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Dynamics of Na^+,K^+,$2Cl^-$ Cotransporter and Na^+,K^+-ATPase Expression in the Branchial Epithelium of Brown Trout (*Salmo trutta*) and Atlantic Salmon (*Salmo salar*)

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ABSTRACT The dynamics of branchial Na^+,K^+,$2Cl^-$ cotransporter (NKCC) and Na^+,K^+-ATPase (NKA) expression were investigated in brown trout and Atlantic salmon during salinity shifts and the parr-smolt transformation, respectively. In the brown trout, Western blotting revealed that NKCC and NKA abundance increased gradually and in parallel (30- and ten-fold, respectively) after transfer to seawater (SW). The NKA hydrolytic activity increased ten-fold after SW-transfer. Following back-transfer to fresh water (FW), the levels of both proteins and NKA activity decreased. The NKCC immunostaining in the gill of SW-acclimated trout was strong, and mainly localized in large cells in the filament and around the bases of the lamellae. In FW-acclimated trout, immunostaining was less intense and more diffuse. Partial cDNAs of the secretory NKCC1 isoform were cloned and sequenced from both brown trout and Atlantic salmon gills. Two differently sized transcripts were detected by Northern blotting in the gill but not in other osmoregulatory tissues (kidney, pyloric caeca, intestine). The abundance in the gill of these transcripts and of the associated NKCC protein increased four- and 30-fold, respectively, during parr-smolt transformation. The abundance of NKA α-subunit protein also increased in the gill during parr-smolt transformation though to a lesser extent than enzymatic activity (2.5- and eight-fold, respectively). In separate series of in vitro experiments, cortisol directly stimulated the expression of NKCC mRNA in gill tissue of both salmonids. The study demonstrates the coordinated regulation of NKCC and NKA proteins in the gill during salinity shifts and parr-smolt transformation of salmonids. *J. Exp. Zool. 293:106–118, 2002.*

Anadromous salmonids are modestly euryhaline throughout their life, which means that they tolerate transfers between freshwater (FW) and seawater (SW) of moderate salinity. However, the euryhaline capacity is greatly enhanced in most species during a narrow period in the spring of their first or second year. This occurs through a process termed the parr-smolt transformation, which normally precedes or concurs with the onset of downstream migration. The transformation involves several morphological, physiological, and biochemical changes which have been the subject of numerous studies (Boeuf, '93). A major focus has been put on the preparative osmoregulatory changes associated with transition from FW to SW, and it is well known that the gill is transformed from a hyper- into a hypo-osmoregulatory organ. A key enzyme in salt-transport in the two operative modes of the gill is the Na^+, K^+-ATPase (NKA), the protein and mRNA

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expression of which are considerably increased during SW-acclimation (brown trout: e.g., Madsen et al., '95) and parr-smolt transformation (brown trout: e.g., Nielsen et al., '99; Atlantic salmon: e.g., D'Cotta et al., 2000; Seidelin et al., 2001). The regulation is largely accomplished by hormonal modulation of NKA gene expression, and it is well documented (McCormick, '95). Whereas NKA is believed to be involved in both ion uptake and excretion, the role of another transport protein, the secretory-type $Na^+,K^+,2Cl^-$ cotransporter (NKCC), is more specifically associated with the ion secretory mode of the teleost gill. The branchial expression and regulation of this protein have as yet only been addressed in a few studies. The NKCC is presumably involved in the basolateral entry step of chloride into the chloride cell and several other ion secreting cells using the electrochemical gradient for Na^+, established by the primary action of the NKA (Evans et al., '99; Haas and Forbush, 2000). This is supported by the fact that loop-diuretics (which inhibit the transport function of the protein) inhibit the short-circuit current across the opercular epithelium of killifish (Degnan et al., '77; Eriksson et al., '85). Also, SW-acclimation of rainbow trout increases bumetanide-sensitive Rb^+-uptake five-fold in the gill (Flik et al., '97), which agrees with the five- to 20-fold increase in NKCC protein abundance found by Behnke et al. ('96). SW-acclimation and smoltification have recently been reported to increase the level of the NKCC protein in Atlantic salmon chloride cells (Pelis et al., 2001). The main purpose of this study was to evaluate the dynamics in the expression of the branchial NKCC at both mRNA and protein levels during salinity shifts in the brown trout and during the parr-smolt transformation in the Atlantic salmon.

MATERIALS AND METHODS

Animals

One year old brown trout (*Salmo trutta*; 40–60 g; mixed sex; post-smolt) were obtained from the Vork Hatchery (Egtved, Denmark) in July 2000 and kept in an indoor tank with running tap water (in mM: 1.4 Cl^-, 1.5 SO_4^{2-}, 1.5 Na^+, 0.16 K^+, 3.0 Ca^{2+}, 0.6 Mg^{2+}, pH 8.3) at the Odense University Campus (15°C, 12:12 hr light:dark artificial photoperiod) for three weeks before experimentation. SW-acclimation was accomplished by direct transfer of a batch of fish to 28 ppt natural, recirculated, and filtered SW (15°C). Upper mode Atlantic salmon parr (*Salmo salar*; >13 cm; 20–40 g; one year old first generation hatchery fish of the Irish Burrishoole River stock) were obtained in January 1997 from the Salmon Rearing Station (Randers, Denmark) where they had been hatched and reared in indoor tanks under simulated natural photoperiod and water temperature (minimum winter temperature 4°C). The fish were brought to Odense University Campus and held outdoors in 500-l flow-through FW tanks supplied with Odense tap water. Further details of the parr-smolt transformation of these fish are published in Seidelin et al. (2001). Brown trout and Atlantic salmon used in the in vitro experiments (see below) were obtained from the same sources as above (1997 and 2000, respectively), and they were reared and held under similar conditions. Brown trout were used for experimentation in February, and Atlantic salmon were used in April. Fish used in all of the above studies were fed commercial trout pellets equaling 2% of their body weight three times per week.

Sampling

After stunning the fish with a blow to the head, blood was drawn from the caudal blood vessels into a heparinized syringe, and the plasma was immediately separated by centrifugation at 5,000 *g* for 3 min. The fish were then killed by cutting the spinal cord and pithing of the brain, and the gill arches were excised. For mRNA analyses, one first-, two third-, and two fourth-gill arches were pooled and immediately homogenized in 2.5 ml ice-cold denaturing solution (in mM: 4,000 guanidinium thiocyanate, 25 sodium citrate, 100 β-mercaptoethanol, 0.5% sarcosyl, 0.3% antifoam, pH 7.0). For protein analyses, one second-gill arch was sampled and placed in SEI-buffer (in mM: 300 sucrose, 20 Na_2-EDTA, 50 imidazole, pH 7.3). All samples were subsequently frozen in liquid nitrogen and stored at −80°C. Following this, a piece (0.5 g) of paraxial muscle was sampled from the caudal region.

Acclimation of brown trout to salinity changes

Acclimation of brown trout from FW to SW and from SW to FW was examined by sampling at 0 hr, 4 hr, one day, three days, seven days, and 60 days after transfer to SW, and one and ten days after transfer back to FW. Fish were nonfed through the experiment except between days seven and 60 after transfer to SW, where they were fed the above ration.

Smoltification in Atlantic salmon

In Atlantic salmon, progress of the parr-smolt transformation was examined by sampling every 2–3 weeks (February 5, February 26, March 12, March 25, April 9, April 22, May 5, May 25, and June 9). Feeding was stopped four days prior to sampling.

In vitro cortisol study

The effect of cortisol in vitro on gill NKCC mRNA expression was examined in gill biopsies from eight brown trout and six Atlantic salmon smolts according to McCormick and Bern ('89). Gill biopsies were incubated in salmon Ringer's solution (in mM: 140 NaCl, 15 $NaHCO_3$, 2.5 KCl, 1.5 $CaCl_2$, 1.0 KH_2PO_4, 0.8 $MgSO_4$, 5.0 D-glucose, and 5.0 N-2 hydroxyethyl-piperazine propanesulfonic acid, pH 7.8) with or without cortisol (10 μg/ml^{-1}; Na hydrocortisone hemisuccinate; Sigma, St. Louis, MO) in an atmosphere of 99% O_2, 1% CO_2 for 6 hr (trout) or three days (salmon).

Cloning of gill NKCC

The cloning procedure followed the description by Cutler et al. ('95) as modified by Cutler et al. ('97). Total RNA was extracted from gills of both FW-acclimated brown trout and Atlantic salmon as described by Chomczynski and Sacchi ('87). First-strand cDNA synthesis from 5 μg total RNA was done using Superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD) for 5 hr at 45°C. Using degenerate primer pairs (Table 1), cDNA fragments of the gill NKCC1 were amplified by Polymerase Chain Reaction (PCR). The PCR consisted of 2 min at 94°C, 40 × (5 sec at 92°C, ½ min at 55°C, 1 min at 72°C), and 10 min at 72°C. The primer-pair used was designed as degenerate primers, the sequences of which were taken from two regions of amino acids that were identical to published sequences of the NKCC1. The degenerate primers have inosine/cytosine wobbles incorporated at positions of nucleotide uncertainty as previously described by Cutler et al. ('95). Complementary DNA fragments of approximately 741 base-pairs in size (equal to the size of the orthologue sequence for the human NKCC1, see Table 1) were purified from agarose gels using the Geneclean kit (Bio101, Carlsbad, CA), ligated into a pCR-Blunt II-TOPO vector using the zero Blunt TOPO PCR Cloning kit (Invitrogen, Carlsbad, CA), and sequenced by the dideoxy chain termination method using the Big Dye Terminator sequencing kit (Perkin Elmer, Shelton, CT). The sequence of three clones from each species was determined to give the precise sequence shown in Fig. 1. Comparisons to known DNA and deduced protein sequences were performed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast/).

Analyses

Osmolality and muscle water content (MWC)

Plasma osmolality was measured by freezing point depression osmometry (osmomat 030; Gonotec, Berlin, Germany), and MWC was measured gravimetrically after drying at 105°C for at least 48 hr.

Na^+,K^+-ATPase enzymatic activity

Gill samples were homogenized in ice-cold SEIDM buffer (in mM: 300 sucrose, 20 Na_2-EDTA, 50 imidazole, 10 β-mercaptoethanol, 0.1% sodium deoxycholate, pH 7.3). Following centrifugation at 5,000 *g* for 30 sec, NKA activity was assayed according to McCormick ('93) in the supernatant at 25°C using a microplate reader (SPECTRAmax PLUS, Molecular Devices, Sunnyvale, CA). Protein content was measured by a micro-assay based on the method of Lowry et al. ('51), and enzymatic activity was normalized to protein content and expressed as μmol ADP/mg protein $^{-1}$/hour $^{-1}$.

Northern blot analysis

The level of gill NKCC mRNA was analyzed as described by Seidelin et al. ('99). In short, total

TABLE 1. The pair of degenerate primers used during the PCR amplification of the NKCC cDNA fragments from gills of Atlantic salmon and brown trout[1]

Na^+,K^+,2Cl^- cotransporter	
Sense (aa: 781–790)	$5'GA^{A}/_{G}GA^{C}/_{T}CA^{C}/^{A}_{T}/_{G}T^{I}/_{C}AA^{A}/_{G}AA^{C}/_{T}T^{A}/^{C}_{T}/^{A}_{T}/_{C}G^{I}/_{C}CC^{I}/_{C}CA^{A}/_{G}TG\ 3'$
Antisense (aa: 1019–1028)	$5'A^{A}/_{G}CCA^{I}/^{C}_{C}/_{T}A^{I}/_{C}A^{C}/^{A}_{T}/_{G}TC^{I}/_{C}AT^{I}/_{C}GT^{I}/^{C}_{C}/^{C}_{T}/^{C}_{T}/_{T}TT^{I}/_{C}CC^{C}/_{T}TG\ 3'$

[1]The amino acid (aa) number noted for each primer represents the position of the primer within the amino acid sequence of human bumetanide-sensitive NKCC (NKCC or SLC12A2: Genbank acc.no. NP001037). Inosine nucleotides (**I**) are marked in **blood** type.

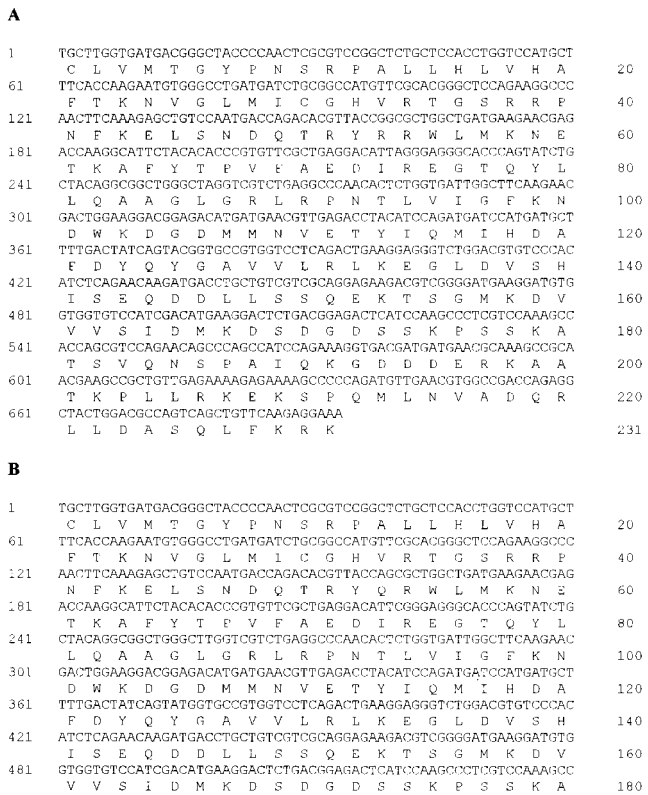


Fig. 1. Nucleotide and deduced amino acid sequences of the gill NKCC1 cDNA fragments from Atlantic salmon (**A**) and brown trout (**B**). The cDNA fragments were amplified by PCR using the degenerate primers provided in Table 1. Nucleotides are numbered from one on the left hand side, amino acids from one on the right hand side. The cDNA sequences have been submitted to the EMBL Data Library under the accession numbers: Atlantic salmon (AJ 417890), brown trout (AJ 417891).

RNA (20 μg) from the gills was submitted to formaldehyde gel electrophoresis (all samples from one experiment on the same gel) and transferred by capillary blotting to a nylon membrane (Zeta probe, Biorad, Hercules, CA). Subsequently, membranes were hybridized with the appropriate radiolabeled specific brown trout or Atlantic salmon gill cDNA-probes of the NKCC1. To adjust for unequal loading, the NKCC mRNA data were normalized to β-actin mRNA content, visualized with a radiolabeled rainbow trout β-actin cDNA probe (Pakdel et al., '89). The hybridization signals were quantified by phosphor imaging (Storm, Molecular Dynamics, Sunnyvale, CA) using ImageQuaNT software (Molecular Dynamics). Molecular size estimation was performed by comparing migration to that of a commercial 0.24–9.5 kb RNA ladder (Gibco BRL).

Preparation of samples and Western analysis

Gill tissue was homogenized in ice-cold SEI buffer with protease inhibitors (in mM: 0.008 leupeptin, 0.4 Pefabloc; Roche, Mannheim, Germany) using a handheld glass homogenizer. The homogenate was centrifuged at 1,000 *g* for 20 min (4°C) to remove intact cells and larger cell fragments. The supernatant was then centrifuged at 50,000 *g* for 30 min (4°C) to isolate the membrane fraction. The pellet was re-suspended and protein content measured by the method of Lowry et al. ('51). Sample buffer and sample-reducing agent (both NuPAGE, NOVEX, San Diego, CA; final concentration in the loaded samples in mM: 141 Tris base, 106 Tris HCl, 73 LDS, 0.5 EDTA, 50 1,4-dithiothretiol and: 8% glycerol (v/v), 0.019% serva blue G250 (w/v), 0.006% phenol red (w/v)) were added prior to heating at 65°C for 10 min. An equal quantity of membrane protein (10 μg) was loaded in all lanes. Proteins were separated by gel electrophoresis using 4–12% bis-tris gels (NuPAGE system), and MOPS/SDS-buffer (in mM: 50 3-(N-morpholino)-propanesulfonic acid, 50 Tris, 3.5 SDS, 1 Na_2-EDTA) with addition of antioxidant (NuPAGE). Molecular size was estimated by including a prestained kaleidoscope marker (Biorad, Hercules, CA).

Following electrophoresis, the gel was soaked for 30 min in transfer buffer (in mM: 25 Tris, 192 glycin) and immunoblotted onto nitro-cellulose membranes (Hybond ECL, Amersham, Uppsala, Sweden) by semidry-blotting for 2 hr at 0.8 mAmp cm^{-2} (Hoefer Scientific Instruments, San Francisco, CA). Membranes were blocked in 2% TWEEN in TBS (in mM: 40 Tris, 1 NaCl, pH 7.5) and washed in 0.05% TWEEN in TBS. Immunological detection of NKA and NKCC were obtained by incubating overnight at room temperature with the α5 anti-NKA and the T4 anti-NKCC primary antibodies, respectively. Both were diluted 1:500 in 0.05% TWEEN in TBS. Following washing, membranes were incubated 1 hr with the secondary antibody (Alkaline Phosphatase-conjugated goat anti-mouse IgG antibody; Zymed, San Francisco, CA) diluted 1:3,000. Washing and 5 min incubation with 0.1 M ethanolamine preceeded the visualization with BCIP-NPT (5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium) sub-

strate (Kirkegaard and Perry, Gaithersburg, MD). The colorimetric reaction was terminated by immersion in milli-Q water. Membranes were scanned by a desk-scanner and band intensities analyzed using Quantiscan software (Biosoft, San Francisco, CA). Protein abundance was expressed in arbitrary units relative to the mean at the first date of sampling.

Immunohistochemistry

NKCC immunoreactive cells were visualized in gills of FW- and SW-acclimated (> 60 days) brown trout. Gills were fixed for 24 hr at 5°C in 4% paraformaldehyde, 0.9% NaCl, 5 mM NaH_2PO_4, pH 7.8 and then stored in 70% ethanol until dehydrating and embedding in paraffin. Five-micrometer saggital sections were stained with the monoclonal T4 antibody as described by Seidelin and Madsen ('99). The primary and secondary antibodies were used at 1:40 and 1:200 dilution, respectively.

Antibodies

The monoclonal α5 is directed against the chicken NKA α_1-subunit, is specific for a cytosolic epitope, and reacts with all isoforms of the α-subunit of distant species. It was developed by D.M. Fambrough (Johns Hopkins University, Baltimore, MD; see Takeyasu et al., '88). The monoclonal T4 antibody is directed against the human colon NKCC, is specific for NKCC, and reacts with an epitope on the carboxy-terminus conserved between NKCC1 and NKCC2 in distantly related species. It was developed by C. Lytle (University of California, Riverside, CA; see Lytle et al., '95) and B. Forbush III (Yale University School of Medicine, New Haven, CT). Both primary antibodies were obtained from the Developmental Studies Hybridoma Bank developed under auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA.

Statistics

Data were analyzed by one-way ANOVA and subsequently compared by the Tukey's honestly significant difference (HSD) procedure. A Tukey-Kramer adjustment of the Tukey's HSD Test was performed when the dataset contained unequal numbers in treatment groups. When necessary, transformations were performed to meet the parametric ANOVA assumption of homogeneity of variances (evaluated by residual-plots). In the brown trout in vitro experiment, the data were evaluated with a paired *t*-test. All statistical analyses were performed using Systat 7.0 (Evanston, IL) and significant differences were accepted when $P < 0.05$.

RESULTS

Characterization of NKCC mRNA and protein

The sequences of the NKCC cDNAs of brown trout and Atlantic salmon (Fig. 1) show high homology to the secretory NKCC1 isoform. Using the homologous cDNA probes, two transcripts of sizes 7.5 and 4.3 kb were detected in the gill of Atlantic salmon smolt (Fig. 2A). In the brown trout, transcripts of sizes 8.4 and 3.6 kb were detected in the gill but only insignificantly expressed in kidney, pyloric caeca, middle and posterior intestine of FW- and SW-acclimated fish (Fig. 2B). In both trout and salmon gills (Fig. 3), the T4 anti-NKCC antibody revealed three bands with apparent molecular weights (MW) of 156/160, 209/214, and 223/224 kDa (brown trout/Atlantic salmon, respectively). The α5 anti-NKA antibody revealed one band of 112/116 kDa in brown trout/Atlantic salmon (Fig. 3). For NKCC protein and mRNA analyses, all bands described above were used in the quantification.

Acclimation of brown trout to salinity changes

Seawater-transfer induced a rapid increase in plasma osmolality from ca 300 to 400 mOsm kg^{-1} after 4–24 hr (Fig. 4A). Osmolality stayed high until day three, and decreased after seven and 60 days in SW to ca 340 mOsm kg^{-1}. MWC decreased within 24 hr after SW-transfer and gradually returned to the pre-transfer level at day 60 (Fig. 4B). MWC was not affected by back-transfer to FW.

The abundance of NKCC protein (Fig. 5A) increased seven days after transfer to SW. The abundance of NKA protein (Fig. 5B) also increased though less pronounced. The abundance of both proteins was further increased at day 60 after transfer. Following return to FW, NKCC abundance decreased after ten days, whereas NKA abundance was unchanged. The NKA activity (Fig. 5C) was significantly higher on day three after SW-transfer and continued to increase on day seven and day 60. Back-transfer to FW reduced enzyme activity after ten days. The

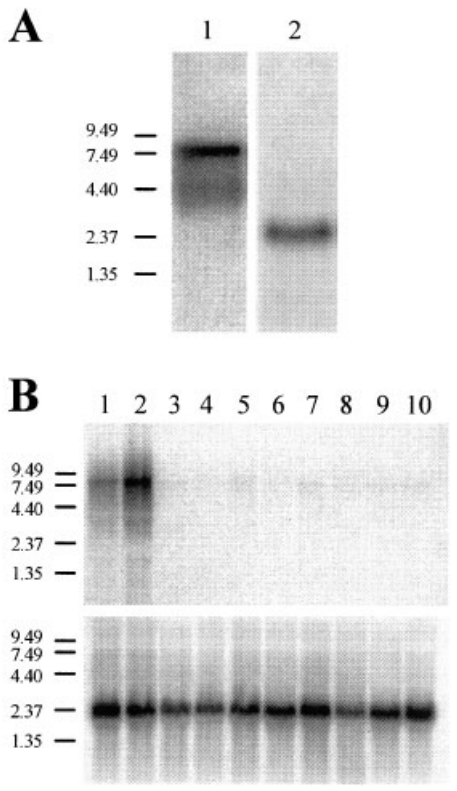


Fig. 2. Northern blot analysis of the NKCC1 isoform and β-actin expression from Atlantic salmon gills (**A**) and various tissues from brown trout tissues (**B**). A: In gills from FW Atlantic salmon, the cloned NKCC1 cDNA hybridized to two transcripts of 7.5 and 4.3 kb in size (lane 1). Beta-actin control hybridization is shown in lane 2. B: Various tissues from brown trout in FW (lanes 1, 3, 5, 7, 9) or acclimated to 28 ppt SW for > 30 days (2, 4, 6, 8, 10) were analyzed for expression of the NKCC1 isoform (upper) and β-actin (lower). Total RNA from gills (lanes 1, 2), kidney (lanes 3, 4), pyloric caeca (lanes 5, 6), middle intestine (lanes 7, 8), and posterior intestine (lanes 9, 10) were loaded in individual lanes. The cloned NKCC1 cDNA hybridized to two transcripts of 8.4 and 3.6 kb in size. Twenty micrograms of total RNA were loaded into each lane. Bars indicate molecular size markers.

expression of NKCC1 mRNA in the gill was higher in SW- than in FW-acclimated trout (Fig. 2B).

In gills of SW-acclimated brown trout, positive NKCC immunostaining was found in large spheric epithelial cells situated in the filament and around the bases of the lamellae (Fig. 6A) and in few cells on the distal part of the lamellae. The immunostaining was less intense and more diffuse in FW-acclimated brown trout (Fig. 6B).

Smoltification in Atlantic salmon

During the parr-smolt transformation, NKCC expression increased at both mRNA and protein level in the gills (Fig. 7). The relative mRNA level increased in March–April, whereas the protein level increased in April–May. Messenger RNA abundance decreased abruptly in June, whereas the protein level fluctuated in late May and June. The abundance of NKA α-subunit and NKA activity both increased in the gill during parr-smolt transformation (Fig. 8). Alpha-subunit abundance increased in March–April to a level ca 2.5-fold higher than the level in February. This level was maintained throughout the sampling period until June. The NKA activity already showed an early increase at the end of February, was unchanged through March, and increased steeply through April and May. The peak level in May was ca eight-fold higher than the level in February and was followed by an abrupt decrease in June.

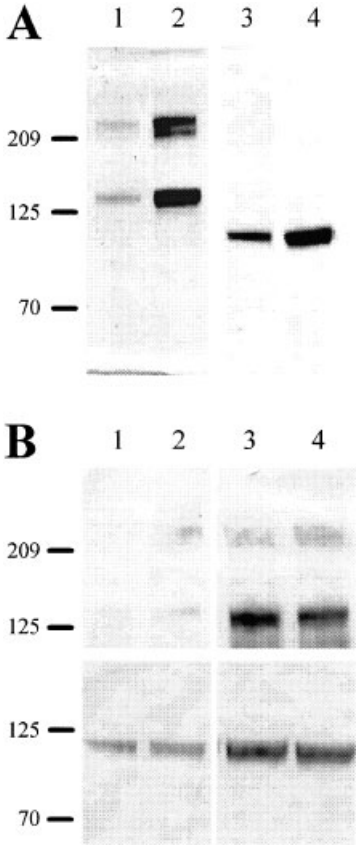


Fig. 3. Western blots recognizing the NKCC and NKA proteins in gill preparations of brown trout (**A**) and Atlantic salmon (**B**). In A, FW (lanes 1, 3) and seven-day SW (lanes 2, 4) preparations were probed with anti-NKCC (lanes 1, 2) or anti-NKA (lanes 3, 4) antibodies. In B, parr (lanes 1, 2) and smolt (lanes 3, 4) were probed with anti-NKCC (upper) and anti-NKA (lower) antibodies. Molecular weight standards are indicated with bars.

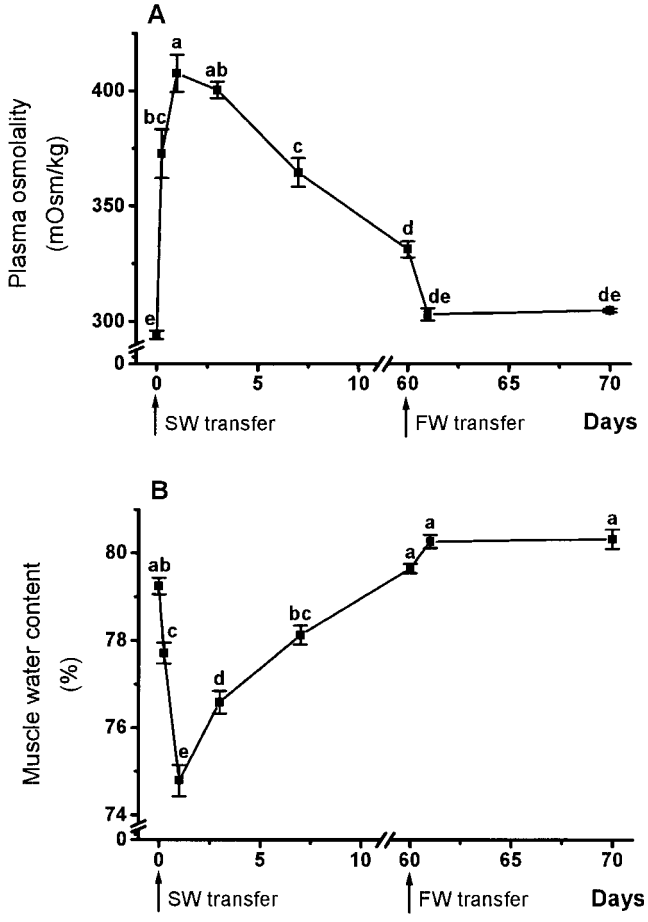


Fig. 4. Plasma osmolality (**A**) and muscle water content (**B**) in brown trout following transfer to 28 ppt SW at day zero and back-transfer to FW on day 60. Different letters indicate significant differences ($P<0.05$). Values are means $\pm$SEM (n=8).

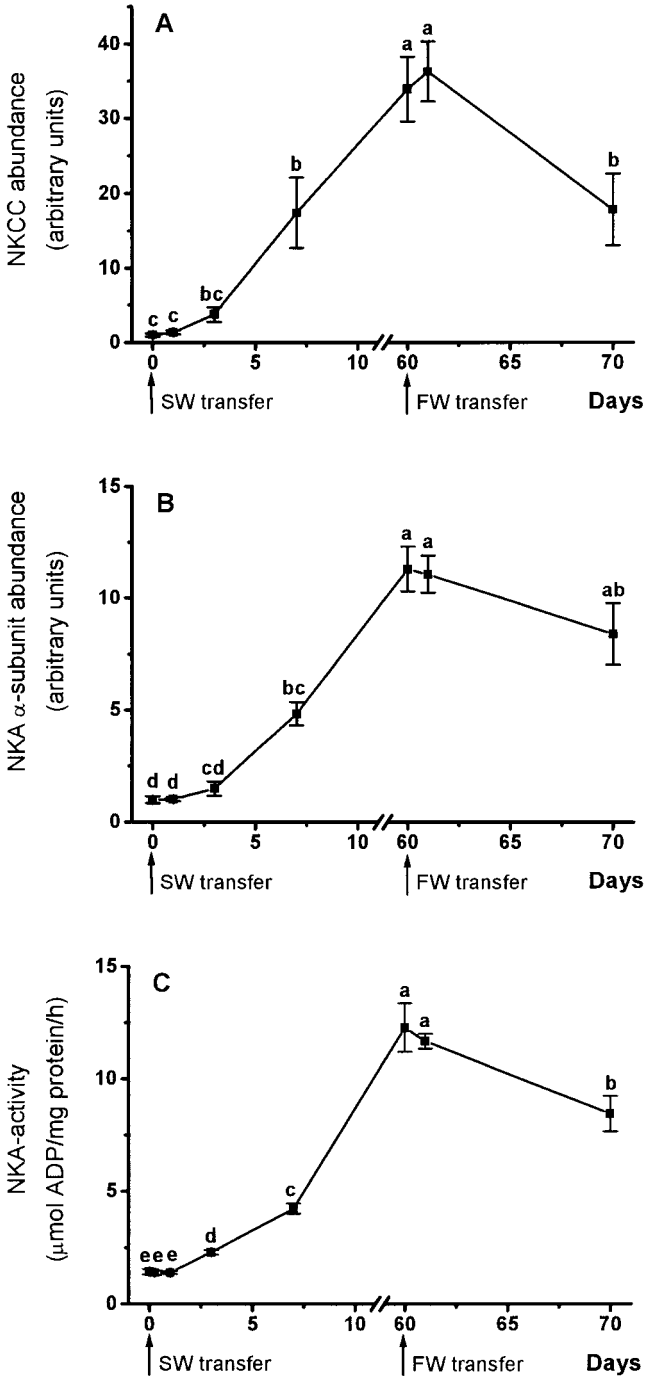


Fig. 5. Abundance of NKCC protein (**A**; arbitrary units normalized to initial value on day zero), NKA protein (**B**; arbitrary units normalized to initial value on day zero), and the NKA-activity (**C**) in brown trout following SW-transfer on day zero and back-transfer to FW on day 60. Different letters indicate significant differences ($P<0.05$). Values are means $\pm$ SEM (n=6 and 8 for protein levels and NKA activity, respectively).

Cortisol effects in vitro

Incubation of gill tissue in vitro with cortisol significantly increased the NKCC mRNA level in both brown trout (6 hr incubation) and Atlantic salmon (three days; Table 2).

DISCUSSION

Cloning of NKCC cDNAs

The NKCC belongs to the family of electroneutral cation-chloride cotransporters. The salmon and trout cDNA sequences only differed in four out of the 693 nucleotides, equivalent to a

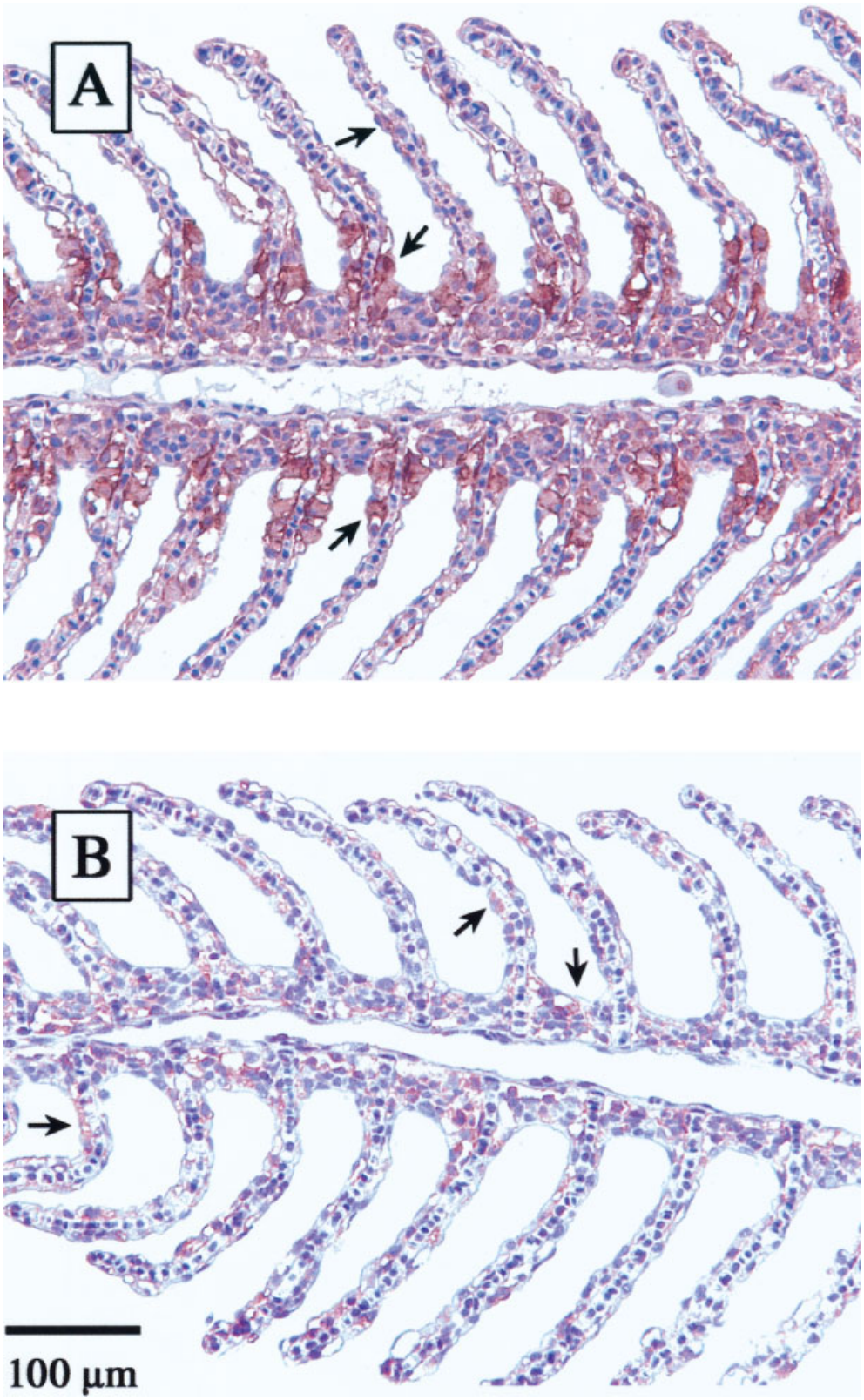
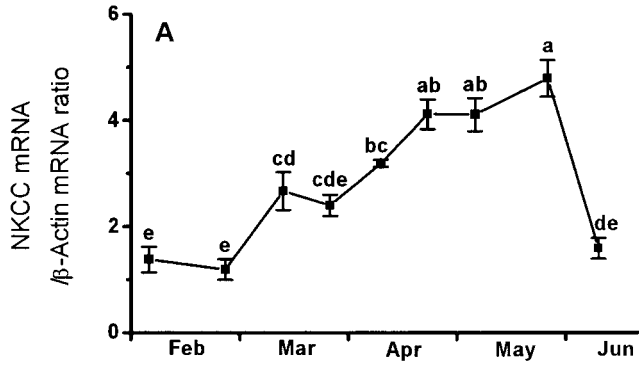


Fig. 6. Saggital sections of gills from SW-acclimated (**A**) and FW-acclimated (**B**) brown trout, stained with anti-NKCC antibody. Arrows indicate immunoreactive cells.



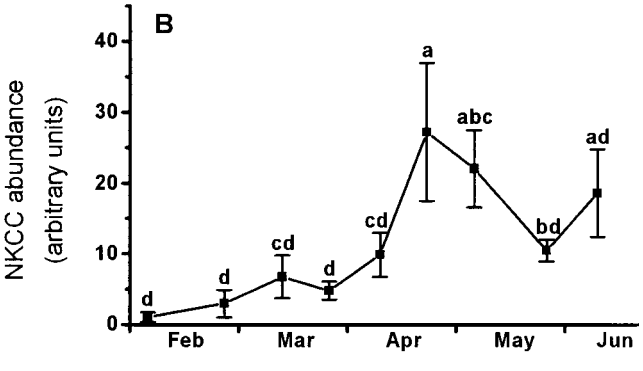
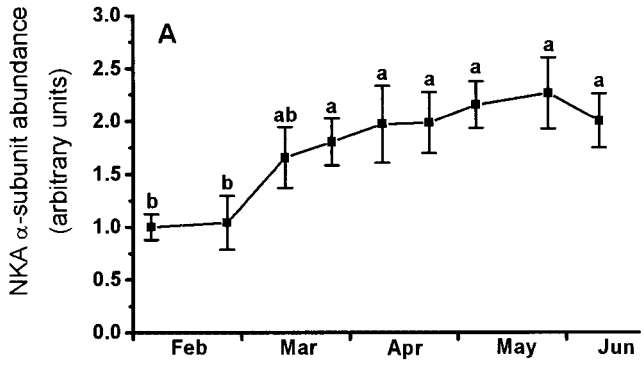


Fig. 7. Abundance of NKCC mRNA (**A**; normalized to β-actin mRNA) and NKCC protein (**B**; arbitrary units normalized to initial value on February 5) in Atlantic salmon during smoltification. Different letters indicate significant differences ($P<0.05$). Values are means ±SEM (n=4 and 5 for mRNA and protein levels, respectively).



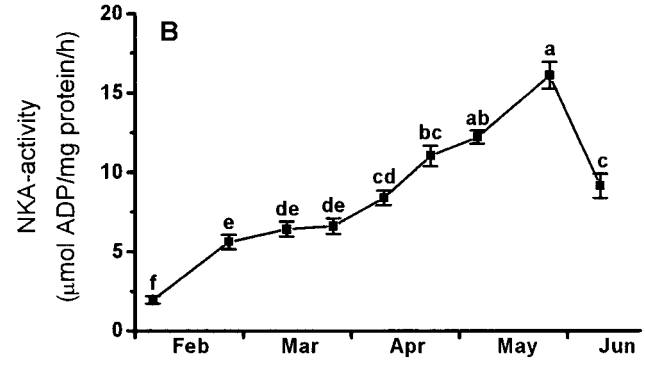


Fig. 8. Abundance of NKA protein (**A**; arbitrary units normalized to initial value on February 5) and NKA activity (**B**) in Atlantic salmon during smoltification. Different letters indicate significant differences ($P<0.05$). Values are means ±SEM (n=6 and 20 for NKA protein abundance and NKA activity, respectively).

99.4% nucleotide sequence identity. This difference only gave rise to one amino acid (aa) difference in the deduced protein sequence of the fragment cloned, thus giving an aa identity of 99.6%. The cloned cDNAs were most possibly salmonid orthologue genes to the bumetanide-sensitive NKCC published for other vertebrate phyla. The salmonid-deduced aa sequence showed 62–63% aa sequence identity to the bumetanide-sensitive NKCC from two shark species (spiny dogfish, *Squalus acanthias*: accession no. AAB60617.1; spotted catshark, *Scyliorhinus canicula*: accession no. CAB86885.1), as well as human NKCC1 (accession no. NP001037). The aa identity to other cotransporter isoforms published was only minor. There was only 35–36% identity to the absorptive NKCC2 form in humans (accession no. NP000329). The first 140–143 aa's of the deduced sequence showed quite high identity to the thiazide-sensitive cotransporter of human (50–51%, NCCT: accession no. NP000330) and winter flounder (47–48%, *Pseudopleuronectes americanus*: accession no. P55019) though no significant identity was observed to the latter 80 amino acids of the sequence.

TABLE 2. In vitro effect of 10 μg ml^{-1} cortisol on NKCC mRNA levels (normalized to β-actin mRNA) in gill samples of brown trout (6 hr incubation) and Atlantic salmon smolts (3 day incubation)[1]

	NKC mRNA/β-actin mRNA
Brown trout:	
Control	1.64 ± 0.18
Cortisol	2.24 ± 0.15*
Atlantic salmon:	
Pre-incubation	0.29 ± 0.03 (a)
Control	0.05 ± 0.01 (b)
Cortisol	0.25 ± 0.03 (a)

[1]Asterisk and different letters indicate significant difference. Values are means ± SEM (n=8 and n=6 in the trout and salmon experiment, respectively).

Interestingly, two recently published expressed sequence tags (ESTs) from teleost species are clearly orthologues to the present salmonid cDNA fragments. One of them is from the Japanese medaka (*Oryzias latipes*) sharing a 78 aa-overlap (aa's 1–78) with 70% identity to the salmonid sequences (EST-accession no. AU171008). The other one is from zebrafish (*Danio rerio*) which share a 135 aa-overlap (aa's 77–231) with 79% identity to the salmonid species (EST-accession no. BG892408).

Characterization of NKCC mRNA and protein

The transcripts encoding NKCC1 in trout and salmon had apparent sizes of 8.4 and 3.6 (trout) and 7.5 and 4.3 (salmon) kilobases. There are relatively few reports of NKCC sequence and size. In the shark (*Squalus acanthias*) rectal gland, the size of the NKCC1 was reported to be 7.4 kb (Xu et al., '94), in bovine aortic endothelium it is 7.5 kb (Yerby et al., '97), and in the human kidney, colon, lung, and stomach it is 7.2–7.5 kb (Payne et al., '95). The size of the rabbit renal (presumably absorptive-type) NKCC transcript was reported to be 4.6–5.1 kb (Payne and Forbush, '94; Gamba et al., '94), and the flounder thiazide-sensitive Na^+,Cl^- cotransporter transcript was found to be 3.0–4.4 kb (Gamba et al., '93, '94). Immunodetection using the anti-NKCC antibody identified three proteins with apparent MW of 156/160, 209/214, and 223/224 kDa in both salmonids (brown trout/Atlantic salmon, respectively). This agrees well with reported weights of NKCC1 in chloride-secreting epithelia ranging from 146–205 kDa (Lytle et al., '95). The NKCC1 has a core mass of ca 130 kDa, and different degrees of glycosylation could explain differences observed in protein molecular weight. On the other hand, a recent study found that the NKCC1 of the rat parotid gland exists as dimers, which may be separated by detergents (Moore-Hoon and Turner, 2000). The present MW similar to 220 kDa does not suggest the presence of a dimer, but this or the association with a smaller peptide cannot be excluded. Experiments involving an initial deglycosylation step, differing solvents and denaturing conditions should clarify whether there are two differently sized NKCCs or an association between two peptides in these two *Salmo* species. In both salmonids, the α5 anti-NKA antibody identified a NKA α-subunit band similar to 112/116 kDa (brown trout/Atlantic salmon, respectively), which is in good agreement with the general vertebrate size of this protein (Blanco and Mercer, '98).

Acclimation of brown trout to salinity changes

Transfer of trout to SW resulted in a rapid increase in plasma osmolality and decrease in MWC within the first 24 hr. Interestingly, the succeeding stabilization (osmolality) and increase (MWC) on day three occurs without changes in branchial NKCC and NKA abundance, suggesting rapid activation of additional regulatory mechanisms. The return in plasma osmolality and MWC to near pre-transfer levels from day seven and onward, on the other hand, correlates with major increases in NKCC and NKA abundance and NKA activity. Back-transfer to FW only affects water- and salt balance insignificantly with a new FW-level attained within 24 hr. The NKCC and NKA abundance and NKA activity are all reduced after ten days, although not back to the initial FW-values. Thus, NKCC and NKA abundance are regulated in a coordinated manner during salinity shifts in trout. This suggests co-localization of the proteins and is probably reflecting chloride cell dynamics in the gill.

The chloride cell is the primary site of chloride extrusion in SW fish (Foskett and Scheffey, '82), where the main location is in the filament at the base of the lamellae. One of the major biochemical characteristics of the secretory chloride cell is the very high abundance of NKA in the basolateral tubular systems (e.g., Wilson et al., 2000). As for other secretory epithelia, it is assumed that the concerted action of basolaterally located NKA and NKCC in conjunction with apically located chloride channels (cystic fibrosis transmembrane conductance regulator-like; Singer et al., '98) is responsible for the active transcellular chloride transport, with sodium passing passively through paracellular pathways. In accordance with this model, Wilson et al. (2000) and Pelis et al. (2001) recently demonstrated co-localization of NKA and NKCC in chloride cells in mudskipper (*Periophthallmodon schlosseri*) and Atlantic salmon, respectively. Our study showed that SW-transfer increased NKCC immunoreactivity in large spherical cells largely present at the bases of the lamellae. Even though the T4 anti-NKCC antibody recognizes both secretory and absorptive NKCC subtypes (Lytle et al., '95), the immunoreactive cells are most likely chloride cells, as their location and shape are similar to that of cells

stained with Zn-Osmium (Madsen, '90) and Na^+,K^+-ATPase antibodies in the brown trout (Seidelin and Madsen, '99). In FW fish, immunoreactivity was less intense and was also present in cells on the lamellae. The present distribution pattern on both filament and lamellae is largely in accordance with the findings by Pelis et al. (2001) in Atlantic salmon. However, they found no difference in cell staining intensity between fish acclimated to FW and SW, which is in contrast to our findings in brown trout (Fig. 6). The present data suggests that the major increase in NKCC expression after SW-transfer was due to increased abundance in individual cells. Behnke et al. ('96) and Pelis et al. (2001) also reported increased cotransporter abundance in the gills of SW-acclimated rainbow trout and Atlantic salmon. Likewise, Flik et al. ('97) reported a five-fold increase in bumetanide-sensitive $^{86}Rb^+$-uptake in gill vesicular preparations from SW-transferred rainbow trout. Though this last study also indicated the presence of a thiazide-sensitive Na^+,Cl^- cotransporter, the NKCC seems to be of predominant importance when salmonids acclimate to SW.

The changed NKCC abundance is much more profound ($\times$ 3) than that of NKA α-subunit and activity. This may reflect a more specific role of NKCC in salt secretion, whereas NKA plays an additional role in salt-uptake in the FW gill (Evans et al., '99). The NKA activity closely follows the abundance of NKA, which indicates no change in enzymatic capacity of the protein during acclimation. Ten days after transfer of the long-term, SW-acclimated trout to FW, the abundance of both ion-transporters decreased by 30–50%. Thus, a longer period is required for complete return to FW status. Nevertheless, this study demonstrates that the branchial osmoregulatory apparatus is highly dynamic in the brown trout.

Smoltification in Atlantic salmon

A major characteristic of the parr-smolt transformation is the reversible development of hypo-osmoregulatory capacity while the fish resides in FW (Boeuf, '93). This was also evident in the salmon used in the present study, where peak hypo-osmoregulatory capacity was found in mid-April through mid-May (see Seidelin et al., 2001). The surge in SW tolerance is accompanied by almost synchronous surges in NKA α- and β-subunit mRNA levels, NKA activity (Seidelin et al., 2001), NKA abundance, and NKCC mRNA and protein levels (present study). A three-fold increase in NKCC mRNA level is paralleled by a 30-fold increase in NKCC protein level indicating the importance of both transcriptional and post-transcriptional mechanisms during development. The more specific SW-adaptive function of NKCC relative to NKA is once again indicated by the magnitude of changes observed during smoltification. The NKA abundance increased ca 2.5-fold during the period, whereas NKCC increased 30-fold. By comparison, NKA activity increased eight-fold, suggesting an increased hydrolytic activity of individual enzymes. This could be explained by differences in isoform composition of the holoenzyme or by post-translational regulatory mechanisms. There is emerging evidence for both possibilities. Alpha subunit isoform patterns may change during SW-acclimation (tilapia: Lee et al., '98). Post-translational mechanisms modulating NKA activity have been demonstrated in gills of the stenohaline Atlantic cod (Crombie et al., '96), as well as in the euryhaline eel (Marsigliante et al., '97) and brown trout (Tipsmark and Madsen, 2001). Interestingly, no similar discrepancies between NKA abundance and activity appear during the course of osmoregulatory adjustments in the brown trout of this study.

Cortisol effects in vitro

In addition to being a general metabolic and stress-responsive hormone, cortisol is known as an osmoregulatory hormone in many teleosts (Hazon and Balment, '98). This is based on correlative evidence of increased plasma cortisol levels in response to SW- or FW-transfer concurrent with increased hypo- or hyper-osmoregulatory ability, respectively. Cortisol levels also increase during parr-smolt transformation concurrent with development of hypo-osmoregulatory mechanisms (Boeuf, '93). Direct evidence showing a specific role of cortisol in stimulating development of branchial filament chloride cells and NKA protein abundance and activity have come from several groups using adrenalectomized fish, in vivo injection studies, and in vitro studies with isolated gill filaments (McCormick, '95). In addition to its well-documented role as a hypo-osmoregulatory hormone, cortisol may also induce proliferation of lamellar chloride cells, which are suspected to play a role in ion uptake during acclimation to ion-poor FW (Laurent and Perry, '90) and stimulate vacuolar-type H^+-ATPase activity in gill tissue (Lin and Randall, '93). Thus, overall gill NKA

activity is not an adequate criterion to evaluate the hypo- or hyper-osmoregulatory nature of cortisol effects, as NKA is responsible for both ion-uptake and ion-excretion (cf. Introduction). Evaluation of gill NKCC1 expression levels may be a better criterion, as this transport protein presumably is specifically localized to secretory-type chloride cells. Thus, the present stimulation (trout, six hours) and stabilization (salmon, three days) of the NKCC1 mRNA by cortisol suggest a specific role as a SW-adapting hormone in the two salmonids. This is in accordance with Pelis and McCormick (2001), who found that cortisol injections stimulated gill filament cotransporter immunoreactivity (using the T4 anti-NKCC antibody) in Atlantic salmon. Cortisol seems to be directly responsible for equipping secretory-type chloride cells in salmonids with the two primary transport proteins: the NKA and the NKCC. Future studies should focus on whether this is due to cortisol acting as a specific transcription factor in existing (immature) chloride cells, due to a general proliferative effect on the chloride cell population or both.

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