and CFTR are all regulated with seawater exposure, although there is some variation between studies and species.

There is very little information on whether a rapid regulation of one or more of these transporters upon salinity change confers improved osmoregulatory performance. Northern populations of killifish had increased gill Na^+/K^+ ATPase mRNA expression and activity, reduced plasma Cl^- imbalance, and lower mortality than southern populations following transfer from seawater to freshwater (Scott et al., 2004a). Singer et al. (2002) reported significant differences in plasma Cl^- and Na^+ concentrations and gill CFTRII gene expression between two aquaculture strains of Atlantic salmon following seawater exposure. Further examination of the same strains implanted with cortisol revealed differences in gill Na^+/K^+ ATPase activity and CFTRI mRNA levels, but not in the mRNA levels of Na^+/K^+ ATPase α-subunit or CFTR II (Singer et al., 2003). In our previous study, we found that families of Atlantic salmon smolts vary in their ability to osmo- and iono-regulate following abrupt transfer to cold seawater (Mackie et al., 2005). The family with the lowest osmolality at 24 h also displayed the lowest plasma Cl^- concentrations, as well as the highest gill Na^+/K^+ ATPase activity. These findings suggest that osmoregulatory performance between salmon families is coupled to gill ion transport proteins.

In the present study, we extend our previous research to test whether interfamily differences in osmoregulatory performance may be associated with differences in the regulation of NKCC and/or CFTR ion transporters in the gill. The first aim of the study was to isolate a gill cDNA containing the complete coding region of an NKCC isoform (NKCC1a) previously reported as a partial transcript by Tipsmark et al. (2002). Our second objective was to determine if interfamily differences occur in the mRNA levels and abundance of NKCC, as well as the mRNA expression of CFTRI and II, following transfer to seawater. For this purpose we selected three of the eleven families examined by Mackie et al. (2005) that exhibited high, intermediate, and low osmolality levels following seawater exposure, quantified NKCC protein abundance and mRNA expression levels for NKCC1a, CFTRI, and CFTRII, and compared the results with plasma osmolality and Cl^- concentration measured previously. The families used in the present study and our previous study (Mackie et al., 2005) were produced at the Atlantic Salmon Broodstock Development Program (Chamcook, New Brunswick) using full-sibling crosses of commercial strains developed from wild Bay of Fundy salmon (Saint John River). For further details see Mackie et al. (2005).

2. Materials and methods

2.1. Animals

The families used in this study were selected from the eleven families tested for osmoregulatory performance following cold seawater challenge in Mackie et al. (2005). Significant differences in osmolality between seawater exposed families were only observed at 24 h post seawater transfer and these results were used to select three families for further investigations: family K, which had the lowest average osmolality, family H, which had the highest average osmolality, and family I, which had a value between K and H, but was not significantly different from either (see Table 1 in Mackie et al., 2005).

2.2. Experimental protocol

The experimental protocol for this study was identical to that outlined in Mackie et al. (2005). Briefly, fish were fasted for 24 h before sampling and the water supplying the tanks to the seawater exposed group was switched from fresh water to filtered seawater (33 ppt, pH=8.0, 1.9–4 °C, dissolved O_2=12.2 mg L^{-1}, flow rate 10 L min^{-1}) so that within 1.5 h full strength seawater was achieved (0 h). To assess the timeline of physiological changes due to seawater exposure, fish held in freshwater were sampled at 0, 24, 96 h and 30 days and an equal number of seawater exposed fish were sampled at 24, 96 h and 30 days following full-strength seawater exposure. To test for differences between families within each treatment, ten individuals were sampled per family. To rule out tank effects, five of the individuals were collected from each of two tanks for the same treatment. The fish were anesthetized with MS-222 (150 mg L^{-1}), weighed and fork length was measured. Fish were killed by a sharp blow to the head. The gill arches were removed, immediately frozen (liquid nitrogen) and stored (−80 °C) for later protein and mRNA expression analysis.

2.3. Molecular cloning and sequencing

2.3.1. Initial cloning

Degenerate PCR primers were designed from the multiple alignment of cDNA sequences coding for vertebrate NKCC isoforms (Table 1). Trizol was used to extract total RNA from gill tissue of seawater exposed Atlantic salmon following the manufacturer's protocol. First-strand cDNA was synthesized using the protocol described in Mackie et al. (2005). HotStar Taq DNA polymerase (Qiagen, Mississauga, ON, Canada) was used for the PCR. The 25 μl reaction consisted of 2.5 μl of reaction buffer (without $MgCl_2$), 1 mM $MgCl_2$, 0.2 mM dNTP (MBI Fermentas, Burlington, ON, Canada), 0.2 μM each of forward and reverse primer, 1 U of Taq and 5 μl of cDNA diluted 10-fold with DNase/RNase free water. DNA was amplified with the following conditions: 94 °C for an initial

Table 1
List of primers used for molecular cloning, gene mapping, and gene expression analysis

Primer	Sequence (5′→3′)	Orientation	Application
NKCCF	AAVATVTGGGGTGTSATGCT	Forward	Initial cloning
NKCCR	TAVGANGCMARGAARAARTT	Reverse	Initial cloning
NKCC1-F	GGAATCTCGGAAGAGTTGCGGCA	Forward	RACE cloning; full-length cloning
NKCC2-R	GGGGCTTGGTGTTGATGTCTC	Reverse	RACE cloning
NKCC13	ATCATCATATATCAATGGCTACGG	Reverse	Full-length cloning
NKCC08	TCTGAATTCGAAAGCACCGC	Forward	Gene expression
NKCC09	TAAATGTCCGGCACAACTCG	Reverse	Gene expression
B-actin F	ATGGGCCAGAAAGACAGCTA	Forward	Gene expression
B-actin R	CCAGAGGCGTACAGGGACAA	Reverse	Gene expression

15 min, 35 cycles of 94 °C for 1 min, 53 °C for 1 min, 72 °C for 1 min, followed by a final 72 °C for 10 min. PCR products separated by gel electrophoresis (1% agarose gel in TAE buffer; 40 mM Tris-acetate, 1 mM EDTA, pH 8.0) were cloned into a pGEM-T Easy plasmid vector (Promega, Madison, Wisconsin, USA). Clones were sequenced in both directions using Big Dye Terminator Cycle Sequencing Kit version 3.0 (Applied Biosystems) and found to be most similar to the mammalian (*Mus musculus*; GenBank accession no. U13174), spiny dogfish (*Squalus acanthias*; GenBank accession no. U05958) and eel (*Anguilla anguilla*; accession no. AJ486858) NKCC transporters by BLAST analysis (Altschul et al., 1997) against the GenBank protein database.

2.3.2. Full-length cloning

To obtain the full coding sequence of the target cDNA, the SMART™ Race cDNA amplification kit was used (Clontech, BD Sciences, San Jose, California, US plasmid vector A) and various NKCC specific primers were designed to amplify 5′ and 3′ RACE PCR products from the constructed cDNA library (Table 1). However, RACE-PCR consistently yielded a truncated fragment lacking the 3′ end of the cDNA and an alternative strategy was therefore used to obtain the complete coding region. An expressed sequence tag (EST) was identified from rainbow trout (GenBank accession no. CA382971) that overlapped with the 3′ portion of the cloned fragment and extended into the 3′ UTR of the cDNA. This was used as a template to design an alternative reverse primer (NKCC13) for RT-PCR and amplify across the entire coding region (Table 1). Cloning and sequencing of the RACE fragments were all performed as described above and Hi-Fidelity Taq (Invitrogen) was used for RT-PCR under the following conditions: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 4 min, and a final extension of 72 °C for 10 min.

2.4. Sequence analysis

Primer design, contig assembly, and other sequence manipulations were carried out using Vector NTI Advance (Invitrogen). Similarity searches were preformed with BLAST programs at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Transmembrane protein domains were predicted using TMHMM, version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

2.5. Phylogenetic analysis

Phylogenetic analyses were performed in MEGA, version 3.1 (Kumar et al., 2004) using a set of protein sequences coding for NKCC isoforms expressed in European eel (*Anguilla anguilla*), Mozambique tilapia (*Oreochromis mossambicus*), seabass (*Dicentrarchus labrax*), humans (*Homo sapiens*), rat (*Rattus norvergicus*), mouse (*Mus musculus*), spiny dogfish (*Squalus acanthias*), and tobacco hornworm (*Manduca sexta*) downloaded from the GenBank database (see legend of Fig. 2 for accession numbers). Sequences were aligned using default CLUSTAL parameters and a Neighbor-Joining (NJ) tree was constructed assuming a Poisson model with equal substitution rates among sites and complete deletion of gaps. The robustness of nodes in the NJ tree was estimated by bootstrap analysis with a total of 1000 replicates.

2.6. Semi-quantitative reverse transcriptase polymerase chain reaction

Preparation of gill tissue for mRNA analysis is described above. PCR was performed on gill cDNA using primers designed to amplify a 162 bp fragment of the NKCC1a gene located in the 5′ UTR region (Table 1). Details of the primers used to isolate fragments of the Atlantic salmon CFTR I and CFTR II isoforms were previously described by Singer et al. (2002). Taq DNA polymerase (5 U μl^{-1}; Invitrogen Canada Inc, Burlington, Ontario) was used in all PCR reactions (Master Gradient Thermocycler, Eppendorf AG, Hamburg, Germany). The thermal cycling protocol to amplify the NKCC gene consisted of 2 min at 94 °C, followed by 27 cycles of 30 s at 94 °C, 30 s at 58 °C, and a 1 min at 72 °C. A final step of 72 °C for 10 min completed the product extension of annealed primers. The PCR protocol for CFTRI and CFTRII included both primers in a single reaction and a thermal cycling profile consisting of 2 min at 94 °C, followed by 25 cycles of 30 s at 94 °C, 30 s at 60 °C, and a 1 min at 72 °C, with a final extension of 72 °C for 10 min. β-actin (299 bp) was used as a control gene for all PCR reactions. Preliminary experiments showed that the expression of β-actin in the gills did not change between freshwater and seawater-exposed fish ($P > 0.05$). The PCR protocol was optimized to ensure the number of PCR cycles was within the linear range for the genes of interest and β-actin. Each sample was run in duplicate.

2.7. SDS-page and Western Blotting

NKCC protein abundance was quantified in the gill using sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blotting following the protocol by Pelis and McCormick (2001) with modifications. The gill filaments from the first and fourth gill arches were prepared using partial membrane purification (Zaugg, 1982). Approximately 1 g of tissue was added to 10 vol. of ice-cold homogenization buffer (150 mM sucrose, 10 mM EDTA, 50 mM Imidazole) treated with Complete mini-protease inhibitor cocktail tablets (Roch Diagnostics; 1 tablet per 10 ml solution). The tissue was homogenized by hand using a glass homogenizer to remove the soft tissue from the cartilage and centrifuged at 3000 rpm for 7 min at 4 °C. The supernatant was discarded and the pellet resuspended in 0.5 ml of ice-cold homogenization buffer with protease inhibitor tablets and 0.1% deoxycholic acid. The re-suspension was homogenized by hand, centrifuged at 3000 rpm for 6 min at 4 °C, and the supernatant was collected. Protein concentration of the supernatant was quantified using a commercial BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, Illinois). The remaining supernatant was placed in Laemmli sample buffer, heated in a water bath at 65 °C for 15 min and stored at −80 °C.

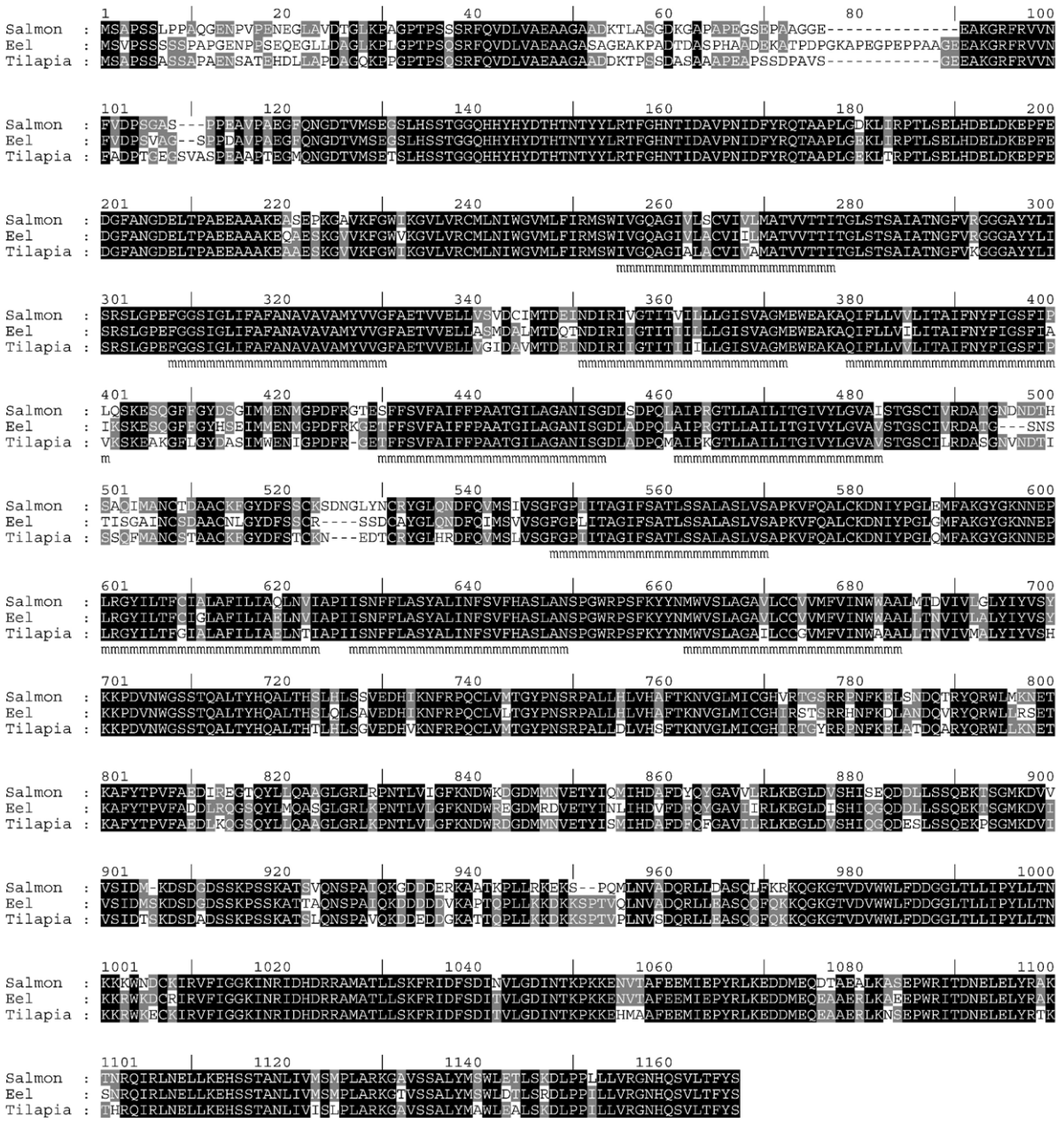

Fig. 1. Amino acid alignment of NKCC1a isoforms found in Atlantic salmon (DQ864492), European eel (CAD31111), and Mozambique tilapia (AAR97731). Residues shaded in black represent amino acids conserved across all three species, while those in grey shade are shared by only two of the three species. Dashes indicate gaps in the alignment. Lowercase m's indicate the location of putative transmembrane domains along the salmon sequence as predicted by TMHMM.

The membranes were loaded on a two-phase SDS-PAGE mini gel (top phase: 4% stacking gel; bottom phase: 6% separating gel) at 10 μg of protein per lane. The gels were run for approximately 1.5 h in running buffer (0.025 M Tris base, 0.192 M Glycine, 0.01% Sodium dodecyl sulfate wt./vol. in deionized water adjusted to pH 8.3) followed by overnight transfer to immobilon P (polyvinylidene fluoride (PVDF)) membranes (Millipore, Bedford, Massachusetts) in transfer buffer (0.025 M Tris base, 0.192 M Glycine, pH 8.3). The PVDF membranes were rinsed with distilled water and soaked in blocking buffer (5% skim milk wt./vol. in PBS containing 10% Triton X-100 (PBST)) for 1 h, then rinsed three times in PBST and incubated in T4-primary antibody (600 μg/ml; Pelis et al., 2001) for the $Na^+/K^+–2Cl^-$ cotransporter diluted 1:1000 in

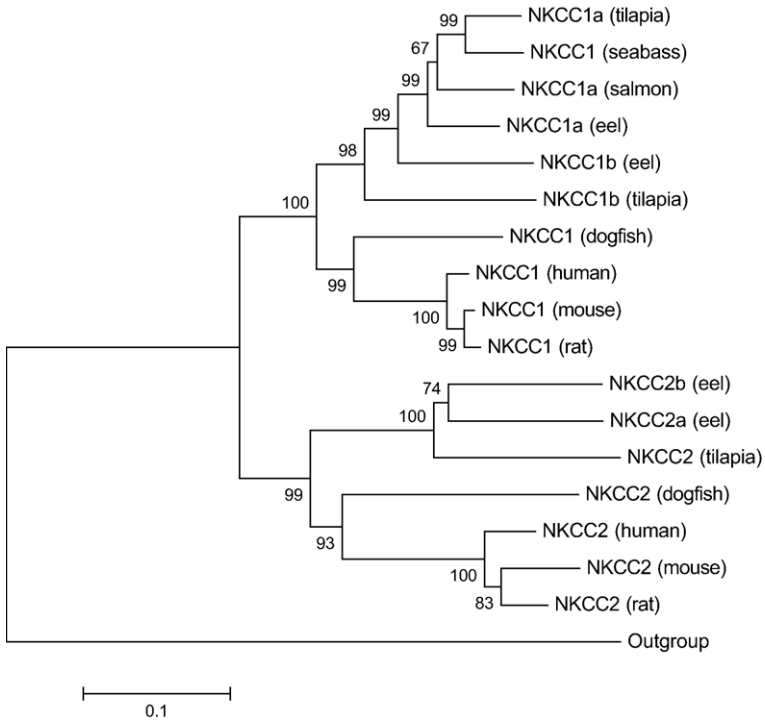

Fig. 2. Neighbor-joining phylogenetic tree depicting evolutionary relationships among NKCC isoforms expressed in spiny dogfish (NKCC1: AAB60617; NKCC2: AAM74966), human (NKCC1: NP_001037; NKCC2: NP_00032), mouse (NKCC1: NP_033220; NKCC2: NP_035519), rat (NKCC1: NP_113986; NKCC2: NP_062007), Mozambique tilapia (NKCC1a: AAR97731; NKCC1b: AAR97732; NKCC2: AAR97733), European eel (NKCC1a: CAD31111; NKCC1b: CAD31112; NKCC2a: CAD92100; NKCC2b: CAD92101), seabass (NKCC1: AY954108) and Atlantic salmon (NKCC1a: DQ864492). The outgroup sequence used to root the tree was derived from tobacco hornworm (AAA75600). Numbers at each node indicate bootstrap values from 1000 replicates.

PBST for 1 h. Membranes were rinsed three times in PBST and incubated in peroxidase-labeled goat anti-mouse IgG (H+L) (2 μg/ml; Kirkgaard & Perry Laboratories, Gaithersburg, MD) diluted 1:1000 in PBST. The membranes were again rinsed three times in PBST followed by a final rinse in PBS. Immunoreactivity was detected with DAB buffer (3,3′ Diaminobenzidine tetrahydrochloride (DAB; Pierce, Rockford, Illinois).

To obtain staining intensity, membranes were scanned and band intensity was measured using ImageJ 1.34 s (Wayne Rashband, National Institutes of Health, USA (2004); http://rsb.info.nih.gov). The area selected for densitometry measurements was constant for every lane on an individual gel. Band intensity was plotted as the pixel intensity against distance along gel lane. The area under the resulting curve was used to compare band density between samples. An internal control was also added to one lane on every gel to standardize protein density measurements across all gels. Bands were compared to a standard protein ladder (Precision protein — broad range, BioRad Laboratories, Hercules, California) to estimate the size of the isolated protein.

2.8. Statistics

All data are expressed as the mean±the standard error of the mean (SE). Analysis of variance was used to compare means with SAS 8.2 statistical software (SAS Institute Inc., Cary, North Carolina). A one-way analysis of variance was used to determine whether there were initial differences among families in fresh water. A three-way analysis of variance was used to test main effects and their interactions. Least square means was used to determine if there was a significant tank effect. Tank effect was not significant and the data from tanks A and B were pooled. If main effects (family, salinity or time) or interactions were significant an analysis of variance followed by Student–Newman–Keuls test was used to test the null hypothesis that there were no differences between the means of the families within a treatment group (freshwater or seawater) at a specific time period. The Brown and Forsythe test was used to assess homogeneity of the means and the Shapiro–Wilks test assessed normality. All means were compared using a 95% confidence interval.

3. Results

3.1. Molecular cloning of NKCC1a in Atlantic salmon

Cloning experiments yielded a cDNA of 4040 bp (GenBank accession no. DQ864492) comprised of a 5′-UTR (491 bp), an uninterrupted open reading frame (3444 bp), and a short 3′-UTR (105 bp). The putative coding region was predicted to encode a 1147 amino acid protein containing ten potential transmembrane domains in the central portion (Fig. 1). Sequence comparisons

with the partial cDNA sequence previously reported by Tipsmark et al. (2002) revealed a nearly perfect match, with the exception of a single non-synonymous substitution (A→G) at position 2815, causing a glutamine residue to be replaced by arginine in the protein sequence.

Pair-wise sequence comparisons of the coding region with a wide range of NKCC isoforms found in other vertebrates indicated that the sequence isolated from Atlantic salmon shares 81–92% and 73–75% amino acid similarity with members of the NKCC1 and NKCC2 subfamilies, respectively. To investigate these results further, we performed a phylogenetic analysis with isoforms found in human, mouse, rat, Mozambique tilapia, European eel, and spiny dogfish (Fig. 2). As expected, NKCC1 and NKCC2 co-transporters were separated into two different lineages with the salmon isoform grouping with other NKCC1 sequences. Furthermore, the salmon sequence formed a separate cluster with NKCC1a isoforms, which represent one of two NKCC1 transcripts expressed in tilapia and eel (NKCC1a/b). Indeed, comparisons of the salmon protein (thereafter denoted NKCC1a) with those of tilapia and eel revealed remarkably high amino acid conservation, although substantial variation was observed in the N-terminal region (Fig. 1).

3.2. Initial expression of transporters in fresh water

There were no clear differences in initial fresh water levels of gill NKCC1 mRNA levels among families (Fig. 3, $p=0.36$). Gill NKCC protein levels in fresh water smolts were initially higher in family K than family H, though these differences were not statistically significant (Fig. 4). The levels of gill CFTR I mRNA were highest in family K and lowest in family H though these differences were significantly different only at $p=0.08$ (Table 2). There were no clear differences in initial fresh water levels of gill CFTR II mRNA levels among families (Table 2, $p=0.35$).

3.3. Seawater transfer: family differences in gill ion transporters

After 24 h in seawater, family K had significantly lower plasma osmolality (340±4.0 mOsm), relative to family H (357±5.5 mOsm) and the values for family I (351±3 mOsm) were midway between the other two families ($p>0.05$). Plasma osmolality was also measured 4 and 30 days after transfer to seawater but no family differences were detected (Mackie et al., 2005). Following transfer to seawater, family H had significantly higher gill NKCC1 mRNA levels compared to family I or K at 96 h (Fig. 3). The abundance of NKCC protein was not significantly influenced by family, though the absolute levels of NKCC protein were higher in family K than family H (Fig. 4). There was a significant interaction between time and salinity on the expression of CFTRI and CFTRII. After 24 h in seawater families K and I had significantly higher CFTRI mRNA compared to family H (Table 2).

3.4. Seawater transfer: time frame of response

Family H had significantly higher gill NKCC1a mRNA levels after 24 and 96 h of seawater exposure relative to freshwater controls (Fig. 3). As well, the time course of gill NKCC1a mRNA levels in family H changed over time. A peak in gill NKCC1a mRNA levels occurred at 96 h post seawater transfer, with levels decreasing again after 30 days of exposure (Fig. 3). There were no significant differences in NKCC1a

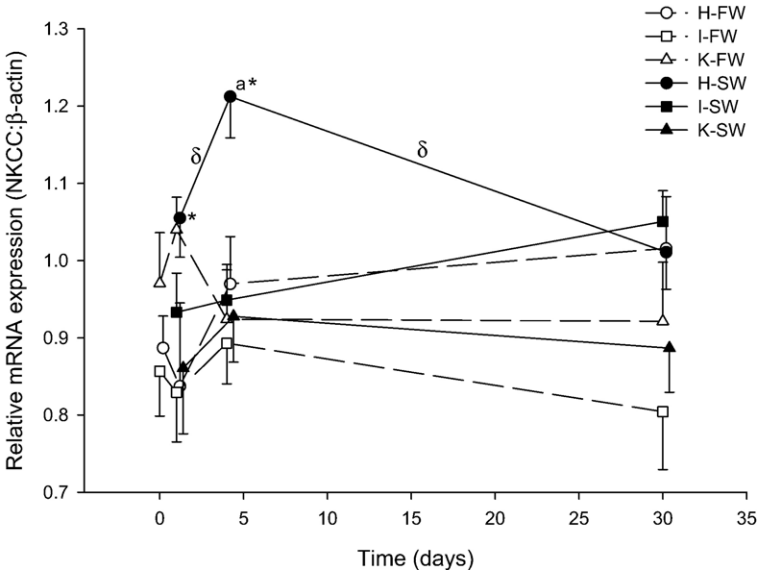

Fig. 3. Relative gill NKCC1 mRNA levels (NKCC1:β-actin) of families K (triangle), I (square), and H (circle) in freshwater (dashed lines) and seawater (solid lines) sampled 0 h, 1 day, 4 days and 30 days post transfer. Plots were shifted slightly along the X-axis to clarify standard error bars. Lower case letters denote significant differences between families within a treatment (freshwater or seawater) and sample time ($P<0.05$). An asterisk indicates a significant difference between freshwater and seawater fish within a family and time period ($P<0.05$). δ on a line indicates a significant change in expression between the two time periods (before and after the symbol) within a family and treatment ($P<0.05$). Values are means±standard error of the mean ($n=8$).

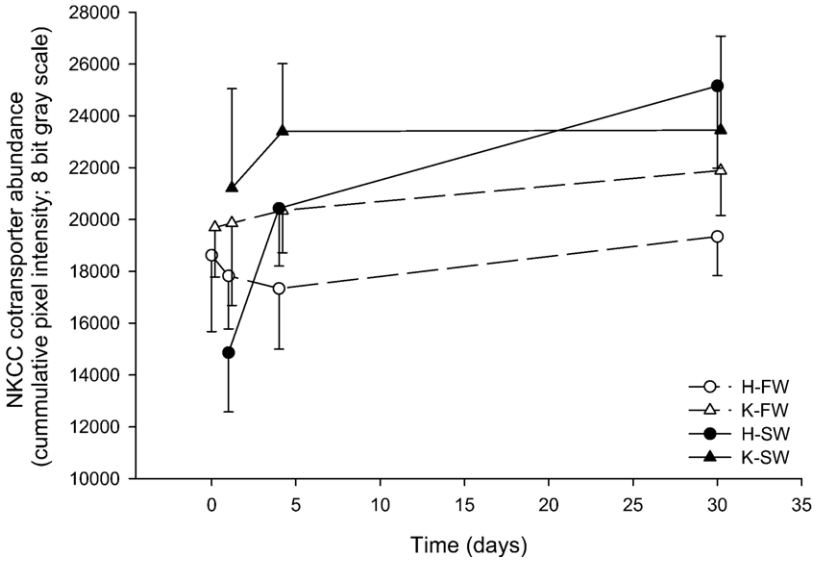

Fig. 4. Gill NKCC cotransporter abundance of families K (triangle) and H (circle) in freshwater (dashed lines) and seawater (solid lines) sampled 0 h, 1 day, 4 days, and 30 days post transfer. Plots were shifted slightly along the X-axis to clarify standard error bars. Values are means±standard error of the mean (n=9).

mRNA levels in the other families in either seawater or freshwater over time. The abundance of gill NKCC protein did not change over time (Fig. 4). Although there was a significant interaction between salinity and time on gill CFTRI and II mRNA levels, there was no consistent pattern of change in response to salinity (Table 2).

4. Discussion

4.1. NKCC isoforms in Atlantic salmon

Prior to this study, molecular data on NKCC isoforms expressed in Atlantic salmon were limited to a partial cDNA sequence reported to encode NKCC1 (Tipsmark et al., 2002). Sequence information for this isoform has now been extended in both the 5′ and 3′ directions to include the entire coding region. Using sequence comparisons and phylogenetic analysis, we confirmed that it is indeed a member of the NKCC1 subfamily and further determined that it most likely represents the evolutionary counterpart of the NKCC1a isoforms previously described in eel and tilapia. Incidentally, our phylogenetic analysis also indicated that the NKCC1 isoform recently isolated by Lorin-Nebel et al. (2006) in seabass is most closely related to NKCC1a in other

Table 2
Relative gill mRNA expression of CFTR I an CFTRII (CFTR: β-actin) of families H, I, and K collected at 0, 1, 4 and 30 days following transfer to freshwater and seawater

		Time (days)				
		Family	0	1	4	30
CFTRI	Freshwater	H	2.11±0.38	2.69±0.54	2.31±0.45	4.96±0.85
		I	2.48±0.46	2.68±0.56	2.54±0.26	3.38±0.99
		K	3.89±0.73	3.65±0.75	2.50±0.54	4.00±0.49
	Seawater	H		1.81^{a}±0.17	3.89±0.99	2.55±0.24
		I		2.88^{b}±0.37	3.34±1.07	2.74±0.47
		K		3.17^{b}±0.22	3.42±0.69	3.87±0.59
CFTRII	Freshwater	H	1.29±0.25	1.19±0.12	1.02±0.07	1.14±0.26
		I	0.95±0.16	0.73±0.07	1.07±0.22	1.22±0.19
		K	1.28±0.10	1.62±0.47	1.20±0.16	1.08±0.13
	Seawater	H		1.39±0.10	1.31±0.15	0.92±0.05
		I		1.66±0.42	1.72±0.73	1.19±0.19
		K		1.71±0.25	1.40±0.12	1.55±0.27

Values are means±standard error of the mean (n=6). Lower case letters indicate significant differences between families within a treatment (freshwater or seawater) and sample time (P<0.05).

teleosts (Fig. 1), which is consistent with its role in seawater adaptation. Though we did not attempt to isolate other NKCC isoforms potentially expressed in Atlantic salmon, we did obtain preliminary data from *in silico* analysis of EST sequences in GenBank suggesting that NKCC1b and NKCC2 are also expressed in this species (K. Gharbi, unpublished results). Nonetheless, current data indicate that NKCC1a is the more interesting seawater gill isoform in teleosts, showing consistent up-regulation with salinity changes in eel (Cutler and Cramb, 2002), tilapia (Hiroi et al., 2005), seabass (Lorin-Nebel et al., 2006), and Atlantic salmon (see discussion below).

4.2. Changes in gill ion transporters with seawater exposure

Smoltification prepares juvenile Atlantic salmon for seaward migration, in particular salinity tolerance increases as a result of increased capacity of the gill to secrete excess NaCl in seawater (Hoar, 1976). Branchial secretion of NaCl in seawater is dependent on three key transporters, Na^+/K^+ ATPase, NKCC, and CFTR (Marshall and Grosell, 2006). In a previous study on the same individuals, we proposed that rapid up-regulation of Na^+/K^+ ATPase upon seawater exposure in Atlantic salmon smolts (0^+) would confer improved osmoregulatory performance (Mackie et al., 2005). In fact, the family with the lowest osmolality at 24 h (family K), had the highest gill Na^+/K^+ ATPase activity. In the present study, we extended our predictions to include the gill NKCC and CFTR transporters. Namely that family K (with the greatest salinity tolerance) would have the highest level of gill NKCC1a mRNA and protein abundance, as well as CFTR I and II mRNA expression relative to family I and H. Indeed, CFTR I mRNA levels were significantly higher in family K (and I) relative to family H after 24 h of salinity exposure. In contrast, however, gill NKCC1a and CFTR II mRNA levels, as well as NKCC abundance were not different between families at 24 h. Interestingly, gill NKCC1a mRNA levels were significantly higher in the family with the lowest salinity tolerance (family H) after 4 days of seawater relative to the other two families. Taking the data together, the improved osmoregulatory performance of family K at 24 h was linked to an up-regulation of the gill CFTR I mRNA levels and Na^+/K^+ATPase activity, but not NKCC1a and CFTR II mRNA levels nor NKCC protein abundance. These findings contrast those of Singer et al. (2002) who found significant differences in gill CFTR II mRNA levels in two Norwegian strains of Atlantic salmon smolts after seawater transfer, but they reported no strain differences in mRNA levels of other gill transporters or Na^+/K^+ ATPase activity. The differences between studies is not surprising given the complexity of gill ion regulatory processes, differences in genetic composition of salmon, and experimental conditions.

In our original study, the mRNA levels of the α1a isoform of Na^+/K^+ ATPase were significantly elevated in the seawater-exposed smolts, while the α1b isoform was higher in freshwater control smolts (Mackie et al., 2005), as previously observed in rainbow trout (Richards et al., 2003). Regardless, gill mRNA levels of Na^+/K^+ ATPase did not vary between salmon smolt families in our earlier study. This data is in agreement with the NKCC1a and CFTR II mRNA data in the present study where levels were not variable between families. The lack of interfamily differences between mRNA levels of Na^+/K^+ ATPase was explained in our earlier paper by the possibility of alternative salinity sensitive isoforms of Na^+/K^+ ATPase and/or that regulation of enzyme activity was not at the level of gene expression (Mackie et al., 2005). In the case of NKCC, the alternative isoform hypothesis cannot be ruled out. We found evidence for NKCC1b and NKCC2 genes in Atlantic salmon (see above) and in eel, NKCC1a but not NKCC1b was expressed in gill tissue (Cutler and Cramb, 2002). Moreover, one might expect parallel changes in NKCC protein abundance if there was an increase in gene transcription of other isoforms, but this was not observed. Therefore, there is no evidence for the alternative isoform hypothesis at present.

It is also possible that NKCC activity is not solely regulated by transcriptional and translational changes. For example, two recent studies indicate that cytosol nitric oxide levels (Ebbesson et al., 2005) and membrane lipid composition (Lingwood et al., 2005) significantly impact gill Na^+/K^+ ATPase activity in salmonids. Similar biochemical variables may also influence NKCC function, but this is currently unknown.

At the protein level, gill NKCC abundance was not altered in seawater-exposed smolts in the present study, whereas gill Na^+/K^+ ATPase activity was significantly enhanced in the same fish in our companion study (Mackie et al., 2005). In other studies, clear correlations between gill NKCC abundance and Na^+/K^+ ATPase activity have been observed in salmon parr transferred to seawater or when pre-smolts developed into smolts (Pelis et al., 2001; Tipsmark et al., 2002). The variation between studies may relate to genetic, developmental (eg. smolt 1^+ or smolt 0^+) or environmental differences.

Out-of-season or 0^+ smolts are induced to prematurely undergo smoltification through photoperiod manipulation (Saunders et al., 1990; Thrush et al., 1994;

Duston and Saunders, 1995) as transfer to sea cages six months earlier not only reduces hatchery time but also provides a year-round supply of Atlantic salmon for aquaculture operations. Environmental factors, such as the low temperatures (2.4–4 °C) and continuous light (24L:OD) used in the present study to mimic 0^+ smolt east coast industry practices, may have had a significant impact on the physiological response to abrupt salinity transfer. Hundeland et al. (2000) reported that Atlantic salmon 0^+ smolts had the least osmoregulatory disturbance at ~9 °C relative to lower (<5 °C) and higher temperatures (~19 °C). In the present study, gill NKCC and Na^+/K^+ ATPase mRNA and abundance/activity levels were lowest in the winter months prior to peaking at the time of the natural smolt migration (May) of Atlantic salmon (1^+) (Tipsmark et al., 2002). The salmon smolts (0^+) under investigation here may have had relatively low levels of gill transporter expression in freshwater and/or may have been less responsive to environmental manipulation in the winter months. For the salmon industry on the eastern coast of Canada, however, the experimental conditions used in our study may provide more realistic data for future selective breeding experiments. Taken together with our previous study, gill Na^+/K^+ ATPase activity and CFTR I mRNA levels, rather than Na^+/K^+ ATPase and NKCC1a mRNA levels or NKCC protein abundance, may be the best indicator of osmoregulatory performance between families. Further experiments using a larger number of families are now necessary to test whether gill Na^+/K^+ ATPase activity and CFTR I mRNA levels can be widely used as a selection index for genetic selection programs to improve stocks of eastern Canadian Altantic salmon for aquaculture.

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