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Differential expression of gill Na^+,K^+-ATPase α- and β-subunits, Na^+,K^+,$2Cl^-$ cotransporter and CFTR anion channel in juvenile anadromous and landlocked Atlantic salmon *Salmo salar*

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Summary

This study examines changes in gill Na^+,K^+-ATPase (NKA) α- and β-subunit isoforms, Na^+,K^+,$2Cl^-$ cotransporter (NKCC) and cystic fibrosis transmembrane conductance regulator (CFTR I and II) in anadromous and landlocked strains of Atlantic salmon during parr–smolt transformation, and after seawater (SW) transfer in May/June. Gill NKA activity increased from February through April, May and June among both strains in freshwater (FW), with peak enzyme activity in the landlocked salmon being 50% below that of the anadromous fish in May and June. Gill NKA-α1b, -α3, -β_1 and NKCC mRNA levels in anadromous salmon increased transiently, reaching peak levels in smolts in April/May, whereas no similar smolt-related upregulation of these transcripts occurred in juvenile landlocked salmon. Gill NKA-α1a mRNA decreased significantly in anadromous salmon from February through June, whereas α1a levels in landlocked salmon, after an initial decrease in April, remained significantly higher than those of the anadromous smolts in May and June. Following SW transfer, gill NKA-α1b and NKCC mRNA increased in both strains, whereas NKA-α1a decreased. Both strains exhibited a transient increase in gill NKA α-protein abundance, with peak levels in May. Gill α-protein abundance was lower in SW than corresponding FW values in June. Gill NKCC protein abundance increased transiently in anadromous fish, with peak levels in May, whereas a slight increase was observed in landlocked salmon in May, increasing to peak levels in June. Gill CFTR I mRNA levels increased significantly from February to April in both strains, followed by a slight, though not significant increase in May and June. CFTR I mRNA levels were significantly lower in landlocked than anadromous salmon in April/June. Gill CFTR II mRNA levels did not change significantly in either strain. Our findings demonstrates that differential expression of gill NKA-α1a, -α1b and -α3 isoforms may be important for potential functional differences in NKA, both during preparatory development and during salinity adjustments in salmon. Furthermore, landlocked salmon have lost some of the unique preparatory upregulation of gill NKA, NKCC and, to some extent, CFTR anion channel associated with the development of hypo-osmoregulatory ability in anadromous salmon.

Key words: smoltification, osmoregulation, ion regulation, cystic fibrosis transmembrane conductance regulator, Salmonid, development.

Introduction

In teleosts, the gill plays a principal role in the maintenance of ion homeostasis in both freshwater (FW) and seawater (SW) acclimated fish (Evans et al., 2005). Euryhaline teleosts, in particular, display a remarkable plasticity when it comes to adjusting ion transport across gill epithelia in response to changes in environmental salinity. This plasticity may arise as acclimation to salinity, or as part of a developmental event (for reviews, see Sakamoto et al., 2001; Evans et al., 2005). Anadromous species, such as Atlantic salmon go through a developmental process, the parr–smolt transformation, which entails a suite of morphological and physiological changes preparing the FW juvenile for subsequent migration into SW as smolts (Hoar, 1988; McCormick et al., 1998). The osmoregulatory challenges anadromous salmonids encounter when migrating into SW require a complete transformation of the gill from an ion-absorbing to an ion-secreting epithelium.

Compensatory ion transport across gill epithelia is achieved

by principal ion transporters primarily located in mitochondria-rich chloride cells and/or pavement cells (Evans et al., 2005). In SW, the basolateral Na^+,K^+-ATPase (NKA) energizes ion secretion by creating an electrochemical gradient used by the Na^+,K^+,$2Cl^-$ cotransporter (NKCC) and apical cystic fibrosis transmembrane conductance regulator (CFTR) to provide transcellular Cl^- secretion, with Na^+ excretion being paracellular (Evans et al., 2005). In FW, the basolateral NKA is probably also involved in driving uptake of NaCl, possibly in conjunction with an apical V-type H^+-ATPase, *via* apical Na^+ channels and Cl^-/HCO_3^- exchangers (Marshall, 2002). Accordingly, the NKA is an essential participant in maintaining ionic concentrations and body fluids within appropriate physiological limits.

The NKA enzyme consist of three subunits; α, β and γ (Blanco and Mercer, 1998). The α-subunit contains binding sites for cations, ATP and ouabain, and is thus responsible for the catalytic and ion regulatory capacity of the enzyme, while the β-subunit appears to be associated with protein maturation and anchoring of the enzyme complex in membranes (Blanco and Mercer, 1998). The γ-subunit appears to modulate affinity of the NKA enzyme for Na^+, K^+ and ATP; however, a γ-subunit has not yet been found in teleosts (Therien and Blostein, 2000; Hirose et al., 2003). In mammals, four α (α1–α4) and four β (β1–β4) subunit isoforms have been identified (Blanco and Mercer, 1998), while teleosts display an even wider repertoire of α- and β-subunit isoforms (Rajarao et al., 2001; Gharbi et al., 2004; Gharbi et al., 2005), many of which are expressed in gills (Richards et al., 2003). Recently, differential expression of α-subunit isoforms in salmonids (Richards et al., 2003; Mackie et al., 2005; Bystriansky et al., 2006) suggests that isoform switching may be an important mechanism by which anadromous species modulate NKA function in response to altered salinity.

Parr–smolt transformation in anadromous salmon is associated with a characteristic preparatory increase in overall gill NKA α- and β-subunit mRNA levels (Seidelin et al., 2001), NKA α-protein abundance (D'Cotta et al., 2000), NKA activity (McCormick, 1995) and NKCC mRNA and protein abundance (Pelis et al., 2001; Tipsmark et al., 2002). Gill CFTR I and CFTR II isoform mRNA levels have also been found to increase in smolts following SW transfer (Singer et al., 2002). However, whether differential expression of α-subunit isoforms and CFTR isoform mRNA levels also occur during parr–smolt development is currently unknown.

In contrast to anadromous salmon, several non-anadromous, landlocked, salmon populations complete their life-cycle in freshwater (McDowall, 1988). It is generally accepted that landlocked forms of Atlantic salmon are derived independently from various local anadromous founder populations, which were later prevented from reaching the upper reaches of watersheds with the elevation of the land post-glaciation (Power, 1958; Behnke, 1972). In the case of Bleke, the landlocked salmon used in the present study, anadromous salmon are presently prevented from reaching lake Byglandsfjord by the Vigelandsfossen waterfall between the sea and the lake (Dahl, 1928; Berg, 1985). Studies of landlocked Atlantic salmon from North America and Europe have shown differences in the capacity of these strains to adapt to SW (Birt et al., 1991; Birt and Green, 1993; Staurnes et al., 1992; Schmitz, 1995). The Bleke has recently been shown to have less preparatory development of gill NKA activity and SW tolerance compared to anadromous strains during the spring parr–smolt transformation period (Nilsen et al., 2003).

Thus, the main objective of this study was to compare changes of NKA α- and β-subunit isoforms, NKCC and CFTR isoforms in anadromous and landlocked strains of Atlantic salmon in order to get a deeper understanding of the preparatory development of SW tolerance in salmonids. Further, we also briefly discuss our results in the context of anadromous *vs* landlocked life histories.

Materials and methods

Fish material and rearing conditions

Juvenile Atlantic salmon *Salmo salar* L. from a non-anadromous, landlocked population (Bleke, lake Byglandsfjord, South-central Norway), and an anadromous population (River Vosso, South-western Norway) were used in the present study. The Bleke and Vosso strains originated from eggs collected from wild broodstock, the eggs were fertilised, incubated and hatched and under controlled hatchery conditions, and the fry were first fed in May. The Bleke strain was reared in the hatchery at Evje (managed by Setesdal Settefisk Ltd, Evje, Norway) at ambient water temperature (3°C in January, increasing to 5°C in mid-May, and reaching a peak of 16°C in August) with pH values ranging between 6.4–6.6. The Vosso strain was reared in the hatchery at Voss (managed by Voss Hatchery Foundation) at ambient water temperature (2–4°C from January through April increasing to 6°C in May). From mid-May, heated water (10°C) was used until ambient water temperature exceeded 10°C, reaching a peak of 20°C in August. The pH was maintained at approximately 6.4 throughout the juvenile stages. Fish from both strains were brought to the Aquatic Laboratory of the Bergen High Technology Center in October and kept separate in 1 m^2 indoor tanks supplied with flow-through, pH adjusted (6.9–7.1) FW, with a constant rearing temperature of 8°C. The fish were exposed to simulated natural photoperiod (SNP; 60°25′N) and fed a commercial dry diet (T. Skretting A/S, Stavanger, Norway) according to Austreng et al. (Austreng et al., 1987) for 8–12 h during the photo-phase. On March 5, a total of 150 anadromous (mean mass 29.4±2.2 g) and 150 landlocked (mean mass 21.7±1.1 g) salmon were transferred into duplicate 1 m^2 indoor tanks (N=75 in each tank) and reared as described above. In mid-May, both anadromous and landlocked salmon were transferred into full-strength SW (natural SW 34‰, 8°C) for assessment of long-term post-smolt performance. Mid-May was chosen as the time for SW transfer of both strains, based on previous findings (Nilsen et al., 2003) that both Vosso and Bleke show peak NKA activity, although of different intensity, at this time. On May 15 mean mass (± s.e.m.) of the Vosso and Bleke was not significantly different (44.6±2.4 g and 39.1±2.2 g, respectively), above the proposed threshold size for smolt development in juvenile Atlantic salmon (Thorpe et al., 1980). Condition factor in May was 1.08±0.02 and 1.14±0.01, respectively; again these did not differ significantly. Fish from both strains were also subjected to SW challenge tests (natural

SW 34‰, 8°C, 96 h, *N*=8 from each strain) on March 9, April 21, May 20 and June 22.

Sampling

Fish (*N*=10) from both strains in FW were quickly dip-netted out of the tanks and anaesthetized directly in 100 mg l^{-1} tricaine methanesulphonate (MS222; Sigma, St Louis, MO, USA) on February 26, April 15, May 15 and June 18. Wet mass and fork length were recorded, and blood collected from the caudal vessels using heparinized syringes, stored on ice less than 30 min before being centrifuged (1500 ***g***, 10 min, 4°C) and plasma aliquots frozen on dry ice. Gill tissue for determination of mRNA and protein levels was quickly dissected out and frozen directly on dry ice. Gill tissue for determination of NKA activity was placed in ice-cold SEI buffer (250 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} imidazole, pH 7.3) and frozen. All samples were stored at –80°C until assayed.

Gill NKA activity and plasma chloride

Gill NKA activity was determined by the method of McCormick (McCormick, 1993). Briefly, this kinetic assay utilizes the hydrolysis of ATP, which is enzymatically coupled to the conversion of NADH to NAD^+ by pyruvate kinase and lactic dehydrogenase with or without the addition of ouabain, a specific inhibitor of NKA. Readings were done at 340 nm for 10 min at 25°C. Protein in homogenate was determined by a bicinchoninic acid method (Smith et al., 1985). The NKA activity is expressed as μmol ADP mg^{-1} protein h^{-1}. Plasma chloride (Cl^-) levels (mmol l^{-1}) were analyzed in duplicate 10 μl samples using a chloride titrator (Radiometer CMT 10, Copenhagen, Denmark).

Total RNA isolation and reverse transcription

Total RNA for cloning was extracted from ~100 mg tissue from several salmon organs using Tri Reagent (Sigma, St Louis, MO, USA) as outlined elsewhere (Chomczynski, 1993). Total RNA was quantified spectrophotometrically, purity assessed (260/280 was ⩾1.8) and integrity checked by 1% agarose/formaldehyde gel electrophoresis. First strand synthesis of cDNA for subsequent use in cloning was generated using 2 μg total RNA, oligo d(T_{15}) and M-MLV RT (Promega, Madison, WI, USA) as described by the manufacturer.

Total RNA for gene expression studies was extracted from ~50 mg gill tissue, quantified and assessed as described above. tRNA was treated with RQ1 RNase-free DNase (Promega) and cDNA reverse transcribed using 0.5 μg tRNA and random nonamers in conjunction with the Reverse Transcription Core kit (EUROGENTEC RT-RTCK-05, Liege, Belgium) following the manufacturer's instructions.

Real-time quantitative PCR

A cohort of expressed sequence tags (EST) encoding partial *Salmo salar* NKA α-subunit sequences were identified by searching the GenBank EST database of published sequences from *Salmo salar* (accession nos AJ250809 and AJ250810), *Oncorhynchus mykiss* [for accession no., see Richards et al. (Richards et al., 2003)] and *Danio reiro* [for accession no., see Rajarao et al. (Rajarao et al., 2001)] using the BLAST algorithm (Altschul et al., 1997). Clones encoding multiple NKA-α subunit isoforms were obtained from the Norwegian Salmon Genome Project (SGP) and sequenced using Big-Dye version 3.1 and ABI 3700 automated sequencer at the University of Bergen. Based on BLAST searches against published sequences in the GenBank and multiple clustalW alignments (Thompson et al., 1994), five NKA-α subunits (α1a, α1b, α1c, α2 and α3) were identified. Given the high sequence similarity and presence of duplicate NKA-α isoform genes in Atlantic salmon (Gharbi et al., 2005), additional cloning using NKA-α isoform specific primers (Primer Express v3.0, Applied Biosystems, Inc., Foster City, CA, USA) designed from partial *Salmo salar* NKA-α1a/i (accession no. AY692142), NKA-α1b/i (accession no. AY692143), NKA-α1c/i (accession no. AY692145), NKA-α2 (accession no. AY692147), *Oncorhynchus mykiss* NKA-α3 (accession no. AY319388) and salmon ESTs were conducted in order to validate nucleotide sequences obtained from sequenced SGP clones. The PCR (50 μl) consisted of 4 μl cDNA, 200 nmol l^{-1} forward and reverse primers, 1.25 mmol l^{-1} dNTPs, 1.5 mmol l^{-1} $MgCl_2$ and 2 U μl^{-1} *Tag* polymerase (Promega) and thermal conditions of 5 min at 95°C, then 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 90 s and final extension at 72°C for 7 min. Subsequent PCR products were separated by 1% agarose gel electrophoresis, bands of appropriate size extracted using QIAEX II gel extraction kit (Qiagen, Crawley, UK) and PCR fragments cloned into a pCR®4-TOPO sequencing vector (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Plasmids were transformed into One Shot® TOP10 chemically competent *E. coli* and grown on ampicillin LB–agar plates. Colonies containing inserts were cultured overnight, purified using QIAGEN Mini Plasmid Kit (Qiagen) and sequenced in both directions.

Real-time quantitative PCR (Q-PCR) primers, 6-FAM labeled MGB probes and GenBank accession numbers are shown in Table 1. Primer specificity was tested by PCR using 10 μl cDNA, 400 nmol l^{-1} of each primer and SYBR Green Universal Master mix (Applied Biosystems Inc.) in a total reaction volume of 25 μl. The thermal cycling protocol consisted of 2 min at 50°C, 10 min at 95°C followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. Melt-curve analysis verified that the primer sets for each Q-PCR assay generated one single product and no primer–dimer artifacts. Expression of CFTR I and II isoforms was analysed using SYBR-based quantative PCR analysis using isoform-specific primers (Table 1) based on the sequences for *S. salar* CFTR I and II isoforms published elsewhere (Chen et al., 2001), and normalised to expression of elongation factor 1A ($EF1A_A$: accession no. AF321836, Table 1). Polymerase chain reactions were done with Brilliant SYBR Green Q-PCR Master Mix (Stratagene, La Jolla, CA, USA) on a Mx3000P (Stratagene). PCR reactions contained 1 μl cDNA (50 ng RNA), 150 nmol l^{-1} of each primer and 12.5 μl Brilliant SYBR Green Master Mix in a total volume of 25 μl. All Q-PCR reactions were performed as follows: 10 min of polymerase activation at 95°C, 40 cycles of 95°C for 30 s and 60°C for 1 min. Melting curve analysis was performed following each reaction to confirm that there was only a single product of the reaction. In addition, representative PCR products were analysed by electrophoresis to verify that only a single band was present.

Table 1. *Nucleotide sequences of real-time PCR primers and probes used to quantify gene expression in gills of Atlantic salmon*

Gene	GenBank accession no.	Forward primer	Reverse primer	6-FAM MGB probes	Size (bp)
NKA α-1a	CK878443	CCAGGATCACTCAATGTCACTCT	GCTATCAAAGGCAAATGAGTTTAATATCATTGTAAAA	ACGATTACATTATAAGGCAATACT	93
NKA α-1b	CK879688	GCTACATCTCAACCAACAACATTACAC	TGCAGCTGAGTGCACCAT	ACCATTACATCCAATGAACACT	91
NKA α-1c	CK 885259	AGGGAGACGTACTACTAGAAAGCAT	CAGAACTTAAAATTCCGAGCAGCAA	ACAACCATGCAAGAACT	85
NKA α-3	CK170270	GGAGACCAGCAGAGGAACAG	CCCTACCAGCCCTCTGAGT	AAGACCCAGCCTGAAATG	58
NKA β1	CK886866	CGTCAAGCTGAACAGGATCGT	CCTCAGGGATGCTTTCATTGGA	CCTTGGCCTGAAGTTG	68
NKCC	AJ417890	GATGATCTGCGGCCATGTTC	AGACCAGTAACCTGTCGAGAAAC	CTCCAGAAGGCCCAACTT	68
EF1A$_A$	AF321836	CCCCTCCAGGACGTTTACAAA	CACACGGCCCACAGGTACA	ATCGGTGGTATTGGAAC	57
CFTR I	AF155237	CCTTCTCCAATATGGTTGAAGAGGCAAG	GAGGCACTTGGATGAGTCAGCAG	Sybr Green	84
CFTR II	AF161070	GCCTTATTTCTTCTATTTGTATGCACTT	GCCACCATGAAAAACTAAAGAGTACCTCAG	Sybr Green	67
EF1A$_A$	AF321836	GAGAACCATTGAGAAGTTCGAGAAG	GCACCCAGGCATACTTGAAAG	Sybr Green	71

Negative control reactions were performed for representative samples using RNA that had not been reverse transcribed to control for the possible presence of genomic DNA contamination. Genomic DNA was present but never constituted more than 1:32768 starting copies. Non-template control reactions were also performed to verify that there was no cDNA contamination or primer–dimer amplification in the reactions. All TaqMan Q-PCR assays were performed in a total volume of 25 μl on the ABI prism 7000 detection system platform (Applied Biosystems) using 5 μl cDNA (25 ng RNA) template, 900 nmol l^{-1} forward and reverse primers, 200 nmol l^{-1} probe and 12.5 μl TaqMan® Universal PCR Master Mix containing AmpErase® uracil N-glycosylase. The thermal cycling protocol consisted of 2 min at 50°C, 10 min at 95°C, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. Omission of reverse transcriptase in the RT reaction resulted in a shift in threshold cycle (C_t) values of ⩾13 cycles in all assays, which shows that interference from residual DNA in RNA samples after DNase treatment was negligible. Validation experiments (Applied Biosystems User Bulletin #2) using cDNA generated from twofold serial dilutions of RNA gave log input cDNA *vs* C_t plots with R^2>0.99 and ΔC_t<0.1 for all target genes (NKA-α1a, NKA-α1b, NKA-α1c, NKA-α3, NKA-β1 and NKCC) in relation to elongation factor 1A ($EF1A_A$) (Olsvik et al., 2005). Results are presented as relative expression according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using $EF1A_A$ as an internal control and anadromous parr (February 26) as calibrator. All TaqMan Q-PCR assays were used within a C_t range where the log input cDNA *vs* C_t plots were found to be linear over 5 log phases with R^2>0.98. Non-template controls were included on all plates.

Western blots

Gill NKA α-subunit and NKCC protein abundance were determined by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as outlined elsewhere (Pelis et al., 2001), with a few modifications (Stefansson et al., 2007). Briefly, NKA and NKCC abundance was detected using a mouse monoclonal antibody specific for chicken α-subunit (Takeyasu et al., 1990) and a mouse monoclonal antibody directed against 310 amino acids at the carboxyl terminus of human colonic NKCC1, respectively. The NKA (α5; developed by D. M. Fambrough, Johns Hopkins University, MD, USA) and NKCC (T4; developed by Christian Lytle and Bliss Forbush III) antibodies were obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Department of Biological Sciences, Iowa City, IA, USA. Thawed gill tissue was homogenized using an ice-cold glass homogenizer in SEI buffer containing protease inhibitors (1 complete-mini tab per 10 ml SEI, Roche Diagnostics Corporation, Indianapolis, USA) and centrifuged at 2060 ***g*** for 7 min. The resulting pellet of subcellular material was resuspended in 5 volumes of SEI buffer containing 0.1% sodium deoxycholate. After centrifugation at 2060 ***g*** for 6 min, supernatant was diluted with Laemmli's buffer and heated at 60°C for 15 min. This crude membrane preparation is similar to that used by Zaugg (Zaugg, 1982) and results in fourfold enrichment of membrane-bound proteins. A sample volume of 10 μg total protein was separated by 7.5%

and 6% SDS-PAGE for NKA and NKCC, respectively. After 2 h, the gels were blotted onto Immobilion P (PVDF) membranes (Millipore, Bedford, MA, USA) overnight on ice and incubated in blocking buffer [PBS containing 0.05% Triton X-100 (Tx) and 2% skimmed milk] for 1 h at room temperature. After rinsing of membranes in PBS-Tx, membranes were incubated with anti-NKA (α5; 1:2000) or anti-NKCC (T4; 1:1000) antibodies. Membranes were rinsed and incubated with secondary peroxidase-conjugated antibodies (1:1000) for 1 h and reacted with diaminobenzidine solution until bands were visible. Colour development was stopped with deionised water, membranes dried and digital photographs taken. Band staining intensity was quantified using ImageJ image processing and analysis software (see Pelis et al., 2001).

Statistics

All statistical analyses were performed with Statistica 6.0. (StatSoft, Inc., Tulsa, OK, USA). The homogeneity of variance was tested using the Hartley *F*-max test. When necessary, data were log-transformed to meet the parametric assumptions of ANOVA (Zar, 1996). Comparisons of NKA, NKCC and CFTR were performed using a nested ANOVA with duplicate tanks nested within time and strain, whereas a two-way ANOVA was used to test for overall differences within strains between SW and FW, and between strains in SW. A two-way ANOVA was used to test for overall differences in NKA-α and NKCC protein levels. Significant ANOVAs were followed by Tukey unequal N HSD *post hoc* tests. Data are presented as mean ± standard error of the mean (s.e.m.) and considered significant at the $P<0.05$ level.

Results

Gill NKA activity and hypo-osmoregulatory capacity

FW gill NKA activity levels in the anadromous strain increased significantly from April to May, with enzyme activities being fivefold higher in May and June than those observed in February (Fig. 1A). In the landlocked strain, FW gill NKA activity levels increased significantly from April to May, with enzyme activities being twofold higher in May and June than those observed in February (Fig. 1A). Gill NKA activity in FW was significantly lower among fish in the landlocked than the anadromous strain in May and June.

Gill NKA activity in the anadromous salmon was not significantly affected by 4 days of SW exposure in May, while it was significantly elevated in landlocked salmon (Fig. 1A). In June, after 1 month in SW, gill NKA activity was significantly higher in the landlocked strain compared with levels in FW fish (Fig. 1A).

Improved short-term hypo-osmoregulatory capacity, measured as the ability to regulate plasma Cl^- levels after 4 days in 34‰ SW, was observed in both strains from February to May (Fig. 1B). In April, hypo-osmoregulatory capacity was significantly greater in the anadromous strain than in the landlocked strain. In June, after 1 month in SW, both strains retained low plasma Cl^- levels.

Gill NKA α-isoform mRNA levels

FW gill NKA α-1a isoform mRNA levels in the anadromous strain decreased continuously from February through April, May and June, with levels in June being fourfold lower than those observed in February (Fig. 2A). The landlocked strain showed a twofold decrease in NKA α-1a mRNA levels from February to April and remained stable in May and June, resulting in NKA α-1a levels being significantly higher than those of the anadromous strain in May and June. In May and June, after 4 days and 1 month of SW exposure, respectively, gill NKA α-1a mRNA levels were significantly lower in both strains compared with corresponding FW fish (Fig. 2A), with no significant differences between strains.

FW gill NKA α-1b isoform mRNA levels in the anadromous strain increased significantly from February through April and May, with relative mRNA levels in May being sixfold higher than those observed in February, followed by a significant decrease in June (Fig. 2B). In contrast, only a twofold increase

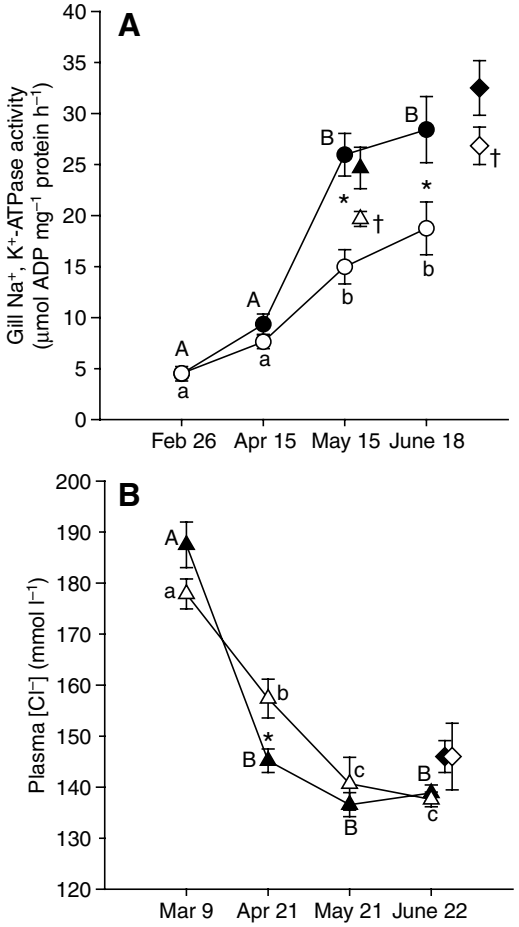

Fig. 1. (A) Gill Na^+, K^+-ATPase activity in anadromous (closed circles) and landlocked (open circles) Atlantic salmon in FW and SW from February 26 through June 18; (B) plasma [Cl^-] in SW from March 9 through June 22. Symbols for anadromous (closed triangles) and landlocked salmon (open triangles) after 96 h SW (34‰) exposure, and anadromous (closed diamonds) and landlocked salmon (open diamonds) in mid-June after 1 month in SW are offset for clarity. Values are means ± s.e.m. (N=8–10). *Significant difference between strains in FW ($P<0.05$). Different capital and small letters denote differences ($P<0.05$) between time points within anadromous and landlocked salmon, respectively. †Significant differences between FW and SW in landlocked salmon.

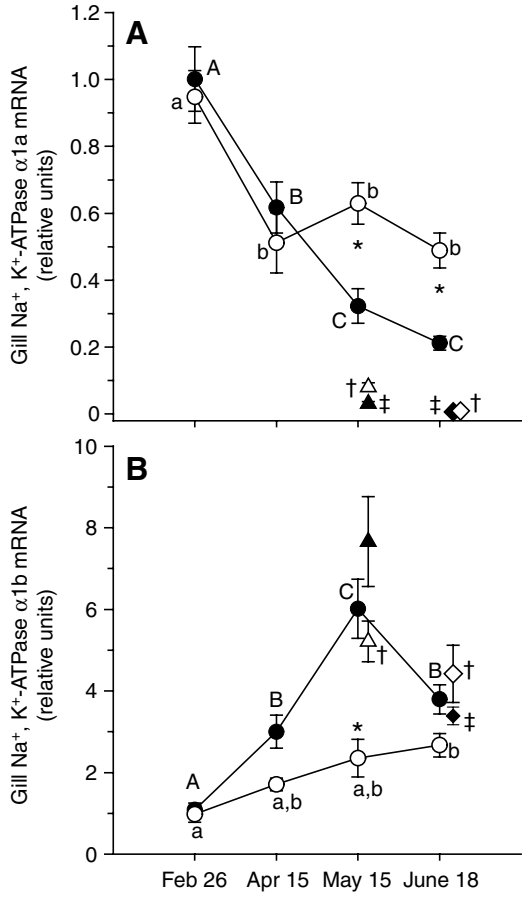

Fig. 2. Gill Na^+, K^+-ATPase α1a (A) and α1b (B) mRNA levels in anadromous (closed circles) and landlocked (open circles) Atlantic salmon in FW from February 26 through June 18. Symbols for anadromous (closed triangle) and landlocked salmon (open triangle) after 96 h SW (34‰) exposure, and anadromous (closed diamond) and landlocked salmon (open diamond) in mid-June after 1 month in SW are offset for clarity. Values are means ± s.e.m. (N=8–10). *Significant difference between strains in FW (P<0.05). Different capital and small letters denote differences (P<0.05) between time points within anadromous and landlocked salmon, respectively. Significant differences between FW and SW are indicated by †(landlocked) ‡(anadromous) salmon.

in gill NKA α-1b mRNA levels was observed in the landlocked strain from February through June (Fig. 2B), yet significantly lower than peak smolt levels in anadromous fish. In May, after 4 days of SW exposure, NKA α-1b levels increased significantly in the landlocked strain. In June, after 1 month in SW, mRNA levels in the anadromous strain were significantly lower than those of FW smolts in May, whereas NKA α-1b levels in the landlocked strain were significantly higher in SW than FW (Fig. 2B).

Gill NKA α-1c isoform mRNA levels did not change significantly in either strain in FW from February through June, or following SW exposure (Table 2). NKA α-2 mRNA was not detected in gills.

FW gill NKA α-3 isoform mRNA levels in the anadromous strain showed a transient increase in May (Fig. 3A). In contrast, gill NKA α-3 levels remained low in the landlocked strain throughout the study. NKA α-3 mRNA levels were not influenced by SW exposure (Fig. 3A).

Gill NKA β1-subunit mRNA levels

FW gill NKA β1 subunit mRNA levels in the anadromous strain showed a significant transient increase from February to peak levels in April, followed by a reduction to parr levels in June (Fig. 3B). In contrast, no significant increase of gill NKA β1 mRNA occurred from February through June in the landlocked strain. After 4 days in SW, NKA β1 levels in the anadromous and landlocked strains were approximately the same as in corresponding FW fish (Fig. 3B). In June, after 1 month in SW, NKA β1 mRNA levels in both strains were significantly lower compared to fish in FW.

Gill NKA α-protein abundance

FW gill NKA α-protein abundance in the anadromous strain increased significantly from parr levels in February to peak levels in May, with protein levels remaining high in June (Fig. 4). The landlocked strain showed a similar, though not significant, increase in protein levels between February and May. In June, after 1 month in SW, protein levels in both strains were lower compared to fish in FW. No significant differences in gill NKA α-protein abundance were observed between the two strains, either in FW or SW (Fig. 4).

Table 2. *Gill NKA-α1c and CFTR II mRNA levels in anadromous and landlocked Atlantic salmon in freshwater, and NKA-α1c after 4 days (May 19) and 1 month (June 18) in seawater*

	Gill mRNA levels (relative units)			
	NKA-α1c		CFTR II	
Date	Anadromous	Landlocked	Anadromous	Landlocked
February 26 (FW)	1.08±0.06	1.08±0.08	9.92±0.96	10.39±1.13
April 15 (FW)	1.01±0.09	1.03±0.05	12.44±1.31	9.35±1.04
May (FW)	1.01±0.11	1.04±0.11	12.74±1.18	13.09±1.69
June 18 (FW)	1.11±0.08	1.08±0.07	11.14±1.46	9.99±1.69
May 19 (SW)	1.15±0.11	1.12±0.08	–	–
June 18 (SW)	0.88±0.04	0.97±0.06	–	–

FW, freshwater; SW, seawater; NKA-α1c, Na^+,K^+-ATPase α1c; CFTR II, cystic fibrosis transmembrane conductance regulator II. CFTR II was not measured following seawater transfer.

Values are mean ± s.e.m. (N=8–10). No significant differences were observed.

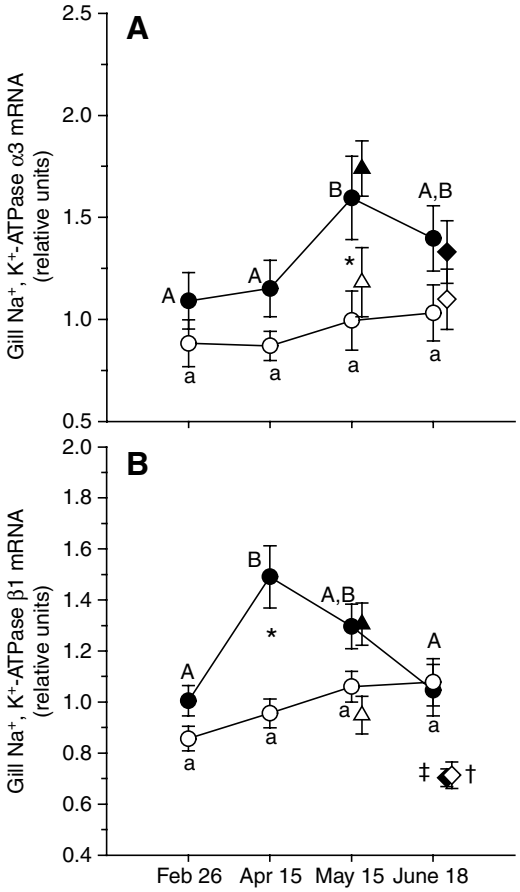

Fig. 3. Gill Na^+, K^+-ATPase α3 (A) and Na^+, K^+-ATPase β1 (B) mRNA levels in anadromous (closed circles) and landlocked (open circles) Atlantic salmon in FW from February 26 through June 18. Symbols for anadromous (closed triangle) and landlocked salmon (open triangle) after 96 h SW (34‰) exposure, and anadromous (closed diamond) and landlocked salmon (open diamond) in mid-June after 1 month in SW are offset for clarity. Values are means ± s.e.m. (N=8–10). *Significant difference between strains in FW (P<0.05). Different capital and small letters denote differences (P<0.05) between time points within anadromous and landlocked salmon, respectively. Significant differences between FW and SW are indicated by †(landlocked) ‡(anadromous) salmon.

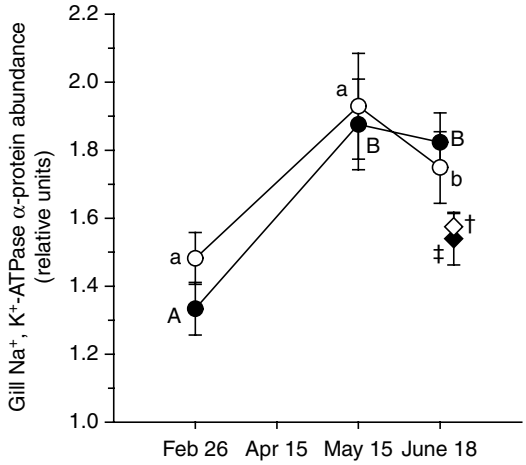

Fig. 4. Gill Na^+, K^+-ATPase α-protein abundance in anadromous (closed circles) and landlocked (open circles) Atlantic salmon in FW from February 26 through June 18. Symbols for anadromous (closed diamond) and landlocked salmon (open diamond) in mid-June after 1 month in SW are offset for clarity. Values are means ± s.e.m. (N=5–6). Different capital and small letters denote differences (P<0.05) between time points within anadromous and landlocked salmon, respectively. Significant differences between FW and SW are indicated by † (landlocked) and ‡ (anadromous) salmon.

Gill NKCC mRNA levels

FW gill NKCC mRNA levels in the anadromous strain increased threefold from February through April and May, decreasing in June (Fig. 5A). In contrast, the landlocked strain exhibited 50% increase in NKCC mRNA levels from February to April, remaining stable through June. After 4 days in SW, gill NKCC mRNA levels were significantly elevated in landlocked, but not anadromous fish (Fig. 5A). In June, after 1 month in SW, relative NKCC mRNA levels were approximately the same as observed for fish in FW (Fig. 5A).

Gill NKCC protein abundance

FW gill NKCC abundance in the anadromous strain increased significantly from February to peak levels in May, followed by a decrease in June. The landlocked strain exhibited no significant increase of NKCC abundance in May, but NKCC abundance was significantly higher in June than in February (Fig. 5B). After 1 month of SW exposure, NKCC levels were similar among fish in both strains and did not differ from levels in FW.

Gill CFTR I and II mRNA levels

Gill CFTR I mRNA levels in anadromous salmon increased significantly from February to April, remained stable in May, followed by a slight increase in June (Fig. 6). Gill CFTR I mRNA levels in landlocked salmon increased from February to April and remained higher through June (Fig. 6), yet levels were significantly lower than those of anadromous salmon in April and June.

Gill CFTR II mRNA levels did not change significantly in either strain from February through June (Table 2). No significant differences in CFTR II levels were observed between the two strains.

Discussion

Parr–smolt transformation in anadromous salmon is associated with a characteristic preparatory increase in gill NKA activity, which is largely completed while salmon are still in FW, allowing the smolt to move rapidly from FW to full-strength SW with minimum osmotic disturbance (Hoar, 1988). Here we provide evidence that this hypo-osmoregulatory development, as judged by changes of transcriptional, translational and activity levels of key ion-regulatory proteins, is dampened during the spring smoltification period in landlocked compared to anadromous Atlantic salmon.

Consistent with the recent findings in rainbow trout (Richards et al., 2003), we found four NKA α-isoforms (α1a, α1b, α1c and α3) to be present in salmon gills, while α2 was not detected. Present findings of a transient upregulation of gill α1b mRNA

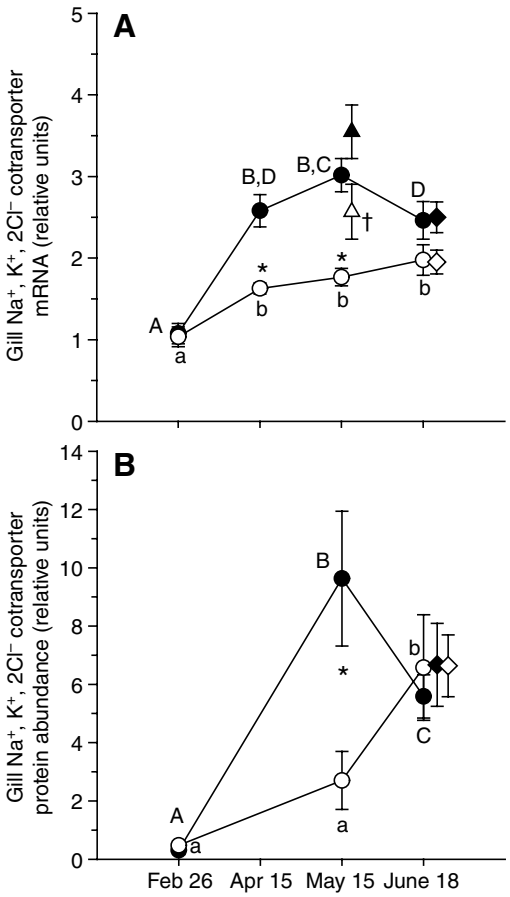

Fig. 5. Gill Na^+ K^+, $2Cl^-$ cotransporter mRNA levels (A) and protein abundance (B in anadromous (closed circles) and landlocked (open circles) Atlantic salmon in FW from February 26 through June 18. Symbols for anadromous (closed triangle) and landlocked salmon (open triangle) after 96 h SW (34‰) exposure, and anadromous (closed diamond) and landlocked salmon (open diamond) in mid-June after 1 month in SW are offset for clarity. Values are means ± s.e.m. (N=8–10). *Significant difference between strains in FW (P<0.05). Different capital and small letters denote differences (P<0.05) between timepoints within anadromous and landlocked salmon, respectively. †Significant differences between FW and SW in landlocked salmon.

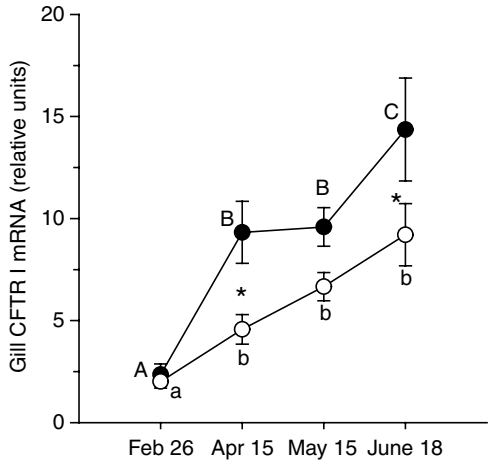

Fig. 6. Gill cystic fibrosis transmembrane conductance regulator I (CFTR I) mRNA levels in anadromous (closed circles) and landlocked (open circles) Atlantic salmon in FW from February 26 through June 18. CFTR I was not measured following SW transfer. Values are means ± s.e.m. (N=8–10). *Significant difference between strains in FW (P<0.05). Different capital and small letters denote differences (P<0.05) between time points within anadromous and landlocked salmon, respectively.

levels in anadromous salmon, concurrent with a continuous decrease of α1a, suggest that reciprocal expression of these two isoforms not only represents a mechanism through which salmonids can modulate gill NKA in response to altered salinity (Richards et al., 2003; Mackie et al., 2005; Bystriansky et al., 2006), but also constitutes an important feature underlying the preparatory increase of gill NKA activity occurring in anadromous salmon prior to SW entry. Consequently, as gill α1b mRNA is the principal isoform upregulated in anadromous smolts in the present study, showing a relative 20-fold higher upregulation than α1a from parr to smolts in May, it is likely that the preparatory increase in overall gill NKA α-subunit mRNA levels previously reported in salmon (D'Cotta et al., 2000; Seidelin et al., 2001) actually may have been a result of specific α1b isoform upregulation. In contrast to anadromous salmon, no apparent smolt-like increase of gill α1b levels occurred in landlocked salmon. On the other hand, a slight increase of α1b in juveniles landlocked during spring parallels a lower temporal increase in enzyme activity of these fish compared with anadromous smolts. Elevated NKA activity is, however, not necessarily dependent on increased α1b isoform mRNA levels. In contrast to studies on salmonids, including the present, a transient α1a upregulation was found in killifish following transfer from brackish water (BW) to SW (Scott et al., 2004a). However, α1a was also upregulated upon transfer from BW to FW, and this increase was larger and more prolonged than from BW to SW. Scott et al. further found (Scott et al., 2004b) that the differences in mortality observed between Northern and Southern killifish upon transfer from BW to FW correlated well with gill NKA activity and α1a mRNA levels. With the exception of the transient increase in SW, both studies by Scott and colleagues concur with the hypothesis of α1a having kinetic properties associated with successful ion regulation in FW (Richards et al., 2003), but the reciprocal shift between α1a and α1b isoforms seems to be specific for salmonids. Although an overall transient upregulation of gill NKA α1b mRNA, concurrent with an abrupt, sustained decrease of α1a levels in both anadromous and landlocked salmon following SW transfer, is consistent with recent findings in salmonids (Richards et al., 2003; Mackie et al., 2005; Bystriansky et al., 2006), the differences in magnitude by which these two strains respond to SW exposure illustrate an important trait associated with the development of hypo-osmoregulatory ability in salmonids; the presence and magnitude of responses to salinity changes is dependent on their euryhaline capacity prior to SW entry. For instance, despite higher NKA α1b mRNA levels in anadromous salmon following SW transfer, landlocked salmon display a higher induction of α1b levels, compared with their corresponding FW values. It is therefore likely that landlocked salmon may compensate the lack of preparatory changes through higher *de novo* synthesis of α1b

following SW transfer, as further indicated by a higher relative induction of enzyme activity among these fish in SW. Similar differences were recently observed between rainbow trout, Arctic char *Salvelinus alpinus* and Atlantic salmon following SW transfer (Bystriansky et al., 2006). In the case of NKA α1c, however, no apparent changes occurred in either anadromous or landlocked salmon in the present study, supporting the suggestion of a 'housekeeping' function of α1c in branchial tissue of salmonids (Richards et al., 2003).

The role of the NKA α3 subunit isoform in salinity acclimation appears less important than α1 isoforms. In fact, studies in heterologous expression systems have shown that mammalian NKA isozymes show distinct affinities for Na^+ and K^+, with the α3 isoform possessing higher K_m values for Na^+ than the α1 and α2 NKA isozymes (Jewell and Lingrel, 1991; Blanco and Mercer, 1998; Crambert et al., 2000). Thus, a lower Na^+ affinity of α3 isozymes suggests that isozymes comprising this isoform may be less efficient in transporting Na^+ when the intracellular Na^+ concentration is low. However, although of a lower magnitude than α1b, a distinct transient increase of gill α3 mRNA levels in anadromous, but not landlocked salmon, suggests a significant role of this isoform during parr–smolt transformation. Consistent with findings in rainbow trout (Richards et al., 2003), neither anadromous nor landlocked salmon showed any significant increase of α3 levels following SW exposure. In tilapia, *Oreochromis mossmabicus*, gill α3 (twofold) and α1 (fivefold) mRNA levels increase following transfer from FW to SW (Feng et al., 2002). Taken together, these results suggest that differential expression of α1 subunit isoforms is more pronounced than for α3 isoforms, probably because the α1 isoforms have kinetic properties more favorable for the differential ion transport processes of chloride cells in FW and SW (Richards et al., 2003; Evans et al., 2005). Nevertheless, further studies are necessary to ascertain the relative importance and specific roles of α1 and α3 in ion regulation.

The NKA β1-subunit is necessary for protein maturation and anchoring of the enzyme in cell membranes. Thus, co-expression of both α and β subunits are essential for NKA function (Blanco and Mercer, 1998). The transient increase of gill NKA β1 mRNA levels in anadromous salmon in the present study is largely in accordance with previous findings (Seidelin et al., 2001). On the other hand, the lack of a preparatory increase of β1 mRNA levels in landlocked salmon, despite elevated enzyme activity in May and June, and further, decreasing levels of β1 mRNA at peak smoltification in anadromous salmon and in both strains after SW transfer, suggest additional mechanisms by which β subunits may be regulated, possibly through various osmoregulatory and/or hormone response elements (Kolla et al., 1999; Deane and Woo, 2004) or differential expression of multiple β subunit isoforms. For instance, in the European eel *Anguilla anguilla*, expression of gill β_{233}, a duplicate copy of the NKA β1-isoform, has been found to be dependent upon the developmental stages of these fish, as upregulation only occurs in migratory silver eels, and not in adult non-migratory yellow eels following SW transfer (Cutler et al., 2000). Recently, *in silico* analysis of Expressed Sequence Tags has identified at least four NKA β subunit isoforms in salmonids (Gharbi et al., 2004; Gharbi et al., 2005). Assuming that multiple β subunit isoforms are present in gills, it is possible that differential expression of putative gill β1 isoforms may be similar to isoform switching of gill α1a and α1b during salmon smoltification and salinity acclimation. Alternatively, β-subunit abundance could be regulated at post-transcriptional levels, and thus differ from regulation of α-subunit synthesis.

Changes at the transcriptional level are often assumed to parallel increased protein abundance. As such, one would expect differential regulation of α-subunit isoforms at the transcriptional level to bring about differences in α protein abundance. Overall, a good correspondence between total NKA α-subunit mRNA, protein abundance and enzyme activity in the present study would suggest a coordinated regulation at the transcriptional and translational levels. This was not always the case, however, as both anadromous and landlocked salmon displayed a similar transient increase in gill α protein abundance, despite total α-subunit mRNA levels in May being 2.5-fold higher in anadromous than landlocked salmon, based on an estimation of all four α-subunit isoforms. Similar differences in overall α-subunit mRNA and protein abundance have been found in anadromous salmonids (D'Cotta et al., 2000; Seidelin et al., 2001; Tipsmark et al., 2002), killifish (Scott et al., 2004b) and tilapia (Lee et al., 1998; Lee et al., 2003). Thus, the transient increase of α-subunit mRNA and protein abundance, with peak levels in May, concurrent with sustained elevated enzyme activity in June, indicate the importance of both transcriptional and post-transcriptional mechanisms in modulating NKA activity. Post-transcriptional mechanisms have been shown to modulate gill NKA activity in brown trout *Salmo trutta* (Tipsmark and Madsen, 2001), and could explain a sustained enzyme activity in June, despite a decrease in corresponding α-subunit protein and mRNA levels. The temporal switching of gill α1a and α1b mRNA between anadromous and landlocked salmon contrasts the similar transient upregulation of α-subunit protein in these two strains. Assuming that upregulation of α-isoform mRNA levels are, in fact, associated by translational changes in putative NKA α-isoform abundance, it is conceivable that the α5 antibody, which is based on conserved regions of multiple α-subunit isoforms in several vertebrate species (Takeyasu et al., 1990), may recognize all putative α-subunit isoforms, and thus account for some of the discrepancies observed in the present study. Further investigations should verify differential expression of putative α-isoforms at the translational level in order to ascertain their physiological role in ion regulation.

While gill NKA is an essential participant in both ion secretion and uptake in gills, the basolateral NKCC and apical CFTR anion channel are considered to be primarily involved in ion secretion (Evans et al., 2005). Present findings of a preparatory transient increase of gill NKCC mRNA and protein levels in anadromous salmon are largely in accordance with previous studies in salmon (Pelis et al., 2001; Tipsmark et al., 2002). Interestingly, landlocked salmon appear to have lost the preparatory upregulation of gill NKCC mRNA associated with the parr–smolt transformation. However, like the anadromous salmon, landlocked salmon have the capacity to upregulate this transcript following SW transfer. This suggests that our TaqMan assay most likely is specific for the secretory NKCC isoform.

On the other hand, two secretory isoforms, the NKCC1a and NKCC1b, have been identified in European eel, and only gill NKCC1a is upregulated following SW transfer (Cutler and Cramb, 2002). Thus, one cannot exclude the possibility of more than one secretory isoform being present in salmon gills, and that these may be differentially regulated. As with NKA, there was no straightforward correspondence between NKCC mRNA and protein levels, in either anadromous or landlocked salmon, as increased NKCC protein abundance was more profound than NKCC mRNA levels, possibly reflecting a lower turnover of this protein in salmon gills. Similar differences have been observed in anadromous salmonids (Tipsmark et al., 2002) and killifish (Scott et al., 2004b). On the other hand, a distinct upregulation of NKCC protein in landlocked salmon between May and June contradicts the apparent lack of a preparatory increase at the transcriptional level in these fish. Some of the discrepancies observed in present and other studies may be ascribed to the use of the T4 antibody, as it most likely recognizes both the secretory and an absorptive isoforms (Lytle et al., 1995).

In the case of CFTR anion channel isoforms CFTR I and CFTR II, our present findings suggest that these two isoforms are differentially regulated during salmon smoltification. The continuous increase of gill CFTR I mRNA levels in anadromous salmon, and to a lesser extent in landlocked salmon, suggests a preparatory increase of this isoform during acquisition of salinity tolerance. Given that CFTR is primarily involved with ion secretion (Evans et al., 2005), it was somewhat surprising that CFTR II mRNA levels remained stable in FW among both anadromous and landlocked salmon. Assuming that both CFTR isoforms are actually inserted into the apical membrane as functional Cl^- channels, it is possible that high CFTR II levels may be important for a rapid activation of CFTR when exposed to higher salinity. In fact, Singer et al. (Singer et al., 2002) found a sustained increase of gill CFTR I mRNA levels in Atlantic salmon smolts following SW, while CFTR II mRNA levels increased transiently, peaking after 24 h in SW. Further studies are clearly required to ascertain the physiological role of CFTR I and II in salmon smoltification and SW adaptation.

Salmonids display a remarkable plasticity when it comes to adjusting ion homeostasis in response to changes in environmental salinity. This plasticity may arise as part of a developmental event, or in response to salinity exposure (McCormick, 2001; Evans et al., 2005; Hiroi and McCormick, 2007). Although the landlocked salmon appears to have lost some of the developmental increase in ion transport proteins associated with preparation for SW migration, these fish seem to have retained the plasticity to respond when challenged with SW, as judged by their ability to upregulate key ion transporters and maintain low plasma Cl^- levels similar to the anadromous strain. In contrast to our previous study (Nilsen et al., 2003) where this landlocked strain showed 40% mortality after 16 days in SW, no mortality occurred in the present study. One contributing factor to these contrasting observations may be the larger size of the juvenile Bleke in the present study (mean mass 39.1 g) compared with that of our previous study (mean mass 24.8 g) (Nilsen et al., 2003) when transferred to SW in May. This suggestion is in line with the general view that larger body size corresponds with greater hypo-osmoregulatory capacity in juvenile FW salmonids [see Hoar (Hoar, 1988) and references therein]. However, this gradual, size-dependent increase in salinity tolerance in parr and non-anadromous species is different from the rapid and dramatic increase in salinity tolerance that develops during parr–smolt transformation [characterised by concurrent change in ontogeny, increased developmental rate and increased differentiation (McCormick and Saunders, 1987)]. A threshold size for smolting in the range 9.5 cm (1+ smolts) and 12 cm (2+ smolts) was described in offspring of wild broodstock (Thorpe et al., 1980), supporting our conclusions that fish from both strains were above the critical size threshold for Atlantic salmon smolt development in May (>15 cm, 39–44 g). There is further support for our view that differences between the two strains were not caused by differences in fish size: Bjerknes et al. concluded that fish size did not influence plasma osmolality or muscle water content following SW acclimation of Atlantic salmon >9.5 cm, whereas parr <9.5 cm suffered high mortalities and severe osmotic disturbance (Bjerknes et al., 1992), and Handeland and Stefansson reported higher NKA activity in small than large smolts (approx. 40 g *vs* 55 g), supporting smolt development being less dependent on fish size even within a wider size range than in the present experiment (Handeland and Stefannson, 2001). Finally, with the exception of an increase in NKCC protein abundance, no major changes in ion regulatory parameters were observed in the juvenile Bleke between May and June, despite an increase in fish size to 54.7 (±2.9) and 54.9 (±3.6) g of the Bleke and Vosso, respectively. These observations further support our view that the differences observed in May between Vosso smolts and juvenile Bleke reflect differences between strains, and are not caused by the slight (non-significant) difference in fish size in May.

The apparent loss of the preparatory osmoregulatory changes in the landlocked salmon is likely the result of natural selection, as these changes are no long necessary. Similar mechanisms have been suggested for other traits such as the loss of muscle fibers (Johnston et al., 2005), as energy may be wasted in processes that reduce their overall fitness (McDowall, 1988). In contrast, the ability to respond to SW as a protective mechanism has been retained, due to its importance in exploiting other habitats. This plasticity most likely is under less selection pressure, as the trait is only energy demanding upon SW stimulation. Even though our findings suggest the landlocked salmon do not need a preparatory increase in hypo-osmoregulatory capacity to attain ion homeostasis in SW, it must be kept in mind that these results were obtained in a protective environment with no external stressors that would otherwise be present in the wild. As stated above, the capacity to acclimate to seawater may depend on fish size, as larger fish may be able to withstand a hypersaline environment sufficiently long, allowing time for the SW-stimulated plasticity to occur, whereas the smaller fish may suffer severe salinity stress, leaving them unable to accommodate a plastic change. Support for this view was observed in a sub-experiment when fish from the present study experienced additional stress just prior to SW exposure in May. The majority of the landlocked fish died within days of SW exposure, whereas the anadromous salmon all survived (L.E., unpublished observation). Taken together, these observations indicate that stressed fish with an inadequate

preparatory development of ion transporters are unable to exploit their inherent plasticity upon SW exposure.

In summary, the present study demonstrates that differential expression of gill NKA α1a, α1b and α3 isoform transcripts may, in part, be an important molecular mechanism underlying potential functional differences in NKA, both during preparatory development and during salinity adjustments in salmon. Furthermore, despite having lost some of the unique preparatory upregulation of key ion-secretory proteins associated with parr–smolt transformation, landlocked salmon have retained some hypo-osmoregulatory capacity when exposed to SW during spring.

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