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Development of intestinal ion-transporting mechanisms during smoltification and seawater acclimation in Atlantic salmon *Salmo salar*

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This study investigated the expression of ion transporters involved in intestinal fluid absorption and presents evidence for developmental changes in abundance and tissue distribution of these transporters during smoltification and seawater (SW) acclimation of Atlantic salmon *Salmo salar*. Emphasis was placed on Na^+, K^+-ATPase (NKA) and Na^+, K^+, Cl^- co-transporter (NKCC) isoforms, at both transcriptional and protein levels, together with transcription of chloride channel genes. The nka α1c was the dominant isoform at the transcript level in both proximal and distal intestines; also, it was the most abundant isoform expressed in the basolateral membrane of enterocytes in the proximal intestine. This isoform was also abundantly expressed in the distal intestine in the lower part of the mucosal folds. The protein expression of intestinal Nkaα1c increased during smoltification. Immunostaining was localized to the basal membrane of the enterocytes in freshwater (FW) fish, and re-distributed to a lateral position after SW entry. Two other Nka isoforms, α*1a* and α*1b*, were expressed in the intestine but were not regulated to the same extent during smoltification and subsequent SW transfer. Their localization in the intestinal wall indicates a house-keeping function in excitatory tissues. The absorptive form of the NKCC-like isoform (sub-apically located NKCC2 and/or Na^+, Cl^- co-transporter) increased during smoltification and further after SW transfer. The cellular distribution changed from a diffuse expression in the sub-apical regions during smoltification to clustering of the transporters closer to the apical membrane after entry to SW. Furthermore, transcript abundance indicates that the mechanisms necessary for exit of chloride ions across the basolateral membrane and into the lateral intercellular space are present in the form of one or more of three different chloride channels: cystic fibrosis transmembrane conductance regulator I and II and chloride channel 3.

Key words: cystic fibrosis transmembrane conductance regulator; fluid absorption; Na^+/K^+-ATPase; $Na^+/K^+/2Cl^-$ co-transporter; osmoregulation; salmonid.

INTRODUCTION

The anadromous life cycle of Atlantic salmon *Salmo salar* L. 1758 entails migration of juveniles from freshwater (FW) habitats into the marine environment, where they

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grow and become sexually mature before returning to their natal river to reproduce (Hoar, 1988). Juvenile *S. salar* go through parr–smolt transformation (smoltification), a developmental process that involves morphological, physiological, endocrine and neural changes during the spring, preparing smolts for marine life (Hoar, 1988; Ebbesson *et al.*, 2003; Stefansson *et al.*, 2008). The preparatory development of hypo-osmoregulatory ability is a critical feature of smoltification and is associated with remodelling of key osmoregulatory organs, such as the gill, kidney and intestine (Hoar, 1988; Evans *et al.*, 2005; Sundell & Sundh, 2012).

FW and seawater (SW) environments present different osmotic challenges for fishes. Homeostasis in FW is maintained by secretion of large volumes of dilute urine and active uptake of NaCl mainly across the gills, to counteract osmotic gain of water and passive loss of ions, respectively (Marshall, 2002). On the other hand, to counterbalance dehydration in SW, fishes actively drink water which is absorbed through ion-coupled fluid uptake (Loretz, 1995; Grosell, 2011; Sundell & Sundh, 2012). The excess NaCl is secreted across the gills in parallel with the secretion of low amounts of iso-osmotic urine (McCormick, 2012). The intestine has important functions for nutrient absorption and as a barrier to the external environment, and following the switch from FW to SW, the intestine supports an additional function of maintaining the active fluid absorption (Jv) necessary in SW. The increased Jv in SW is associated with elevated Na^+, K^+-ATPase (NKA) activity along the whole intestinal canal from the pyloric caeca (Rey *et al.*, 1991; Seidelin *et al.*, 2000; Veillette *et al.*, 2005) to the proximal (Colin *et al.*, 1985; Sundell *et al.*, 2003) and distal intestines (Colin *et al.*, 1985; Sundell *et al.*, 2003). Thus, as shown for stenohaline SW fishes, the major driving force for the intestinal fluid transport in salmonids is considered to be the basolaterally located NKA (Loretz, 1995; Madsen *et al.*, 2011; Sundell & Sundh, 2012). Jv increases during smolt development (Usher *et al.*, 1991; Veillette *et al.*, 1993; Nielsen *et al.*, 1999; Sundell *et al.*, 2003), and consequently, a developmental increase in NKA activity is expected during smoltification. Previous studies, however, have not been conclusive in this regard, as some studies report developmental and cortisol-induced increases in NKA activity (Madsen, 1990; Rey *et al.*, 1991; Sundell *et al.*, 2003), whereas others do not (Nielsen *et al.*, 1999; Seidelin *et al.*, 1999).

The NKA enzyme is an oligomeric protein comprising α subunits, β subunits and Fxyd proteins (Geering, 2008). The α-subunit contains the binding sites for Na^+, K^+ and ATP, and hence provides the major catalytic and ion-transporting capacity, while the β-subunit is important for stabilizing folding of the α-subunit. The Fxyd protein modulates the NKA enzyme by changing affinity for Na^+ and K^+. In mammals, four α (α1–α4), four β (β1–β4) subunits and seven Fxyd isoforms have been identified (Blanco & Mercer, 1998; Geering, 2008), but heterogeneity of subunit is more complex in salmonids (Richards *et al.*, 2003; Gharbi *et al.*, 2004, 2005). The conservation of isoforms over a large phylogenetic spectrum strongly suggests isoform-specific physiological functions (Blanco, 2005). Several studies on both mammals and fishes suggest that isozyme shifting enables the cells to regulate NKA activity in response to altered physiological requirements through the control of intracellular Na^+ and K^+ levels (Schulte, 2004; Blanco, 2005; Nilsen *et al.*, 2007; McCormick *et al.*, 2009), yet information on the regulation and physiological functions of different isoforms in the teleost intestine is scarce. Based on tissue distribution and relative messenger (m)RNA expression, Tipsmark *et al.* (2010) suggested *nka α1c* to be the major isoform expressed in the intestine of *S. salar*. The mRNA levels for this isoform increased during smoltification

and after SW transfer (Tipsmark *et al.*, 2010). Changes in the mRNA levels of other *nka* isoforms during smoltification, however, were not reported and the expression of the different isoforms at the protein level is currently unexplored in the intestine.

In the marine teleost intestinal brush border membrane (BBM), the uphill transport of K^+ and Cl^- into the enterocytes involves two apical co-transporters: the $Na^+/K^+/2Cl^-$ co-transporter (NKCC) and Na^+/Cl^- co-transporter (NCC) (Loretz, 1995; Grosell, 2006), that together with the NKA, contribute to ion transport and subsequent water absorption (Grosell, 2011). NKCCs are members of the chloride/cation co-transporter family, which mediates electroneutral trans-cellular transport of Na^+, K^+ and Cl^- and is expressed in epithelial cells and in exocrine glands (Haas & Forbush, 2000). There are two main isoforms, the basolateral, secretory NKCC1, and the apical, absorptive NKCC2 isoform (Xu *et al.*, 1994). Two *nkcc2* and *ncc* isoforms have been identified in European eel *Anguilla anguilla* (L. 1758) and Japanese eel *Anguilla japonica* Temminck & Schlegel 1846, with *nkcc2*β and *ncc*β being the most abundant transcripts in the intestine (Cutler & Cramb, 2008; Watanabe *et al.*, 2011; Teranishi *et al.*, 2013). In the intestine of sea bream *Sparus aurata* L. 1758, *nkcc2* mRNA is expressed and Nkcc/Ncc immunostaining is found in the apical membrane (Gregorio *et al.*, 2013). Apical Nkcc/Ncc immunostaining was also found in the intestine of sea bass *Dicentrarchus labrax* (L. 1758) (Lorin-Nebel *et al.*, 2006), whereas the intestine of killifish *Fundulus heteroclitus* (L. 1766) showed presence of Nkcc/Ncc in the apical and the basolateral membrane (Marshall *et al.*, 2002). The presence and expression pattern of NKCC/NCCs in the intestine of *S. salar* are currently unexplored.

The extrusion of absorbed Cl^- occurs across the basolateral membrane of several marine fish species through anion channels such as the cystic fibrosis trans-membrane conductance regulator (CFTR) (Loretz, 1995; Grosell, 2011; Whittamore, 2012). These channels are also located in the apical region of *F. heteroclitus*, *D. labrax* and *S. aurata* enterocytes (Marshall *et al.*, 2002; Bodinier *et al.*, 2009; Gregorio *et al.*, 2013), suggesting a role in chloride secretion. Regarding presence of chloride channels in fish intestines, *clc-3* was demonstrated in the intestine of the green spotted pufferfish *Tetraodon nigroviridis* Marion de Procé 1822 (Tang *et al.*, 2010) and tilapia *Oreochromis mossambicus* (Peters 1852) (Miyazaki *et al.*, 1999). Differential expression patterns of NKCCs and chloride channels have been investigated in the gills during smoltification, but the role of NKCCs and chloride channels in the intestine during smoltification and subsequent SW acclimation has yet to be characterized.

The aim of this study was to investigate the developmental changes in tissue distribution and protein abundance of intestinal ion transporters during smoltification and SW acclimation of *S. salar*. Emphasis was placed on the different NKA and NKCC isoforms, at both transcriptional and protein levels, together with transcriptional levels of chloride channels. The physiological role of the intestinal ion transporters is discussed within the context of smoltification.

MATERIALS AND METHODS

FISH MATERIAL AND EXPERIMENTAL DESIGN

Juvenile *S. salar* were obtained from an anadromous population (River Vosso, south-western Norway). The eggs hatched in the middle of April 2009 and the alvelins were first fed at end of May (250 day ° C post-hatching) in a 2 m^2 tank (1200 l). The fish were maintained on constant

light, ambient water and were fed a standard dry diet (Ewos Norway; www.ewos.com) with rations in excess of those recommended based on temperature and fish size (Austreng *et al.*, 1987). During the first week of September 2009, the fish were transferred from the Voss hatchery to a net pen (20 m × 20 m, depth of 5 m) in Lake Evanger. In early December, *c.* 500 fish were brought to the Aquatic Laboratory of the Bergen High Technology Center and kept at 4° C. On 10 January, 400 parr were randomly distributed among three square 1·2 m^2 tanks (500 l) supplied with flow-through, pH-adjusted FW (7·0, range ±0·1). The fish were gradually acclimated from 4 to 8° C over 6 days and kept on a natural photoperiod (SNP; 60° 25′ N) and 8° C throughout the experiment. On 18 May, smolts were transferred to natural SW (salinity 34).

SAMPLING

A total of 12 fish were randomly dip netted out of the tanks, directly anaesthetized in a lethal dose of buffered tricaine methanesulphonate (200 mg l^{-1} MS-222; Sigma-Aldrich; www.sigmaaldrich.com) on 8 February, 22 March, 17 May, 22 May and 20 June. Mass (M, g) and fork length (L_F, nearest mm) were recorded before blood was collected from the caudal vessels using heparinized syringes. Fulton's condition factor (K) was calculated from $K = 100\,M\,{L_F}^{-3}$. The blood was stored on ice until centrifuged (1500 *g*, 10 min, 4° C), plasma was separated and then frozen in aliquots on dry ice. The second gill arch on the left-hand side was excised, placed in ice-cold sucrose-EDTA-imidazole (SEI) buffer (250 mM sucrose, 10 mM EDTA and 50 mM imidazole, pH 7·3) and stored at −80° C until subsequent analysis of NKA activity. The intestine was dissected out and divided into a proximal and a distal region defined according to the position of the ileorectal sphincter: distal intestine being posterior to the sphincter while the proximal intestine being the region located between the sphincter and the pyloric caeca. A small ring from the upper part of each intestinal region was cut out and fixed at 5° C overnight in MFAA (methanol:formaldehyde 37–40%:acetic acid in volume ration 85:10:5) and transferred and stored in 70% ethanol until further processing. The remaining tissue of each intestinal region was cut open longitudinally and rinsed in phosphate-buffered saline (PBS). The mucosa from the two intestinal regions was scraped off the muscle layer using two glass slides. The scrapings were submerged in RNAlater, stored at 5° C overnight and then stored at −80° C until analyses. On selected samples, the mucosa from the proximal and distal intestines was scraped off, frozen in liquid nitrogen and stored at −80° C until later Western blot (WB) analyses.

GILL NKA ACTIVITY

Gill NKA activity was determined by the method of McCormick (1993). Briefly, this kinetic assay utilizes the hydrolysis of ATP, which is enzymatically coupled to the conversion of nicotinamide adenine dinucleotide (NADH) to NAD^+ by pyruvate kinase and lactic dehydrogenase with or without the addition of ouabain, a specific inhibitor of NKA. The protein concentration in homogenates was determined by a bicinchoninic acid method (Smith *et al.*, 1985) and NKA activity is expressed as μmol ADP mg^{-1} protein h^{-1}.

PLASMA LEVELS OF IONS AND CORTISOL

Plasma ion levels were determined using ion-selective electrodes (EML 105; www.radiometer.com). Plasma cortisol was measured in un-extracted plasma using a radioimmunoassay procedure described by Young (1986) using cortisol antibodies (code: S020; Lot: 1014-180182) purchased from Guildhay Ltd (now out of business) validated by Sundh *et al.* (2011).

RNA ISOLATION AND cDNA SYNTHESIS

Total RNA was isolated from 30 mg intestinal tissue by phenol–chloroform extraction using TRI Reagent (Sigma-Aldrich) as outlined by Chomczynski (1993). Total RNA quantity and integrity were determined by using an ND-1000 spectrophotometer (NanoDrop Technologies; www.nanodrop.com) and the Agilent 2100 expert bio-analyser (Agilent Technologies;

www.genomics.agilent.com), respectively. Total RNA was treated with TURBO DNA-free kit (Ambion; www.lifetechnologies.com) and complementary (c)DNA was reversely transcribed using 2 µg of total RNA and Oligo d(T_{15-18}) in conjunction with the SuperScript III kit (Invitrogen; www.lifetechnologies.com) following the manufacturer's instructions.

REAL-TIME QUANTITATIVE PCR

Real-time quantitative polymerase chain reaction (qPCR) was conducted with gene-specific primers in conjunction with SYBR Green Master Mix (Bio-Rad Laboratories Inc.; www.bio-rad.com) using the Bio-Rad MJ Research Chromo 4 System Platform. Forward and reverse primers for the *nka* α (α*1a*, α*1b*, α*1c* and α*3*) and β (β*1*) subunits, *nkcc1a*, *cftrI*, *cftrII* and the *fxyd12* are previously described (Nilsen *et al.*, 2007, 2010; Tipsmark, 2008). Primers for the *nkcc2* (GenBank accession number: NM 001141520) were 5′-CCGCGTGCCCAACATC-3′ (forward) and 5′-GCACGGTTACCGCTCACACT-3′ (reverse), whereas primers for the *clc-3* (GenBank accession number: NM 001173586) were 5′-CGAGGGCATCTACGAATCACA-3′ (forward) and 5′-CTCCTTGGCGTCGAGGAA-3′ (reverse). All qPCR assays were performed using 5 µl diluted cDNA (100 ng RNA), 200 nM of each primer and SYBR Green Master mix in a total reaction volume of 20 µl. The thermal cycling protocol consisted of 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 qPCR assay generated one single product and no primer–dimer artefacts. For each assay, triplicate five-fold cDNA dilution series was used to determine amplification efficiencies (E) calculated as the slope from the plot of $\log_{10}$ RNA concentration *v*. threshold cycle (C_T) values using the following formula: $E = 10^{(-1b^{-1})}$, where b is the slope. This efficiency was used to correct for difference in amplification efficiency when calculating gene expression according to Pfaffl (2004). The reference gene, ribosomal protein L 23 (*rpl23*), was used (Stefansson *et al.*, 2012) and did not change over time or differ between FW and SW-adapted fish in this study. It should be noted that the C_T values of *rpl23* were slightly higher in the proximal than in the distal intestine. Hence, a direct comparison of relative expression levels between the proximal and distal intestines should be made with caution.

PROTEIN EXPRESSION

Antibodies

Antibodies directed against *S. salar* Nkaα1a and Nkaα1b, previously described and validated for gill tissue by McCormick *et al.* (2013) and McCormick *et al.* (2009) respectively, were used in immunohistochemistry (IHC). In order to develop a specific antibody directed against *S. salar*, Nkaα1c, two different amino acid sequences were chosen: sequence nr. 1, the first 13 amino acids of the Nkaα1c protein, MGRGEGREQYELA and nr. 2, the 13 amino acids of the Nkaα1c protein corresponding to the regions selected by McCormick *et al.* (2009) to produce iso-specific antibodies directed against *S. salar* Nkaα1a and Nkaα1b, VHLNKNEGESKHL. Two rabbits *Oryctolagus cuniculus* were given four immunizations each with the combination of the two peptides and serum was collected before, middle and at the end of a 28 day immunization programme (Eurogentec S.A; www.eurogentec.com). Serum from the immunized rabbits was affinity purified for each antibody and the titre was measured by the antibody producer (Eurogentec). The antibody purified against peptide MGRGEGREQYELA was chosen for this study as it resulted in the best immunostaining. The NKA α1c antibody was validated with WB and used in IHC. A mouse *Mus musculus* monoclonal NKA antibody (α5) directed against the α subunit of chicken *Gallus gallus* Nka and believed to cross react with all α isoforms in teleosts, as well as a mouse monoclonal NKCC antibody (T4), directed against the carboxy-terminal of human *Homo sapiens* NKCC Met-902 to Ser-1212, were used for IHC. In at least some species of fishes, the T4 antibody recognizes both Nkcc and Ncc (Hiroi *et al.*, 2008). The α5 and T4 antibodies were developed by D. Fambourgh (John Hopkins University, Baltimore, MD) and C. Lytle (University of California Riverside, Riverside, CA), respectively, and were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development (NICHD; www.nichd.nih.gov) and maintained by the University of Iowa, Department of Biology, Iowa City, IA.

Western blots

WB were performed to determine the specificity of the antibodies made against *S. salar* NKA α1c. Proximal and distal intestinal mucosal scrapings were homogenized in 1 ml ice-cold homogenization buffer (0·25 M sucrose in 0·25 M Tris and 0·01 M $MgCl_2$, pH 7·4) with protease inhibitor cocktail tablets (Roche Diagnostics GmbH; www.roche.com) using a glass–glass homogenizer (Wheaton tapered tissue grinder). The homogenate was centrifuged at 2000 *g* for 20 min at 4° C. The supernatant was then centrifuged at 50 000 *g* for 30 min at 4° C to isolate the membrane fraction. The pellet was re-suspended in 100 µl homogenization buffer and protein concentration was measured with bicinchoninic acid (BCA) protein assay kit (Pierce; www.piercenet.com) at A_{540}, using bovine serum albumin (BSA) as standard. Samples in Laemmli buffer were heated at 65° C for 10 min, 10 µg of protein per lane was loaded and separated by electrophoresis on sodium dodecyl sulphate (SDS) containing 7·5% polyacrylamide gels. Amersham full-range rainbow molecular weight marker (GE Healthcare; www.gelifesciences.com) was used to estimate molecular size. The proteins were blotted onto a polyvinylidene difluoride (PVDF) microporous membrane (Millipore Immobilon-P Transfer Membrane, 0·45 µm pore size; www.merckmillipore.com) in a tris-glycine transfer buffer (25 mM Tris base, 192 mM glycine and 10% methanol) for 60 min at 250 mA.

The membrane was immersed in blocking buffer consisting of 5% non-fat dried milk in Tris-buffered saline (TBS) with 0·1% Tween 20 (TBST) for 1 h at room temperature. Membranes were then incubated with rabbit anti-NKA α1c (0·56 µg ml^{-1}) or mouse α5 primary antibody (1:1000) in TBST for 1 h at room temperature. The membranes were washed six times for 5 min each in TBST and incubated with secondary antibody diluted 1:50 000 in TBST (ECL anti-rabbit IgG or anti-mouse IgG horseradish peroxidase-linked whole antibody, GE Healthcare) for 1 h at room temperature. Visualization, after washing, was obtained using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and X-ray film, developer and fix from Kodak (VWR, https://se.vwr.com/).

Immunohistochemistry

Tissues were dehydrated through an alcohol gradient, cleared in Histolab-clear (Histolab products; www.histolab.se) and embedded in paraffin wax using standard procedures. Sections (7 µm) were sliced with a Shandon finesse microtome (Shandon Scientific; www.thermoscientific.com) from proximal and distal intestines and mounted on 3′-aminopropyltriethoxysilane (APES; Sigma-Aldrich)-coated slides, dried at 37° C for 24 h. Then, the slides were dewaxed in Histolab-clear followed by rehydration in a series of alcohol baths followed by 2 × 5 min in ddH_2O. The slides were then soaked in TBS (6·06 g l^{-1} Tris-HCl, 1·39 g l^{-1} Tris base and 9 g l^{-1} NaCl, pH 7·6) supplemented with 0·05% Tween-20 (TBST) 2 × 5 min. Endogenous peroxidase activity was blocked by immersion in 0·3% H_2O_2 (in TBST:methanol 70:30) for 30 min at room temperature and then washed in TBST 3 × 3 min. The slides were incubated with blocking solution (3% BSA, 5% normal goat *Capra aegagrus* serum and 5% normal donkey *Equus africanus asinus* serum) in TBST for 1 h at room temperature. Excess blocking solution was removed and sections were incubated with 100 µl primary antibody (chicken anti-NKA α1a 1·9 µg ml^{-1}, rabbit anti-NKA α1b 0·22 µg ml^{-1}, rabbit anti-NKA α1c 1·1 µg ml^{-1}, T4 1:800 and α5 1:1000) and diluted in blocking solution, overnight in a humid box at 4° C. Blocking solution without primary antibody served as controls. In order to test the specificity of the different antibodies, pre-absorption tests were performed. Affinity-purified antibodies against NKA α1a, α1b and α1c were pre-incubated at 4° C for 24 h with a 10 times excess by mass of the matching antigen peptide prior to dilution to the working dilution and addition to the sections. Hereafter, all incubations were performed at room temperature. The slides were washed in TBST 3 × 3 min and incubated with a biotinylated secondary antibody (donkey anti-rabbit 1:200, GE Healthcare; donkey anti-mouse 1:1000 or donkey anti-chicken; Jackson ImmunoResearch, https://se.fishersci.com/se/). Thereafter, the slides were washed in PBS (pH 7·2) with 0·05% Tween-20 (PBST) 3 × 3 min and incubated with 100 µl Vectastain Elite ABC (Vector laboratories; www.vectorlabs.com) reagent (according to Vector kit instructions) for 30 min followed by NovaRED (Vector laboratories) peroxidase substrate solution (according to Vector kit instructions) for 5–8 min, then washed again for 10 min in running tap water, dehydrated in alcohol gradient followed by Histolab-clear and

mounted with Pertex (Histolab Products AB). The sections were examined in a Nikon eclipse E1000 microscope, equipped with a Nikon DXM1200 camera (www.nikoninstruments.com). Two non-consecutive sections per fish ($n = 5–8$) and intestinal region were analysed for staining intensity as well as for tissue and cellular localization of each protein. Staining intensity was scored from 0 to 3, where 0 was absence of staining. The total numbers of scores for each protein localization were summarized and presented as representative figures from each sampling point and intestinal region.

Selected sections were used to study co-localization of the NKA isoforms using immunofluorescence (IF). The protocol was similar to the IHC protocol described above. Blocking of endogenous peroxidases, however, was omitted and after a 24 h incubation of the sections with primary antibodies of different combinations (α5:NKA α1c; α5:Nkaα1b and α5:NKA α1a), the sections were rinsed in TBST and incubated with secondary antibodies carrying fluorescent labels (donkey anti-rabbit Alexa 488 1:500, goat anti-chicken Alexa 488 1:1000; Molecular probes, donkey anti-rabbit Cy3 1:800 or donkey anti-mouse Cy3; Jackson ImmunoResearch) in the dark for 1·5 h. Thereafter, the sections were rinsed in TBST and mounted in Vectashield Hard Set mounting medium for fluorescence (Vector Laboratories). The sections were examined in an Axio Observer Z.1 microscope equipped with an AxioCam MRm for fluorescence imaging (Carl Zeiss; www.zeiss.se).

STATISTICS

The homogeneity of variances was tested using Levenes's test and the normal distribution was tested using Shapiro–Wilks test. When necessary, data were transformed to meet the assumptions of the analysis of variance (ANOVA). First, data was analysed in one-way ANOVA with time as main factor and tank nested within time. No significant tank effects were observed ($P > 0·05$) and therefore the fish from the replicate tanks were pooled for all further analyses. Significant differences in the ANOVA were subjected a Student–Neuman–Keuls (SNK) *post hoc* test to determine differences between time points. Values are presented as mean ± S.E. and significance was accepted at $P < 0·05$. IBM SPSS 20 statistical software (SPSS; www.ibm.com/software/se/analytics/spss) was used for all statistical procedures.

RESULTS

GROWTH

At the peak of smoltification, 17 May, $M = 33·3 \pm 2·7$ g (ANOVA: $F_{4,40} = 10·82$, $P < 0·001$) and $L_F = 14·8 \pm 0·3$ cm (ANOVA: $F_{4,40} = 19·73$, $P < 0·001$) had increased compared to the values on 8 February (Table I). This was followed by a further increase in L_F to $15·8 \pm 0·3$ cm after 4 weeks in SW. The value of K in smolts decreased from $1·13 \pm 0·01$, on 8 February, to $1·01 \pm 0·02$ (ANOVA: $F_{4,40} = 20·03$, $P < 0·001$) on 17 May, followed by a further reduction to $0·96 \pm 0·02$ after 4 weeks in SW (Table I).

GILL NKA ACTIVITY

Gill NKA activity increased from parr values ($3·6 \pm 0·23$ μmol ADP mg^{-1} protein h^{-1}) on 8 February, to $9·7 \pm 0·6$ μmol ADP mg^{-1} protein h^{-1} on 22 March (ANOVA: $F_{4,56} = 89·61$, $P < 0·001$) and remained high in smolts on 17 May, followed by a further increase to $21·3 \pm 0·7$ μmol ADP after 4 weeks of SW exposure [Fig. 1(a)].

PLASMA IONS AND CORTISOL

Plasma cortisol levels increased throughout smoltification from $2·5 \pm 0·5$ ng ml^{-1} in the beginning of February to $19·4 \pm 4·7$ ng ml^{-1} on 22 March (ANOVA: $F_{4,39} = 4·42$,

TABLE I. Changes in fork length (L_F), mass (M) and Fulton's condition factor (K) (mean ± S.E., $n = 9$) in freshwater *Salmo salar* from 8 February to 17 May and after 4 days (22 May) and 4 weeks in seawater (20 June). Different superscript lowercase letters denote significant differences ($P < 0{\cdot}05$) between time points

Date	L_F (cm) Mean ± S.E.	M (g) Mean ± S.E.	K Mean ± S.E.
8 February	$12{\cdot}6^{a} \pm 0{\cdot}1$	$22{\cdot}6^{a} \pm 0{\cdot}8$	$1{\cdot}13^{a} \pm 0{\cdot}01$
22 March	$13{\cdot}5^{b} \pm 0{\cdot}1$	$25{\cdot}4^{a} \pm 0{\cdot}5$	$1{\cdot}02^{b} \pm 0{\cdot}02$
17 May	$14{\cdot}8^{c} \pm 0{\cdot}3$	$33{\cdot}3^{bc} \pm 2{\cdot}2$	$1{\cdot}01^{b} \pm 0{\cdot}02$
22 May	$14{\cdot}5^{c} \pm 0{\cdot}4$	$28{\cdot}8^{ab} \pm 2{\cdot}7$	$0{\cdot}91^{c} \pm 0{\cdot}02$
20 June	$15{\cdot}8^{d} \pm 0{\cdot}3$	$40{\cdot}1^{c} \pm 2{\cdot}7$	$0{\cdot}96^{c} \pm 0{\cdot}02$

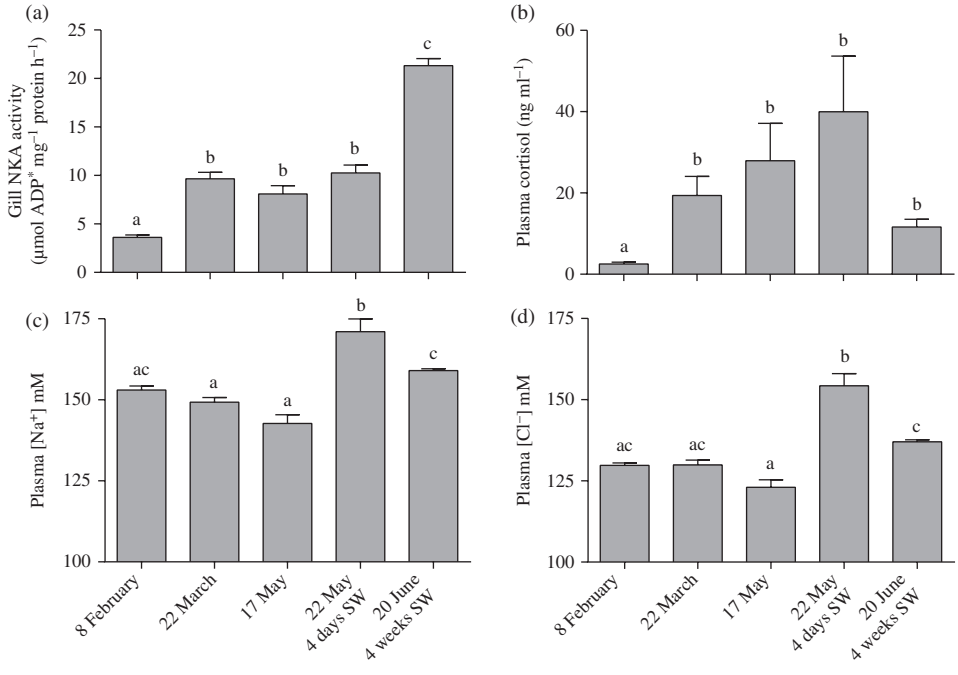

FIG. 1. (a) Gill Na^+, K^+-ATPase (NKA) enzyme activity ($n = 9–14$), (b) plasma cortisol levels, (c) plasma Na^+ and (d) Cl^- levels (d) = 5–8 (mean values ± S.E.) in freshwater *Salmo salar* from 8 February to 17 May, and after 4 days (22 May) and 4 weeks in seawater (SW) (20 June). Different lowercase letters denote significant differences ($P < 0{\cdot}05$) between time points.

$P < 0{\cdot}005$), and reaching $34{\cdot}2 \pm 11{\cdot}4\ ng\ ml^{-1}$ in FW smolts on 17 May [Fig. 1(b)]. After 4 weeks in SW, mean circulating cortisol was reduced to $11{\cdot}6 \pm 1{\cdot}9\ ng\ ml^{-1}$. This was not significantly different from the highest values measured after 4 days in SW ($40{\cdot}0 \pm 13{\cdot}7\ ng\ ml^{-1}$) due to wide variation during smoltification and early SW exposure. Plasma Na^+ and Cl^- levels remained stable throughout smoltification, with

Na^+ levels being $142{\cdot}7 \pm 2{\cdot}7$ mM in FW smolts on 17 May [Fig. 1(c)]. After SW transfer, a transient increase to $171{\cdot}0 \pm 4{\cdot}0$ mM (ANOVA: $F_{4,30} = 19{\cdot}32$, $P < 0{\cdot}001$) on 22 May occurred, followed by a reduction of Na^+ levels to $159{\cdot}0 \pm 0{\cdot}6$ mM after 4 weeks in SW [Fig. 1(c)]. Plasma Cl^- levels similarly increased to $154{\cdot}3 \pm 3{\cdot}7$ mM (ANOVA: $F_{4,30} = 29{\cdot}12$, $P < 0{\cdot}001$) after 4 days of SW exposure, on 22 May, followed by a reduction to $137{\cdot}1 \pm 3{\cdot}7$ mM on 20 June [Fig. 1(d)].

INTESTINAL GENE EXPRESSION

nka *subunit isoforms and* fxd12

In the intestine, *nkaα1c* was the most abundantly expressed subunit among the different NKA isoforms investigated in both proximal and distal intestines [Fig. 2(a)–(c)]. The levels of *nkaα1a* and *nkaβ1* were below the limit of detection and are therefore not shown. In the proximal intestine, no difference in *nkaα1c* [Fig. 2(a)] expression was observed during smoltification, neither in FW nor after SW transfer (ANOVA: $F_{4,36} = 1{\cdot}66$, $P > 0{\cdot}05$). In the distal intestine, *nkaα1c* expression showed significant differences with time (ANOVA: $F_{4,35} = 4{\cdot}53$, $P < 0{\cdot}01$). The following SNK *post hoc* test revealed no differences during smoltification but an up-regulation of the expression was apparent 96 h after SW transfer as compared to 8 February and 17 May and after 4 weeks in SW the expression was higher than the peak of smoltification on 17 May ($P < 0{\cdot}05$).

The expression of *Nkaα1b* [Fig. 2(b)] isoform changed over time in both the proximal (ANOVA: $F_{4,36} = 3{\cdot}10$, $P < 0{\cdot}05$) and distal intestines (ANOVA: $F_{4,35} = 2{\cdot}86$, $P < 0{\cdot}05$). The SNK *post hoc* test did not reveal significant differences with time in the proximal intestine, where a tendency towards a decrease during smoltification could be seen, but the expression levels returned to FW parr levels after 4 weeks in SW ($P < 0{\cdot}05$). In the distal intestine, the *post hoc* test showed an up-regulation during smoltification on 17 May compared to 8 February. The expression was maintained at high levels after 4 weeks in SW as compared to parr at 8 February ($P < 0{\cdot}05$).

The expression of *nkaα3* [Fig. 2(c)] changed over time in the proximal (ANOVA: $F_{4,36} = 3{\cdot}30$, $P < 0{\cdot}05$) but not in the distal (ANOVA: $F_{4,35} = 1.\,29$, $P > 0{\cdot}05$) intestine. A subsequent *post hoc* test did not reveal differential expression during smoltification, but a suppressed expression after 96 h in SW.

For the NKA regulator, *fxyd12* [Fig. 2(d)], expression levels were different over time in the distal (ANOVA: $F_{4,35} = 3{\cdot}13$, $P < 0{\cdot}05$) but not in the proximal intestine (ANOVA: $F_{4,36} = 1{\cdot}02$, $P > 0{\cdot}05$). The differences in time were apparent after the SW transfer, when *fxyd12* expression was up-regulated after 4 weeks in comparison the situation at the peak of smoltification in FW on 17 May ($P < 0{\cdot}05$).

Na^+, K^+, Cl^- co-transporters

The mRNA for *nkcc2* was the most abundant transcript in both proximal and distal intestines among the investigated [Fig. 3(a), (b)]. Further, the expression of *nkcc2* differed over time in both proximal (ANOVA: $F_{4,36} = 8{\cdot}54$, $P < 0{\cdot}001$) and distal (ANOVA: $F_{4,35} = 8{\cdot}73$, $P < 0{\cdot}001$) intestines. In the proximal intestine, SNK *post hoc* test revealed up-regulation of mRNA at 22 March (second sampling point) and at 22 May, after 96 h in SW ($P < 0{\cdot}05$). In the distal intestine, an up-regulation during smoltification was observed at both 22 March and 17 May compared to 8 February.

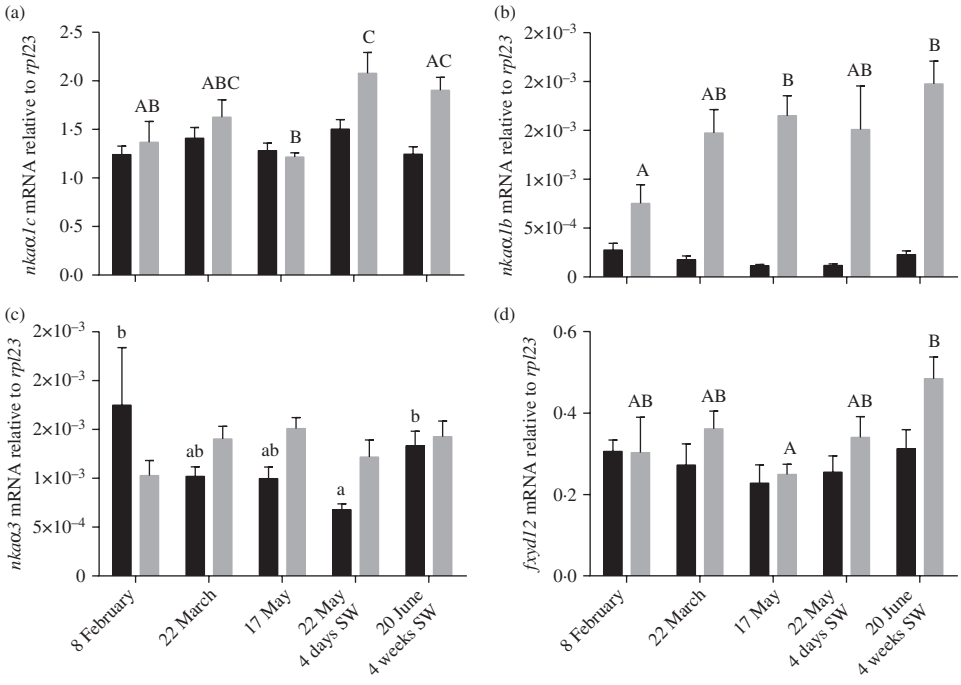

FIG. 2. Changes in proximal (■) and distal (■) intestine (a) *nka*α*1c*, (b) α*1b*, (c) α*3* and (d) *fxyd12* mRNA levels (mean values ± S.E., $n = 5–9$) in freshwater *Salmo salar* from 8 February to 17 May, and after 4 days (22 May) and 4 weeks in seawater (SW) (20 June). Different uppercase and lowercase letters denote significant differences ($P < 0{\cdot}05$) between time points in the distal and proximal intestines. Note that *nka*α*1a* and β*1* subunit isoforms' expression was detected at very low levels and thus could not be quantified by quantitative polymerase chain reaction (qPCR) in a reliable manner.

After SW transfer, the mRNA levels were further increased after 96 h, after which the levels decreased to levels seen during the peak of smoltification but still remained higher than the parr values on 8 February.

The expression of *nkcc1a* did not change with time in the proximal intestine (ANOVA: $F_{4,36} = 0{\cdot}61$, $P > 0{\cdot}05$) but did in the distal intestine (ANOVA: $F_{4,35} = 4{\cdot}01$, $P < 0{\cdot}01$). SNK *post hoc* revealed an up-regulation in mRNA levels after 4 weeks in SW ($P < 0{\cdot}05$), whereas there were no differences during smoltification.

Chloride channels

Both *cftrI* and *cftrII* mRNAs were expressed at the same order of magnitude. For *cftrII* [Fig. 4(a)], the mRNA levels changed with time in both proximal (ANOVA: $F_{4,36} = 16{\cdot}07$, $P < 0{\cdot}001$) and distal intestines (ANOVA: $F_{4,35} = 5{\cdot}98$, $P < 0{\cdot}01$), whereas no changes were seen in *cftrI* mRNA [Fig. 4(b)] (proximal intestine: ANOVA: $F_{4,36} = 0{\cdot}43$, $P > 0{\cdot}05$, distal intestine: ANOVA: $F_{4,35} = 1{\cdot}78$, $P > 0{\cdot}05$). In the proximal intestine, *cftrII* mRNA levels were lower at the peak of smoltification (17 May) and decreased further after 96 h in SW. This was followed by an up-regulation again after 4 weeks in SW to levels comparable to the levels at the peak of smoltification ($P < 0{\cdot}05$). In the distal intestine, gene expression was down-regulated after 96 h in

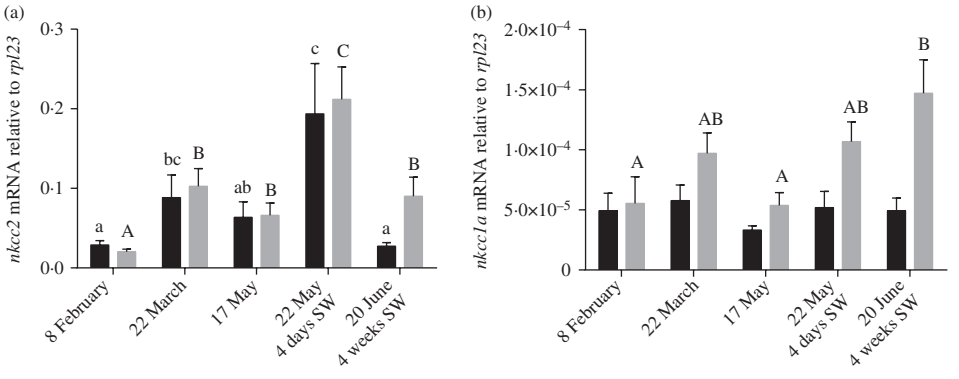

FIG. 3. Changes in proximal (■) and distal (■) intestine (a) *nkcc2* and (b) *nkcc1a* mRNA levels (mean values ± S.E., $n = 5–9$) in freshwater *Salmo salar* from 8 February to 17 May, and after 4 days (22 May) and 4 weeks in seawater (SW) (20 June). Different uppercase and lowercase letters denote significant differences ($P < 0{\cdot}05$) between time points in the distal and proximal intestines.

SW ($P < 0{\cdot}05$). The mRNA expression of the other chloride channel, *clc-3* [Fig. 4(c)], changed with time in the proximal intestine (ANOVA: $F_{4,36} = 3{\cdot}09$, $P < 0{\cdot}05$), where the expression decreased after transfer to SW as compared to mid-smoltification values on March 22 ($P < 0{\cdot}05$).

INTESTINAL PROTEIN EXPRESSION

Western blots

The analysis showed that both the α5 and the NKA α1c antibodies detected a single band with a molecular mass of *c*.100 kDa (Fig. 5). The proteins were expressed in both proximal and distal intestines as well as in fish from both FW and SW. All three fractions were analysed and Nkaα1c was only present in the membrane fraction. To ensure that the affinity-purified NKA α1c antibodies were specific against the peptide sequence used for immunization, WB peptide competition assay was conducted. An *c*. 500-fold molar excess of the NKA α1c peptide was incubated overnight at 4° C with the affinity-purified NKA α1c antibody prior to its use in WB. The NKA α1c peptide completely abolished the Nkaα1c immunoreactive bands (Fig. 5). These results demonstrate that the NKA α1c affinity-purified antibody is specific to its intended peptide sequence.

Immunohistochemistry

Nkaα1c immunostaining was observed in both proximal [Fig. 6(a)–(d)] and distal [Fig. 6(e)–(h)] intestines with the highest abundance in the basolateral part of the enterocytes. The staining was more abundant in the proximal compared to the distal intestine. In the proximal intestine, this staining was observed along the entire mucosal folds, whereas in the distal intestine staining was mainly restricted to the basal part of the mucosal folds, with less or no staining in the upper part. In both intestinal regions, the protein expression was more abundant on 22 March [Fig. 6(b), (f)] and most abundant on 22 May, after 4 days in SW [Fig. 6(d), (h)]. The Nkaα1c immunoreactivity

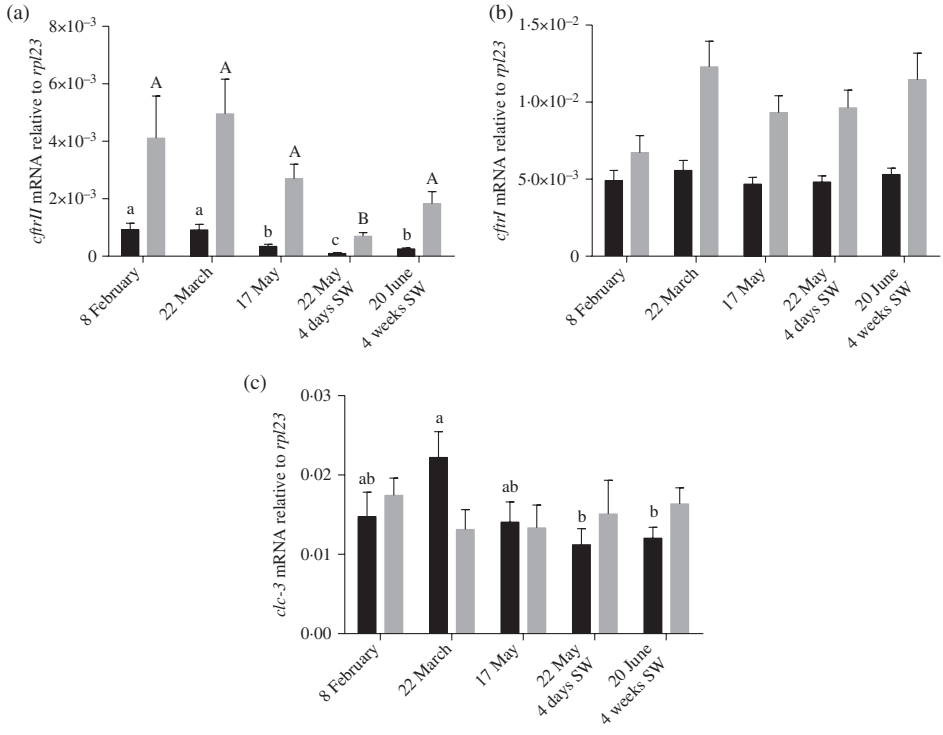

FIG. 4. Changes in proximal (■) and distal (■) intestine (a) *cftr II*,(b) *cftrI* and (c) *clc-3* levels (mean values ± S.E., $n = 5–9$) in freshwater *Salmo salar* from 8 February to 17 May, and after 4 days (22 May) and 4 weeks in seawater (SW) (20 June). Different uppercase and lowercase letters denote significant differences ($P < 0{\cdot}05$) between time points in the distal and proximal intestines.

was completely blocked with pre-absorption of the Nkaα1c peptide [Fig 6(a′), (e′)]. Differences in the localization of the protein along the basolateral membrane of the enterocytes in the proximal [Fig. 7(a)–(d)] and distal [Fig. 7(e)–(h)] intestines were observed with time. Staining in the basal region of the enterocytes was weakly present in the intestinal preparations from the parr stage [Fig. 7(a), (e)], 8 February, the intensity increased during smoltification, 22 March [Fig. 7(b), (f)] and 17 May [Fig. 7(c), (g)], and thereafter a more lateral positioning of the staining along the basolateral membrane of the enterocytes was apparent on 22 May, after 4 days of SW exposure [Fig. 7(d), (h)]. The staining pattern achieved using the non-isoform-specific α5 antibody in the proximal [Fig. 7 (i)–(l)] and distal intestines [Fig. 7(m)–(p)] during smoltification and 4 days after SW transfer resembled the staining achieved with the NKA α1c antibody. A regional difference in the distribution along the mucosal folds, however, was observed. In the proximal intestine, the Nkaα1c and α5 co-localized [Fig. 7(q)–(s)]. In the distal intestine, on the other hand, basolateral immunostaining of the α5 antibody was apparent further up on the mucosal folds as compared to the immunostaining using the NKA α1c antibody which was mainly observed in the lower parts of the mucosal folds [Fig. 7(t)–(v)].

Nkaα1b immunostaining was present in intestinal samples from all developmental stages, in both FW and SW. A basolateral immunostaining with the Nkaα1b antibody

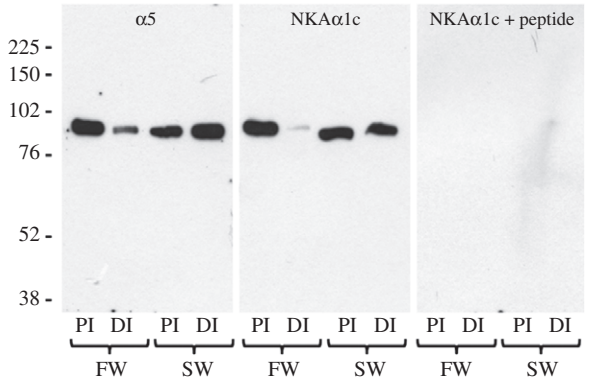

FIG. 5. Western blot analysis of proximal (PI) and distal (DI) intestines from one individual in fresh water (FW) (17 May) and after 4 weeks in seawater (SW) (20 June). The analysis showed that both the α5 and the NKA α1c antibodies revealed one single band with a molecular mass at *c.* 100 kDa. The NKA α1c immunoreactive band was blocked by preabsorption with the NKA α1c peptide.

was observed in both proximal [Fig. 8(a)] and distal intestines [Fig. 8(b)], although this was much weaker compared to Nkaα1c. In addition, Nkaα1b was also observed in the sub-apical region of the enterocytes in close proximity to the apical membrane along the mucosal folds in both proximal and distal intestines. Moreover, the sub-apical expression of α1b was commonly stronger in the proximal compared to the distal intestine suggesting a regional difference in the expression pattern. Expression was also observed in the longitudinal and circular muscle layer of the proximal [Fig. 8(c)] and distal [Fig. 8(d)] intestines. The staining was completely blocked with pre-absorption of the Nkaα1b peptide [Fig. 8(e)–(h)]. Co-localization with the non-isoform-specific α5 was observed for the basolateral, but not for the sub-apical staining [Fig. 8(i)–(k)].

Immunostaining for Nkaα1a was found in both proximal [Fig. 9(a)] and distal intestines [Fig. 9(b)] from all developmental stages. There was no apparent difference

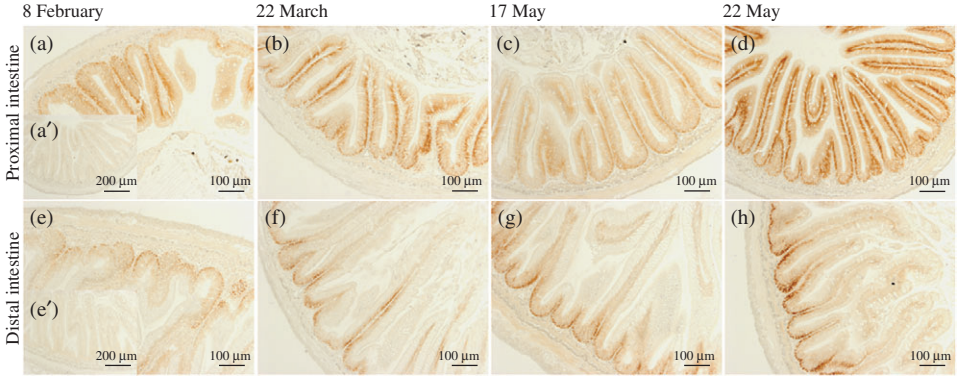

FIG. 6. Nkaα1c immunostaining in the (a–d) proximal and (e–h) distal intestines of *Salmo salar* during smoltification, (a, e) 8 February, (b, f) 22 March, (c, g) 17 May and after (d, h) 4 days in seawater, 22 May. Inserts (a′, e′) show preabsorption control with Nkaα1c peptide.

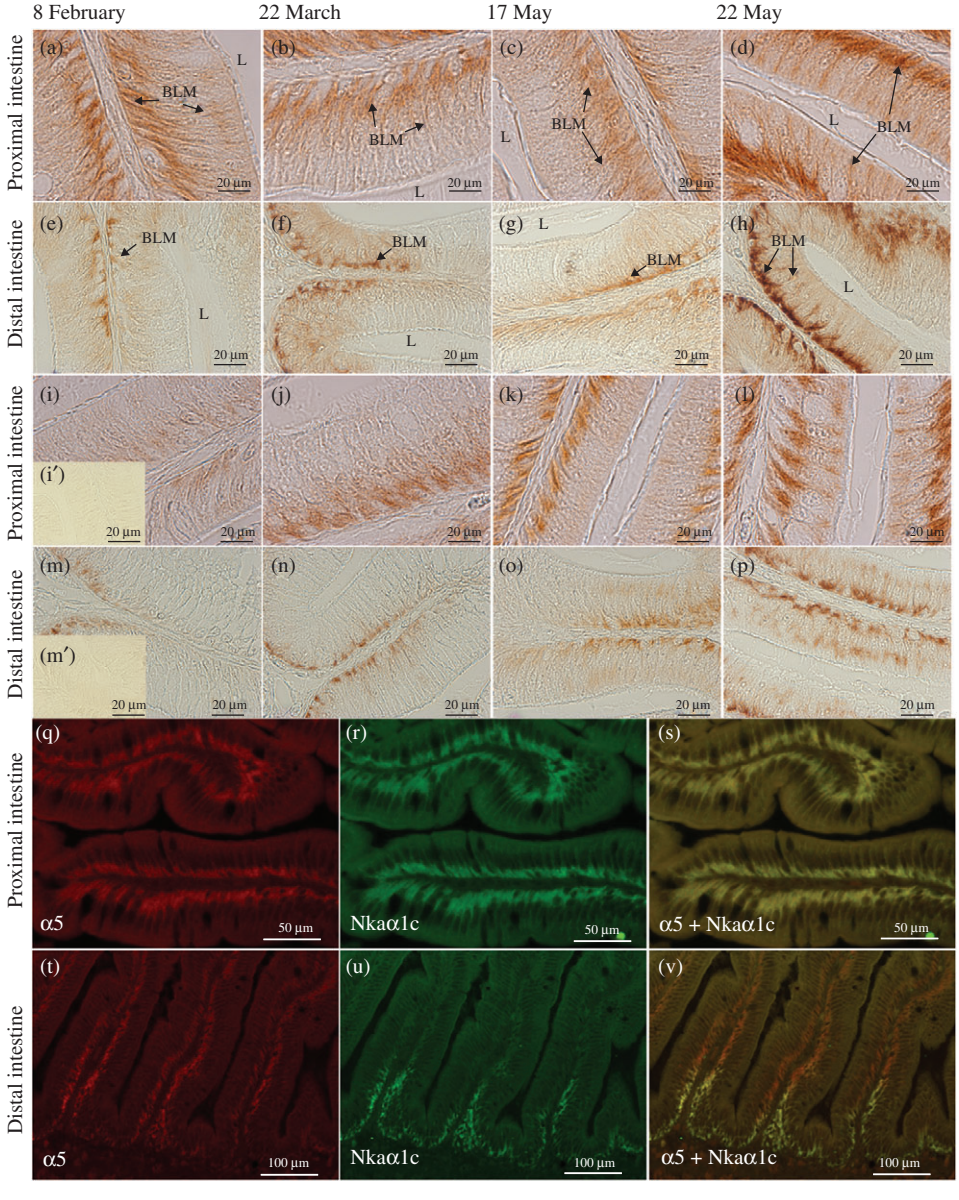

FIG. 7. Detailed micrographs showing immunostaining of Nkaα1c and α5 in the intestine of *Salmo salar* during smoltification and after 4 days in seawater (SW). Nkaα1c immunostaining in the (a–d) proximal and (e–h) distal intestines of *S. salar*, (a, e) 8 February, (b, f) 22 March, (c, g) 17 May and (d, h) after 4 days in SW, 22 May. Immunostaining for α5 in the (i–l) proximal and (m–p) distal intestines of *S. salar*, (i, m) 8 February, (j, n) 22 March, (k, o) 17 May and (l, p) after 4 days in SW, 22 May. Inserts (i′, m′) show immune reactivity after omitting the primary antibody. Co-localization of α5 and Nkaα1c immunostaining in the (q–s) proximal (22 March) and (t–v) distal (22 May) intestines of *S. salar*, respectively. Lumen (L) and the basolateral membrane (BLM) are indicated in the figure.

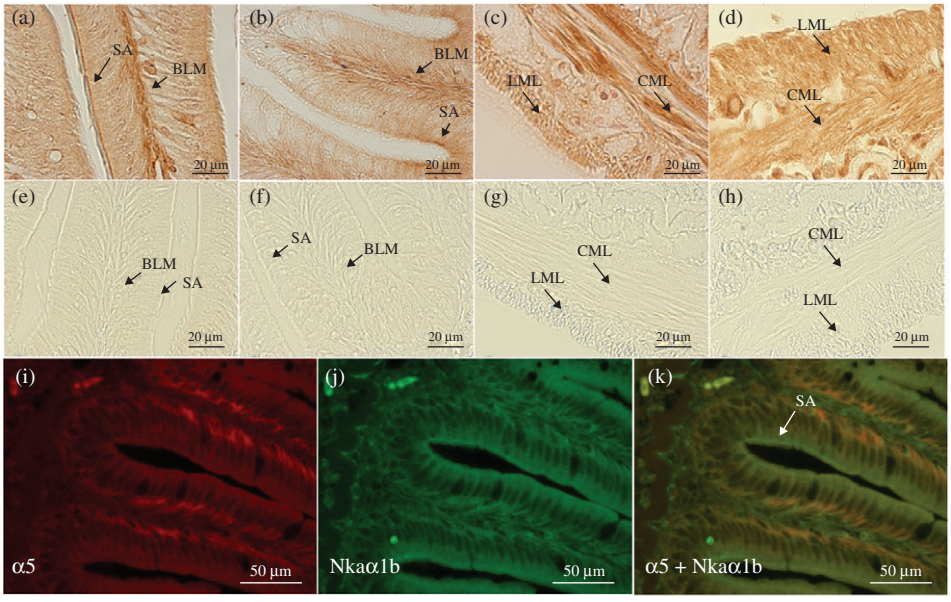

FIG. 8. Nkaα1b immunostaining in the proximal and distal intestines of *Salmo salar* 4 days after seawater (SW) transfer. Basolateral membrane (BLM) and sub-apical (SA) immunostaining of Nkaα1b in the (a) proximal and (b) distal intestines. Immunostaining of the longitudinal (LML) and circular muscle layers (CML) of the (c) proximal and (d) distal intestines. Preabsorption test abolished the basolateral and sub-apical staining in the (e) proximal and (f) distal intestines, as well as the muscle staining in the (g) proximal and (h) distal intestines. Co-localization of α5 and Nkaα1b immunostaining in the (i–k) proximal intestine of *S. salar*. All samples are from 4 days after SW transfer.

in staining intensity with developmental stage. There was weak immune staining in the sub-apical region of the enterocytes and in the basal membrane beneath the enterocytes [Fig. 9(a), (b)]. The most pronounced expression was in the longitudinal and circular muscle layer of the proximal [Fig. 9(c)] and distal [Fig. 9(d)] intestines. The staining was completely blocked with pre-absorption of the Nkaα1a peptide [Fig 9(e)–(h)]. There was no staining in the basolateral region of the enterocytes and there was a low co-localization with the α5 immune reactivity [Fig. 9(i)–(k)].

Immunostaining of Nkcc/Ncc, using the T4 antibody was present in the enterocytes and in the myenteric plexus. Abundant staining was found in the sub-apical region of the proximal intestine [Fig 10(a)–(d)] and in the basal region of the distal intestine [Fig. 10(e)–(h)]. In the proximal intestine, the sub-apical staining, close to the apical membrane, appeared to increase progressively during smoltification in FW [Fig. 10(b), (c)]. After 4 days in SW, the sub-apical staining was most intense [Fig. 10(d)]. No staining was observed in the BBM at any sampling point. In the distal intestine, most staining was present in the basolateral region and the intensity appeared strongest at the peak of smoltification on 17 May [Fig. 10(c)]. Staining in the sub-apical region of the distal intestine was weak and no major differences were observed with time.

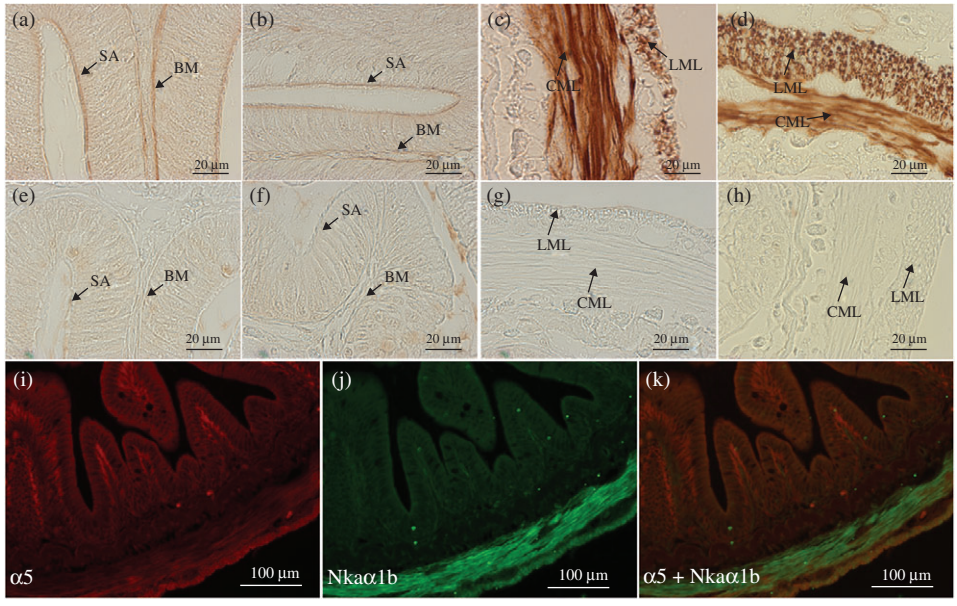

FIG. 9. Nkaα1a immunostaining in the proximal and distal intestines of *Salmo salar* 4 days after seawater (SW) transfer. Basolateral (BLM), basal membrane (BM) and sub-apical (SA) immunostaining of Nkaα1a in the (a) proximal and (b) distal intestines. Immunostaining of the longitudinal (LML) and circular (CML) muscle layers of the (c) proximal and (d) distal intestines. Preabsorption test abolished the basolateral and sub-apical staining in the (e) proximal and (f) distal intestines, as well as the muscle staining in the (g) proximal and (h) distal intestines. Co-localization of α5 and Nkaα1a immunostaining in the (i–k) proximal intestine of *S. salar*. All samples are from 4 days after SW transfer.

DISCUSSION

The gill NKA activity pattern during smoltification and in post-smolts after 4 weeks in SW in this study corresponds well with the ability of the fish to maintain stable plasma Na^+ and Cl^- levels 4 weeks after SW transfer. Further, elevated plasma cortisol levels and decreased *K* during the spring corresponds well with recently reported development of smolt characteristics in fish from the Vosso strain (Handeland *et al.*, 2014). It should be noted, however, that the peak of gill NKA activity in this study as well as in the study by Handeland *et al.* (2014) appeared already to occur in March. This suggests that the timing of the smolt window occurred earlier than observed in previous studies on this strain (Nilsen *et al.*, 2003, 2007, 2008). Since these earlier studies were used to decide the timing for transfer of the fish to SW in this study, it is probable that the transfer occurred towards the end of the smolt window. This is turn may explain why smolts required >4 days to fully adjust their plasma ion levels. The full adjustment was seen conclusively after 4 weeks in SW, reflecting successful development of SW tolerance.

This study indicates that NKA α1c is the predominant isoform of NKA in the intestine and is the main driving force for ion-coupled water transport in the intestine after transfer to SW. The increase in Nkaα1c protein expression occurring during smoltification, while the fish were still in FW, was mainly apparent in the basal parts of the basolateral membrane, whereas after SW transfer, the increase was also apparent in the

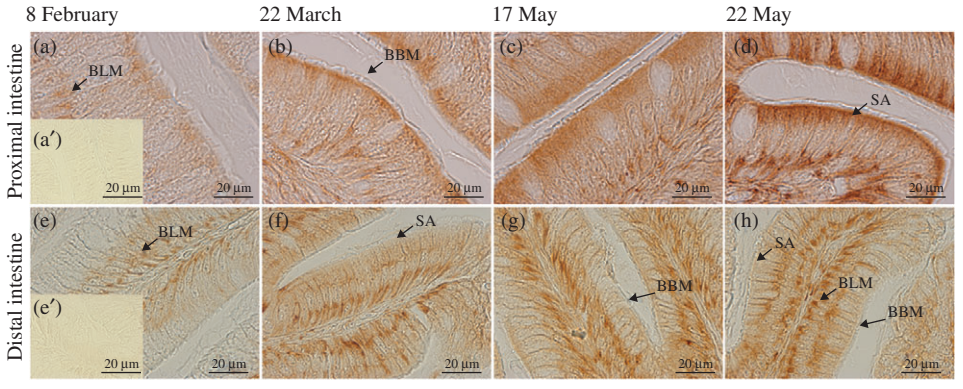

FIG. 10. Nkcc/Ncc immunostaining in the (a–d) proximal and (e–h) distal intestines of *Salmo salar* during smoltification, (a, e) 8 February, (b, f) 22 March, (c, g) 17 May and (d, h) after 4 days in seawater, 22 May. Brush border membrane (BBM), sub-apical (SA) and basolateral membrane (BLM) are indicated in the figure.

lateral parts of the basolateral membrane. This gradual increase ensures the functional build-up of the inwardly directed Na^+ gradient that should drive chloride and potassium ion transport into the enterocytes in the intestine of SW-acclimated fish. It will also contribute to the build-up of the osmotic gradient driving the fluid absorption, by transporting sodium from the cell to the lateral intercellular space (LIS). The cellular localization pattern of newly synthesized Nkaα1c suggests a pre-adaptive mechanism, where the increase of Nkaα1c only in the basal parts of the enterocytes, while the fish is still in FW, allows the incorporation of the necessary number of enzymes in the basolateral enterocyte membrane without creating a build-up of a high osmotic gradient in the LIS. Another transporter essential for the mechanism behind intestinal fluid transport is an apically located NKCC2. The mRNA and protein expression suggest a pre-adaptive role of NKCC in the intestine, as the number of co-transporters increased while the fish is still in FW but is positioned more diffusely in the sub-apical region. After SW entry, the expression increases even further and appears more densely focused to the area just below the BBM. The mRNA expression of three chloride channels, *cftrI* and *II* and *clc-3*, suggests that the necessary mechanisms also for exit of chloride ions across the basolateral membrane may be present. The cellular localization of the different chloride channels, however, was not elucidated in this study.

The finding that the NKA α1c was the most abundant isoform in the intestine is in line with a previous study in *S. salar*, where intestinal *nkaα1c* mRNA levels where more abundant than *nkaα1a*, α*1b*, α*2* and α*3* transcripts (Tipsmark *et al.*, 2010). At the protein level, the NKA α1c antibody generated stronger immune reactivity compared with the NKA α1a and α1b antibodies in the intestinal epithelium. Although this observation does not provide conclusive evidence of NKA α1c as the most abundant isoform, as staining quality and intensity amongst other factors depends on the efficiency and quality of each antibody, the high protein abundance together with high levels of mRNA transcripts indicates a dominating function of NKA α1c in the *S. salar* intestine. Furthermore, the NKA α1a antibody used in this study showed intense staining in other regions of the intestinal wall, such as the muscular layer, indicating a low

abundance of this isoform in the enterocytes, whereas the abundance in the muscular layers was high. In the proximal intestine, Nkaα1c immunostaining also correlated well with immunostaining generated by the α5 antibody, generally acknowledged to recognize all α-subunits in the teleost intestine (Marshall *et al.*, 2002; Grosell *et al.*, 2007; Tresguerres *et al.*, 2010; Madsen *et al.*, 2011). Together, this supports that NKA α1c is the dominant NKA isoform in the proximal intestine of *S. salar*. In the distal intestine, Nkaα1c was most abundant in the lower region of the mucosal folds, similar to immunostaining with α5. In the upper parts of the mucosal folds, on the other hand, there was a lack of co-localization between Nkaα1c and α5, which may suggest significant expression of another NKA α isoforms in this part of the distal intestine. One possible candidate is the α1b, as immunostaining of this isoform was present, basolaterally in the enterocytes, in both the proximal and distal intestines along the whole length of the mucosal folds. The transcript levels of α1b increased in the distal intestine during smoltification and were maintained high after SW acclimation. This may suggest a more important role for this isoform in the distal intestine in a hyperosmotic environment. Even though the α1c and α1b isoforms are the most abundant α subunits in the *S. salar* enterocytes, the possible presence of other α-subunits cannot be excluded. For example, mRNA expression of *nkaα3* has been shown in both rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) (Richards *et al.*, 2003) and *S. salar* where *nkaα2* mRNA was also present (Tipsmark *et al.*, 2010). Thus, other possible candidates for the staining by the α5 antibody are one or both of these isoforms.

Differential expression of α-subunits is an important means by which kinetic properties of the NKA enzyme can be altered in various tissues during development and in response to environmental changes (Geering, 2008). Other potential candidates involved in modulating enzyme activity are members of the Fxyd protein family, which have been found to bind to the α-subunit and modulate enzyme activity through affecting affinities for Na^+ and K^+ (Geering, 2008). Of several Fxyd proteins identified in teleosts, the Fxyd12 is abundantly expressed in the intestine (Tipsmark, 2008; Wang *et al.*, 2008; Yang *et al.*, 2013). Consistent with Tipsmark (2008), this study revealed that Fxyd12 was abundantly expressed in both the proximal and distal intestines of *S. salar*. Based on the presented stable mRNA transcript levels, *fxyd12* may be a constant factor, rather than a factor that modulates the NKA activity during smolt development and/or in response to salinity. As Fxyd12 protein expression was not assessed in this study, however, this remains to be further elucidated.

In addition to a cellular localization typical for NKA involved in epithelial net ion transport, the localization of Nkaα1b and Nkaα1a to the intestinal muscle layers suggests a house-keeping role for these two NKA isoform in excitatory tissues. The sub-apical localization of Nkaα1b, close to the apical membrane, has not been reported previously for fish intestine, but for the mammalian intestine. The enzyme was in position suggested to be functionally involved in apical membrane vesicle formation and regulation of the tight junction permeability (Xie & Cai, 2003; Rajasekaran *et al.*, 2007, 2008). The sub-apical staining in this study, however, did not co-localize with the α5 antibody, and it cannot be ruled out that staining at this location was due to cross reactivity with some other antigen.

The increased intensity of Nkaα1c and α5 immunostaining between February and March suggests that an increased expression of the NKA proteins in the intestine occurs in a pre-adaptive fashion, while the fish is still in FW. This is most likely the result of increased levels of circulating developmental hormones, such as cortisol (Cornell

et al., 1994; Specker *et al.*, 1994; Veillette *et al.*, 1995), which was shown to increase in this study, and growth hormone (GH) (Hoar, 1988; Dickhoff *et al.*, 1990; Björnsson *et al.*, 2011). GH has been shown to increase *nka* α*1c* mRNA levels in the pyloric caeca as well as in the proximal and distal intestines of FW-acclimated *S. salar*, while cortisol increased *nka* α*1c* mRNA levels in the distal intestine (Tipsmark *et al.*, 2010). The protein abundance of Nkaα1c then appeared to be slightly reduced at the peak of smoltification, despite no observed differences in intestinal mRNA transcript levels nor gill ATPase activity. Such a reduction may be a mechanism to protect smolting *S. salar* from executing increased fluid uptake across the intestine while still in FW. This possible mechanism needs to be investigated further. The further increase in both NKA α1c transcript and protein levels 4 days after SW transfer indicate an increased capacity of the enterocytes to drive the ion-coupled fluid transport necessary for a hypo-osmoregulation in SW (Grosell, 2011; Sundell & Sundh, 2012). The increased NKA α1c transcript and protein expression in this study is consistent with several previous studies where higher α-subunit mRNA levels and increased NKA activity are shown in pyloric caeca and intestine of SW-acclimated euryhaline teleosts compared with FW-acclimated animals (Colin *et al.*, 1985; Fuentes *et al.*, 1997; Seidelin *et al.*, 2000; Sundell *et al.*, 2003; Veillette *et al.*, 2005; Chew *et al.*, 2010; Tipsmark *et al.*, 2010).

NKAs are present along the whole basolateral membrane of the enterocytes in the intestine of fishes acclimated to both FW (Wallace *et al.*, 2005; Chew *et al.*, 2010) and SW (Marshall *et al.*, 2002; Grosell *et al.*, 2007; Tresguerres *et al.*, 2010; Madsen *et al.*, 2011), as NKAs have a role in nutrient uptake in all environments (Sundell & Rønnestad, 2011). In SW, however, the amount of NKAs expressed is higher and the current model for fluid uptake predicts a high abundance of NKAs positioned laterally on the enterocytes, in order to create a high osmotic gradient within the LIS that can drive fluid absorption (Grosell, 2010; Sundell & Sundh, 2012). Several studies on SW-acclimated fishes have provided experimental support for this model. The basal as well as lateral staining was more intense in the intestine of marble goby *Oxyeleotris marmorata* (Bleeker 1852) 14 days after SW transfer compared to fish in FW (Chew *et al.*, 2010). Intense lateral immunohistochemical staining for Nka has also been observed in *S. salar* acclimated to SW (Madsen *et al.*, 2011) and *O. mykiss* acclimated to 65% SW (Grosell *et al.*, 2007) as well as in the marine toadfish *Opsanus beta* (Goode & Bean 1880) (Tresguerres *et al.*, 2010) and SW-acclimated *F. heteroclitus* (Marshall *et al.*, 2002). Given the significance of this high Nka expression laterally, for efficient fluid absorption in SW, an increased expression of the protein in this position during the parr–smolt transformation would be expected to be maladaptive while the fish is still in FW. The detailed examination of the localization of NKAs along the basolateral membrane of the enterocytes in this study provides an explanation as to how NKAs can be mobilized pre-adaptively while preventing hypo-osmoregulation until after SW entry. The early increase in Nkaα1c immunostaining was apparent in the basal parts of the basolateral membrane and only at 4 days after transfer to SW it was distributed to more lateral positions. Thus, the increase in expression of NKAs can start during the parr–smolt transformation, building-up a sufficient numbers of NKA enzymes but maintaining them in a basal position of the basolateral membrane with little effect on fluid absorption in FW. After SW entrance, the enzymes are redistributed to more lateral positions in order to gain full functionality in creating high osmolality in the LIS.

Another major component in the ion-coupled fluid uptake is the transport of Cl^- ions (together with K^+ and Na^+) from the intestinal lumen into the enterocytes. This is governed by the Na^+ gradient built-up by NKAs together mainly with the absorptive NKCC2 isoform (Musch *et al.*, 1982; Loretz, 1995; Grosell, 2010). The Cl^- intake, however, has also been reported to occur *via* NCC in euryhaline fishes (Cutler & Cramb, 2008; Watanabe *et al.*, 2011). At the transcriptome level, *S. salar* intestine showed mRNA expression of both the absorptive and secretory forms of the *nkcc*, with the absorptive form, *nkcc2*, being most abundantly expressed. In the proximal intestine, *nkcc2* transcription was up-regulated at the peak of smoltification accompanied by increased abundance of a sub-apical localization of immunostaining by the T4 antibody. The sub-apical localization of the newly synthesized Nkcc/Ncc suggests that these transporters were stored in vesicles sub-apically in the enterocytes for later incorporation into the apical membrane. This type of intracellular trafficking, sub-apical storage of Nkcc2 that can be inserted into the apical membrane in response to external stimuli, has been described in the thick ascending limb of the mammalian kidney (Giménez & Forbush, 2003). Functionally, such an intracellular trafficking system for Nkcc2 in the *S. salar* intestine would constitute an additional preparatory mechanism during the parr–smolt transformation, where the number of Nkcc/Ncc can be increased without exerting their physiological function in the apical membrane, while the fish is still in FW. The external stimuli of entering into SW would then be expected to result in insertion of the storage vesicle containing Nkcc/Ncc into the BBM. Following 4 days of SW exposure, the NKCC2 mRNA levels were even further up-regulated and the immunostaining of Nkcc/Ncc was similarly even more intense. However, the more intense staining was still situated in the sub-apical region of the enterocytes, whereas the BBM appeared negative for Nkcc/Ncc Thus, the possible insertion of Nkcc/Ncc proteins into the apical membrane does not appear to have started to any major extent after such short time as 4 days in SW.

In spite of similar changes in mRNA expression of *nkcc2* in proximal and distal intestines, the sub-apical immunostaining using the T4 antibody was weaker in the distal compared to the proximal intestine. In the *A. japonica*, up-regulation of *nkcc2*β mRNA was found in response to SW acclimation in both anterior and posterior intestines, whereas the rectum showed low levels of *nkcc2*β transcript expression (Watanabe *et al.*, 2011). Instead, mRNA of the other Cl^- co-transporter demonstrated in teleosts, the *ncc*β, was expressed in much higher levels in the rectum compared to in the anterior intestine. Also in *A. anguilla*, *ncc*β, mRNA was solely expressed in the posterior intestine (Cutler & Cramb, 2008). This difference in tissue distribution between *nkcc* and *ncc* in the two *Anguilla* species was suggested to depend on decreasing luminal K^+ levels in the luminal fluid along the intestinal canal (Cutler & Cramb, 2008). As the monovalent cations of the ingested SW will be absorbed along the length of the intestine (Loretz, 1995) and with a relative ratio of Na:K of *c.* 465:10 (full-strength SW), it would probably be more efficient to drive the Cl^- uptake through a co-transport only coupled to Na^+, rather than a co-transporter dependent on both Na^+ and K^+, more distally in the intestine. For *S. salar*, the specificity of the T4 antibody regarding Nkcc and Ncc is not elucidated and it can currently not be excluded that the T4 antibody can distinguish between Nkcc and Ncc as suggested for the alewive *Alosa pseudoharengus* (Wilson 1811) (Christensen *et al.*, 2012). Hence, the weak T4 immunostaining seen in the distal intestine, despite a demonstrated high

Jv in this intestinal region (Veillette *et al.*, 1993), may be due to a presence of Ncc rather than Nkcc2.

This study also demonstrated T4 immunostaining in the basolateral region of the enterocytes. This staining was mainly abundant in the distal intestine and also correlated to the increase in mRNA expression for this NKCC isoform in the distal intestine. Similarly, a basolateral expression of Nkcc was observed in the distal intestine of *F. heteroclitus* by Marshall *et al.* (2002) using the T4 antibody. A basolateral expression as well as mRNA expression of the Nkcc–*nkcc1a* isoform suggest the presence of a secretory form of the NKCC (*i.e.* NKCC1) that may be involved in fluid secretion (Marshall *et al.*, 2002). A mechanism or function for fluid secretion in the intestine of fish acclimated to SW is yet to be established.

The bulk transport of Cl^- from the intestinal lumen to the LIS requires a flux of Cl^- across the basolateral membrane, *i.e.* through chloride channels. The mRNA expression of all three investigated Cl^- channels was demonstrated throughout smoltification and subsequent SW transfer. The *cftrII* mRNA showed the highest degree of regulation, with a down-regulation during smoltification in FW and an even further down-regulation after 4 days in SW. This may indicate a less important role for *cftrII* in SW-acclimated *S. salar*. On the other hand, *clc-3* and *cftrI* showed stable levels of expression throughout the experiment. *clc-3* mRNA expression has similarly been demonstrated in the intestine of *O. mossambicus* (Miyazaki *et al.*, 1999). In the SW-acclimated *F. heteroclitus*, IHC revealed the presence of Cftr channels not only in the basolateral membrane of all enterocytes examined but also in the apical membrane of some enterocytes (Marshall *et al.*, 2002). In *S. aurata* (Gregorio *et al.*, 2013) and *D. labrax* (Bodinier *et al.*, 2009), intestinal Cftr has been localized in both basolateral and apical enterocyte membranes. An apical localization for chloride channels in concert with basolaterally localized Nkcc (Marshall *et al.*, 2002) may be involved in chloride and fluid secretion by the intestine (Marshall *et al.*, 2002; Bucking & Wood, 2006). The cellular localizations and alteration by salinity of all three chloride channels examined in this study will help elucidate their physiological role in the intestine.

In conclusion, this study demonstrated the presence and function of three important ion transporters and channels needed for a successful ion-coupled water transport in the intestine of the SW-acclimated *S. salar*. The pattern of development for the two main transporters, the NKA α1c and the NKCC2/NCC, during smoltification and subsequent SW transfer provides evidence for a functional preparatory adaptation of the ion-coupled fluid transport. During smoltification, there was increased synthesis of Nkaα1c and Nkcc2/Ncc. When the fish are in FW, newly synthesized Nkaα1c were localized in the basal parts of the enterocytes, while new Nkcc/Ncc were localized in a sub-apical position (in ER or vesicles). Four days after transfer to SW, there was a cellular redistribution of Nkaα1c transporters within the enterocyte membranes, from a basal to a more lateral position. There was also redistribution of Nkcc/Ncc, showing a more dense appearance of the co-transporters adjacent to the apical membrane. This results in an enterocyte functionally able to transport and concentrate monovalent ions into the LIS in order to create the necessary osmotic gradient for efficient water transport.

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