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Shifts in the relationship between mRNA and protein abundance of gill ion-transporters during smolt development and seawater acclimation in Atlantic salmon (*Salmo salar*)

Arne K. Christensen[a,b,*], Amy M. Regish[b], Stephen D. McCormick[a,b]

[a] Department of Biology, University of Massachusetts Amherst, Amherst, MA 01003, USA
[b] U.S. Geological Survey, Leetown Science Center, S. O. Conte Anadromous Fish Research Laboratory, Turners Falls, MA 01376, USA

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ABSTRACT

Smolting Atlantic salmon exhibit a seasonal increase in seawater tolerance that is associated with changes in the abundance of major gill ion-transporter transcripts and proteins. In the present study, we investigate how the transcript and protein abundance of specific ion-transporter isoforms relate to each other during smolt development and seawater acclimation, and how each correlates to seawater tolerance. We show that during smolt development both mRNA and protein abundance of gill Na^+/K^+-ATPase α1a subunit (NKAα1a) decreased but the decrease in the mRNA was five-times greater than that of the protein. Gill NKAα1b mRNA levels increased only slightly (1.5-fold) throughout development whereas protein abundance increased 30-fold at its peak. Gill $Na^+/K^+/2Cl^-$ co-transporter 1 (NKCC1) increased at the mRNA and protein level (5- and 12-fold) in smolts. The abundance of a gill ion-transporter's mRNA and protein changed in the same direction through development and after seawater transfer, but the changes were not always strongly correlated: NKAα1a (r = 0.768), NKAα1b (r = 0.40), and NKCC1 (r = 0.898). The maintenance of plasma chloride concentration correlated most strongly with the abundance of NKAα1a mRNA, and the ratio of NKAα1b to NKAα1a mRNA and protein. Growth performance after seawater transfer correlated most strongly with the abundance of NKAα1b protein and the ratio of NKAα1b to NKAα1a protein. Our results indicate that the abundance of ion-transporter mRNA and protein do not always correlate well and a decrease in the abundance of gill NKAα1a mRNA and increase in NKAα1b protein are strong predictors of seawater tolerance and growth performance after seawater transfer.

1. Introduction

Most teleost fish are stenohaline and cannot tolerate large changes in salinity. These fishes are largely limited to habitation at a specific salinity, typically freshwater or seawater. Approximately 5% of teleost fish are euryhaline and are able to adapt to significant changes in salinity. The ability to acclimate to changes in salinity requires alterations in the ion transport properties of osmoregulatory tissues, primarily the gill, gut, and kidney (Edwards and Marshall, 2012). These functional shifts may occur gradually as part of a developmental program preparing the animal for entry into a new osmotic environment, or rapidly in response to abrupt changes in salinity.

Anadromous species, such as Atlantic salmon *Salmo salar* L. 1758, are migratory fish that move from freshwater to seawater and return to freshwater to spawn as part of their life history. In preparation for seawater entry salmon undergo a developmental program known as the parr-smolt transformation which entails a suite of physiological, morphological and behavioral changes that prime the animal for a transition from freshwater to seawater (Hoar, 1988). An important component of the parr-smolt transformation are changes in the type and abundance of ion-transport proteins in the gill epithelium, which effectively transform the tissue from a site of ion absorption in freshwater to one with a high capability for ion secretion in seawater (McCormick, 2013). Most of these changes within the gill epithelium occur in a class of cells called ionocytes (Evans et al., 2005).

In freshwater, gill ionocytes express the ion-transport proteins responsible for net ion influx. Cl^- uptake is thought to be directly coupled to HCO_3^- efflux *via* an anion exchanger. Na^+ uptake may occur according to one or more of three hypothesized mechanisms. In the first mechanism, an apical Na^+/H^+ Exchanger (NHE3) mediates an electroneutral exchange of cytosolic H^+ for environmental Na^+. In the second model, passive Na^+ influx through an apical channel is driven by an electrogenic apical V-type H^+-ATPase. More recently, an additional model for Na^+ uptake has emerged wherein an apical Na^+/Cl^-

* Corresponding author at: School of Professional Studies, Anna Maria College, 50 Sunset Lane, Paxton, MA 01612, USA.
E-mail address: achristensen@annamaria.edu (A.K. Christensen).

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cotransporter (NCC) mediates an electroneutral uptake of Na^+ and Cl^-. Environmental factors, species, and cell type appear to influence which mechanism is likely to mediate Na^+ uptake (Evans et al., 2005; Marshall and Grosell, 2006; Hwang and Lee, 2007; Evans, 2011). The transfer of Na^+ from cells of the gill epithelium into the blood is thought to occur through basolateral Na^+/K^+-ATPase (NKA) (McCormick, 1995), which couples the energy of ATP hydrolysis to the exchange of $3Na^+$ intracellular ions for $2\,K^+$ extracellular ions.

In seawater, the gill is responsible for monovalent ion secretion, in large part through the effort of three major ion transport proteins: NKA, $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1) and the cystic fibrosis transmembrane conductance regulator (CFTR). The primary driving force for ion secretion in seawater ionocytes is the NKA, which maintains the low intracellular Na^+ required for secondary import of Na^+, K^+, and $2Cl^-$ by basolateral NKCC1. Elevated intracellular Cl^- is subsequently extruded through an apical anion channel, CFTR. Na^+ is secreted *via* a paracellular pathway though selectively permeable tight junctions between ionocytes and accessory cells (Sardet et al., 1979; McCormick, 1995; Evans et al., 2005; Marshall and Grosell, 2006; Hwang and Lee, 2007).

Gill NKA is a major provider of the motive force that drives ion absorption in freshwater and ion secretion in seawater. The holoenzyme is composed of an essential α- and β-, and regulatory γ-, subunit each of which has multiple isoforms (Blanco and Mercer, 1998). The α- and β-subunits are required for catalytic activity, and the α-subunit is the site of Na^+ and K^+ binding, and the target of the inhibitory molecule ouabain. Two isoforms of the α-subunit, NKAα1a and NKAα1b, are differentially regulated by salinity in salmonids (Richards et al., 2003; Nilsen et al., 2007). Gill NKAα1b protein abundance, and the number of ionocytes expressing the protein, markedly increase during smolt development and after transfer to seawater. Conversely, gill NKAα1a stays relatively constant during smolt development but decreases dramatically after transfer to seawater (McCormick et al., 2009; McCormick et al., 2013). It is unclear how the highly conserved isoforms function in physiologically opposing roles, but it has been suggested that divergence in critical residues in NKAα1a may lend unique properties for the affinity or type of ions transported (Jorgensen, 2008).

During smolt development, changes in the abundance of gill ion-transporter mRNA and protein extend over many weeks in preparation for seawater entry. The peak of smolt development, when the animal is primed for seawater entry, occurs in the spring and correlates with elevated gill NKA activity and increased abundance of gill ion-transporters (presumably inactive) important for hypoosmoregulation (McCormick and Saunders, 1987; Pelis et al., 2001; Tipsmark et al., 2002; Nilsen et al., 2007; McCormick et al., 2013). Movement into seawater before, or after, peak smolt development results in a greater disruption of internal ion concentrations, reduced growth performance, and increased mortality (Björnsson et al., 1988; Stagg et al., 1989; Berge et al., 1995). After seawater entry, the abundance of gill ion-transporter mRNA and protein rapidly shift over days and weeks to acclimate to the new osmotic environment. Numerous studies have measured either mRNA or protein levels of specific gill ion-transport proteins during smolt development and salinity acclimation, but no studies have examined them simultaneously or compared their relative changes. One aim of the present study is to examine the relationship between the mRNA and protein product of specific ion-transporter isoforms at discrete time points during these preparatory and acclimation periods. Measuring the abundance of gill NKAα1a and NKAα1b mRNA and protein offers a unique opportunity to investigate different levels of gene expression of two similar genes; one of which functions primarily in freshwater leading up to and during preparatory transitions, and one during the subsequent seawater acclimation period. A second aim of the study is to correlate seawater tolerance and growth performance after seawater transfer to the abundance of the mRNA and protein product of important gill ion-transporters: NKAα1a, NKAα1b, and NKCC1.

2. Materials and methods

2.1. Animals and experimental protocols

On 1 October 2009 juvenile Atlantic salmon were obtained from the Kensington State Hatchery (Kensington, CT, USA) and brought to the Conte Anadromous Fish Research Center (Turners Falls, MA, USA). Until the start of the experiment, fish were held in 1.7 m diameter fiberglass tanks (700 l) supplied with flow-through Connecticut River water at a flow rate of $4\,l\,min^{-1}$ and provided with continuous aeration. Fish were maintained at ambient river temperature (1.6–16.0 °C), under natural photoperiod, and fed to satiation with commercial feed (Zeigler Bros, Gardners, PA, USA) using automatic feeders.

At the start of the experiment (February 2010), fish were separated by size into parr [fork length (Lf) ≤ 10.0 cm] and pre-smolt ($Lf \geq 12.1$ cm) groups based on a previously established winter threshold for smolt development in this strain of Atlantic salmon (McCormick et al., 2007). Parr were moved to 2 tanks (700 l) to a density of 250–260 fish per tank. Smolt were moved to 4 tanks (700 l) to a density of 150–160 fish per tank. Fish were maintained at 10 ± 1 °C under natural photoperiod, and fed to satiation with commercial feed (Zeigler Bros) using automatic feeders. All parr had characteristic morphology (dark vertical bands and pink spots on their sides) throughout the study and all smolts had silvering and darkened fin margins when sampled near the peak of smolting.

Six growth trials were performed from February through July wherein smolts were transferred from freshwater to duplicate freshwater recirculation tanks, or to duplicate 35 p.p.t. artificial seawater (Instant Ocean, Spectrum Brands, Blacksburg, VA, USA) recirculation tanks, with particle and charcoal filtration. At the beginning of each growth trial individual smolts were paint marked on their anal fin for identification. For each of the 6 growth trials (20 day) individual weights were recorded at the start of the trial, and after the first (9 day) and second (11 day) growth interval. Fish were terminally sampled at the end of the second interval (day 20). For the first and second growth trial, 16 fish were transferred to each tank. For the third, fourth and fifth trial, 14 fish were transferred to each tank. For the sixth trial, 12 fish were transferred to each tank, with the exception of one SW tank, which received 11. Fish were maintained at 10 ± 1 °C under natural photoperiod and fed daily to satiation. Temperature was held constant throughout the study to remove a variable that is known to affect growth (Handeland et al., 1998). During the first growth trial, there were 14 mortalities in SW; during the first, second, and fifth growth trial there was a single mortality in FW. Specific Growth Rate (SGR) was expressed as the percent change in weight (g) of individual fish per day, and calculated as $(\ln Wt2 - \ln Wt1)/(t2 - t1)\cdot 100$ where W = weight of individual fish at the beginning (t1) or end (t2) of a growth trial interval. At the start of each growth trial 10–12 smolts in freshwater were terminally sampled, and at the start of the fourth growth trial 12 parr were terminally sampled. At the start of each growth trial, a separate seawater challenge (SWC) was performed by transferring smolts from freshwater to duplicate 35 p.p.t. artificial seawater tanks for 24 h followed by a terminal sampling. Twelve fish were used for the first 5 SWCs and 11 fish for the sixth. One mortality occurred during the first SWC.

Food was withheld for 24 h prior to sampling of fish, which occurred between 10:00 and 12:00 h Eastern Standard Time. Fish were anesthetized with MS-222 ($100\,mg\,l^{-1}$, pH 7.0) and blood was drawn from the caudal vessels into a 1 ml ammonium heparinized syringe, spun at 3200 g for 5 min at 4 °C; plasma was then aliquoted and stored at −80 °C for plasma cortisol and chloride analyses. Gill arches were removed, and gill filaments trimmed from the ceratobranchials and placed in a 1.5 ml microcentrifuge tube and frozen immediately at −80 °C for Western blotting analysis and real-time quantitative PCR assays. Four to six gill filaments were placed in 100 µl of ice-cold SEI buffer ($150\,mmol\,l^{-1}$ sucrose, $10\,mmol\,l^{-1}$ EDTA, $50\,mmol\,l^{-1}$

imidazole, pH 7.3) and frozen at −80 °C within 30 min for measurement of NKA activity. All animal procedures were conducted in accordance with the University of Massachusetts, Amherst IACUC-approved protocol #27-02-09.

2.2. Gill and plasma analyses

NKA activity in gill homogenates was determined using a temperature-regulated microplate method (McCormick, 1993). In this assay, ouabain-sensitive ATPase activity was measured by coupling the production of ADP to NADH using lactic dehydrogenase and pyruvate kinase in the presence and absence of 0.5 mmol l^{-1} ouabain. Samples (10 µl) were run in duplicate in 96-well microplates at 25 °C and read at a wavelength of 340 nm for 10 min on a THERMOmax microplate reader using SOFTmax software (Molecular Devices, Menlo Park, CA, USA). A temperature of 25 °C is standard for this assay and allows for a comparison of gill tissue ATPase activity across studies. Protein concentration of the homogenate was determined using a BCA protein assay (Pierce, Rockford, Il, USA). Plasma chloride was analyzed by the silver titration method using a Buchler-Cotlove digital chloridometer (Labconco, Kansas City, MO, USA) and external standards. Plasma cortisol levels were measured by a validated direct competitive enzyme immunoassay as previously outlined (Carey and McCormick, 1998). Sensitivity as defined by the standard curve was 1–160 ng ml^{-1}. The lower detection limit was 0.3 ng ml^{-1}. Using a pooled plasma sample, the average intra-assay variation was 7.2% and the average inter-assay variation was 11.8%.

2.3. Real-time quantitative PCR assays

Total RNA was extracted from approximately 50 mg tissue using TRI reagent (Sigma, St. Louis, MO, USA) as outlined previously (Chomczynski, 1993). The integrity of RNA was visually assessed by agarose gel electrophoresis of a trial preparation, and the quantity and purity of every sample was assessed by the ratio of the absorbance at 260 nm and 280 nm. RNA was DNAse treated (RQ1, Promega Corporation, Madison, WI, USA) and cDNA was synthesized using a reverse transcriptase kit (RT-RTCK-05, Eurogentec, Seraing, Belgium) following the manufacturer's instructions. Real-time quantitative PCR (Q-PCR) assays were performed using the LightCycler 480 System (Roche, Indianapolis, IN, USA) to quantify the abundance of gill NKAα1a, NKAα1b, NKCC1, CFTRI and CFTRII mRNA using previously published primers and cycling conditions (Nilsen et al., 2007). The level of ion-transporter mRNA was reported as a ratio to a normalization gene, elongation factor 1α (EF1α, Olsvik et al., 2005). EF1α did not show significant changes in relation to time or treatment. For each assay, amplification efficiencies (E) were calculated from a fivefold dilution series of cDNA synthesized from total RNA from different groups, each series was run in triplicate. Amplification efficiencies (E) were calculated as the slope of log RNA concentration *versus* threshold cycle = (Ct) values using the following formula: $E = 10(-1/\text{slope})$ to correct for differences in amplification efficiency when calculating gene expression (Pfaffl et al., 2004). The PCR reactions consisted of 5 µl 10-fold diluted cDNA, 250 nM of each primer and SYBRI master mix in a total reaction volume of 20 µl.

2.4. Antibodies

Rabbit anti-ssNKCC1a was raised against a synthetic peptide sequence (CYRQTAAPLGDKLIRPT) corresponding to an N-terminal region of *S. salar* NKCC1a (NM_001123683.1) identified in the GRASP database (Genomic Research on Atlantic Salmon Project; http://web.uvic.ca/grasp/). The sequence was selected because it is immunogenic, well conserved across taxa, and predicted to be unique relative to other NKCC proteins. Peptides were synthesized, purified, and sequenced to verify amino acid composition, and then conjugated to an immune carrier. Two rabbits were given five immunizations and serum was collected from five production bleeds and a final exsanguination (21st Century Biochemicals, Marlborough, MA, USA). Western blotting analysis of gill homogenate found that the antiserum was immunoreactive with three bands of the expected molecular weight (Fig. S1, approximately 120, 160, and 285 kDa) which is in agreement with those observed using the pan-NKCC antibody T4 (Pelis et al., 2001). The signal was abolished when the serum was first adsorbed to the peptide antigen prior to Western blotting analysis. Immunohistochemical and Western blotting immunoreactivity was absent in the intestine suggesting that this antibody did not react with NKCC2. Rabbit anti-NKAα1a and rabbit anti-NKAα1b were developed as previously outlined (McCormick et al., 2009).

2.5. Western blotting analysis

Ion-transporter protein abundance in gill tissue membrane-enriched fractions was quantified by Western immunoblotting as previously outlined (Pelis et al., 2001). Gill tissue was homogenized in 10 volumes of PBS (1.9 mmol l^{-1} NaH_2PO_4, 8.1 mmol l^{-1} Na_2HPO_4, 138 mmol l^{-1} NaCl, pH 7.4) containing 30% sucrose (w/v), 2 mmol l^{-1} EDTA, 1 mmol l^{-1} phenylmethylsulfonyl flouride (PMSF), and Complete Mini protease inhibitor tablets (Roche) and centrifuged at 5000*g* for 10 min at 4 °C. The supernatant was then centrifuged at 20,000*g* for 10 min at 4 °C. The resulting supernatant was centrifuged at 48,000*g* for 2 h at 4 °C. The final pellet was resuspended in homogenization buffer plus 0.1% Triton X-100 (LabChem, Pittsburg, PA, USA). Protein concentration was determined with a bicinchoninic acid (BCA) protein assay (Pierce). Samples were then placed in an equal volume of 2× Laemmli buffer, heated for 15 min at 60 °C and stored at −80 °C. Samples were thawed and proteins were resolved by SDS-PAGE using a 6.75% gel. Two lanes were reserved on each gel for Precision Plus relative molecular weight markers (Bio-Rad Laboratories, Hercules, CA, USA) and a reference gill tissue preparation to control for blot-to-blot variation. Following electrophoresis, proteins were transferred to Immobilon PVDF transfer membrane (Millipore, Bedford, MA, USA) at 30 V overnight in an ice bath in 25 mmol l^{-1} Tris and 192 mmol l^{-1} glycine buffer; pH 8.3. PVDF membranes were blocked with 5% non-fat dry milk in PBST (PBS with 0.05% Triton X-100) for 1 h at room temperature, rinsed in PBST, and exposed to primary antibody in blocking buffer for 1 h at room temperature. After rinsing in PBST, blots were exposed to goat anti-rabbit-HRP (KPL, Gaithersburg, MD, USA) diluted 1:10,000 in blocking buffer for 1 h at room temperature. After rinsing in PBST, blots were incubated for 1 min in a 1:1 mixture of enhanced chemiluminescent (ECL) solution A (396 µmol l^{-1} coumaric acid, 2.5 µmol l^{-1} luminol, 100 mmol l^{-1} Tris, pH 8.5) and ECL B (0.018% H_2O_2, 100 mmol l^{-1} Tris, pH 8.5), then exposed to X-ray film (RPI, Mount Prospect, IL, USA) and scanned. The intensity of each immunoreactive band, or sum of bands within a sample when multimers were present, was measured using ImageJ (National Institutes of Health, Bethesda, MD, USA) and protein abundance was expressed as a cumulative 8-bit greyscale value standardized to the internal reference lanes for each antibody.

2.6. Statistics

All statistical analyses were performed with Statistica 12.0 (StatSoft, Inc., Tulsa, OK, USA). Prior to statistical analyses data were tested for assumptions of normality (Shapiro-Wilk's W test) and homogeneity of variance (Hartley F_{max} test). When group sample sizes (n) were unequal the lower n was used to determine the critical value of F_{max}. When homogeneity of variance was not met data were log-transformed (Figs. 1A, 2A, 3A–E, 5A) to meet the assumptions of parametric models. Differences in plasma chloride of smolts after seawater challenge were compared by one-way analysis of variance (ANOVA) using time as the independent variable. Differences in SGR were compared by three-way

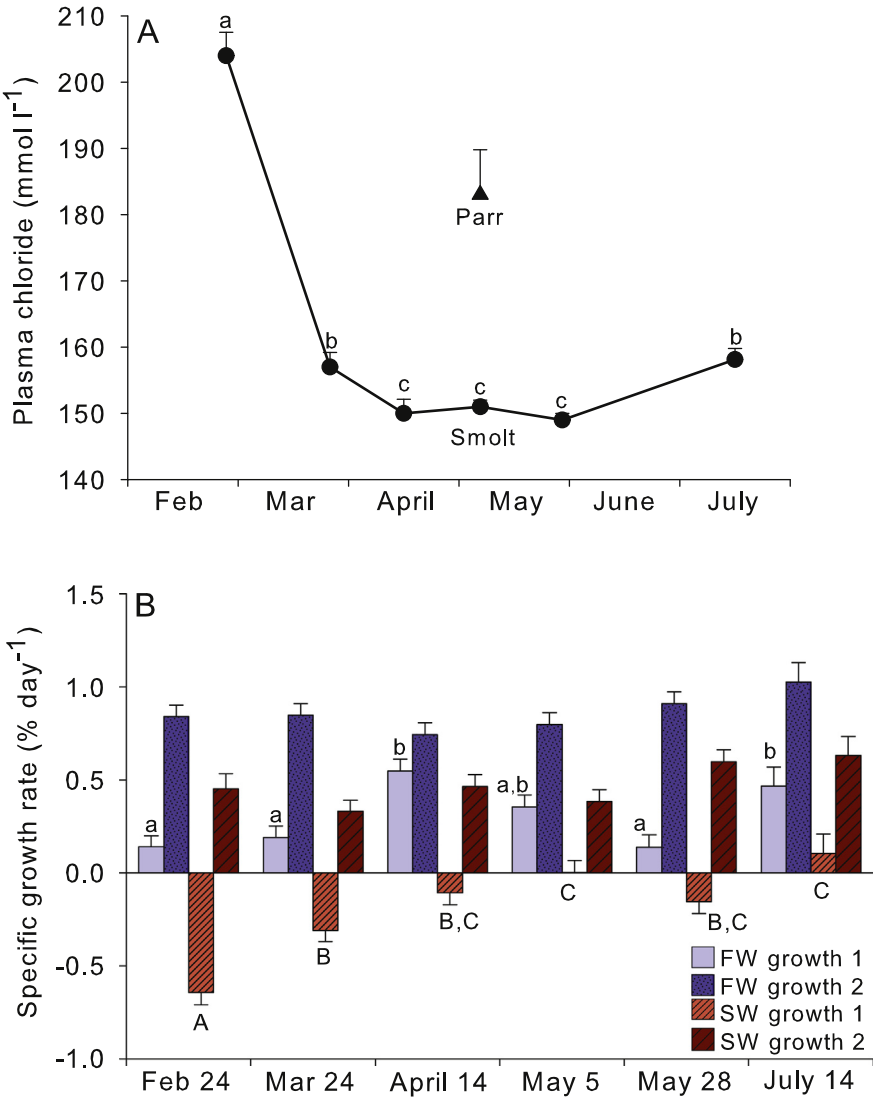

Fig. 1. Plasma chloride (A) of Atlantic salmon smolts (circles) and parr (triangle) after seawater challenge, and specific growth rate (B) of Atlantic salmon smolts after transfer from to freshwater (FW, blue) or freshwater to seawater (SW, red) during growth interval one (Growth 1, days 0–9) or two (Growth 2, days 10–20). Significant differences from the initial February sampling date in plasma chloride in smolts are indicated by letter (one-way ANOVA, Student-Newman-Keuls (SNK) *post hoc* test, $P < 0.05$), values are means ± s.e.m. of 5 parr, or 10 to12 smolts per group ($N = 68$). Significant differences from the initial February sampling date of specific growth rate within each growth interval across trials is indicated by lower case letter for freshwater and uppercase letter for seawater (three-way ANOVA, SNK *post hoc* test, $P < 0.05$). Values are means ± s.e.m. of 10 to 31 fish per group (FW growth 1 $N = 156$; FW growth 2 $N = 156$; SW growth 1 $N = 152$; SW growth 2 $N = 145$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ANOVA using time, salinity, and growth interval as the independent variables. Differences in plasma cortisol, gill NKA activity and gill mRNA were compared by two-way ANOVA using time and salinity as the independent variables. Differences in protein in freshwater were compared by one-way ANOVA using time as the independent variable, and when a comparison between protein abundance in freshwater and after seawater transfer was done, Student's *t*-test was used to determine significance ($P < 0.05$). Significant ANOVAs were followed by a Student-Newman-Keuls (SNK) multiple comparison test to determine differences between experimental groups using $P > 0.05$ as criterion for accepting null hypotheses. Correlations (r) between ion-transporter mRNA and protein abundances, and measured dependent variables to plasma chloride and growth, were determined with Pearson product-moment correlation coefficient. Plasma chloride correlations were performed with values from smolts at the start of each growth trial and May parr. SGR correlations were performed with values from smolts at the start of each growth trial to SGR during the first growth interval (day 0–9).

3. Results

3.1. Seawater tolerance and growth performance

Plasma chloride concentrations in smolts 24 h after transfer from freshwater to seawater were significantly higher in February (204 mmol l^{-1}) than in the spring and summer, and was lowest in late May (149 mmol l^{-1}) indicative of increased salinity tolerance (Fig. 1A). Plasma chloride concentration was approximately 20% higher in parr than in smolts after seawater transfer in early May (183 mmol l^{-1} and 151 mmol l^{-1}, respectively).

SGR of smolts transferred from freshwater to freshwater remained relatively constant throughout the study, whereas SGR of smolts transferred from freshwater to seawater increased throughout the spring and summer (Fig. 1B). Statistically significant differences were only observed in the first growth interval (day 0–9) of each of the six growth trials in freshwater and seawater groups, in contrast to the second growth interval (day 10–20) wherein no differences were detected across either group. SGR in smolts transferred to seawater improved six-fold during the first growth interval from February (−0.64% day^{-1}) to April (−0.11% day^{-1}), then remained relatively constant through May and peaked with a positive value in July (0.11% day^{-1}). There were significant three-way ANOVA effects of time ($P < 0.0001$), growth interval ($P < 0.0001$), and salinity ($P < 0.0001$), and significant interactions among all combinations of independent variables (three-way ANOVA, $P < 0.0500$), except between growth interval and salinity ($P = 0.0728$).

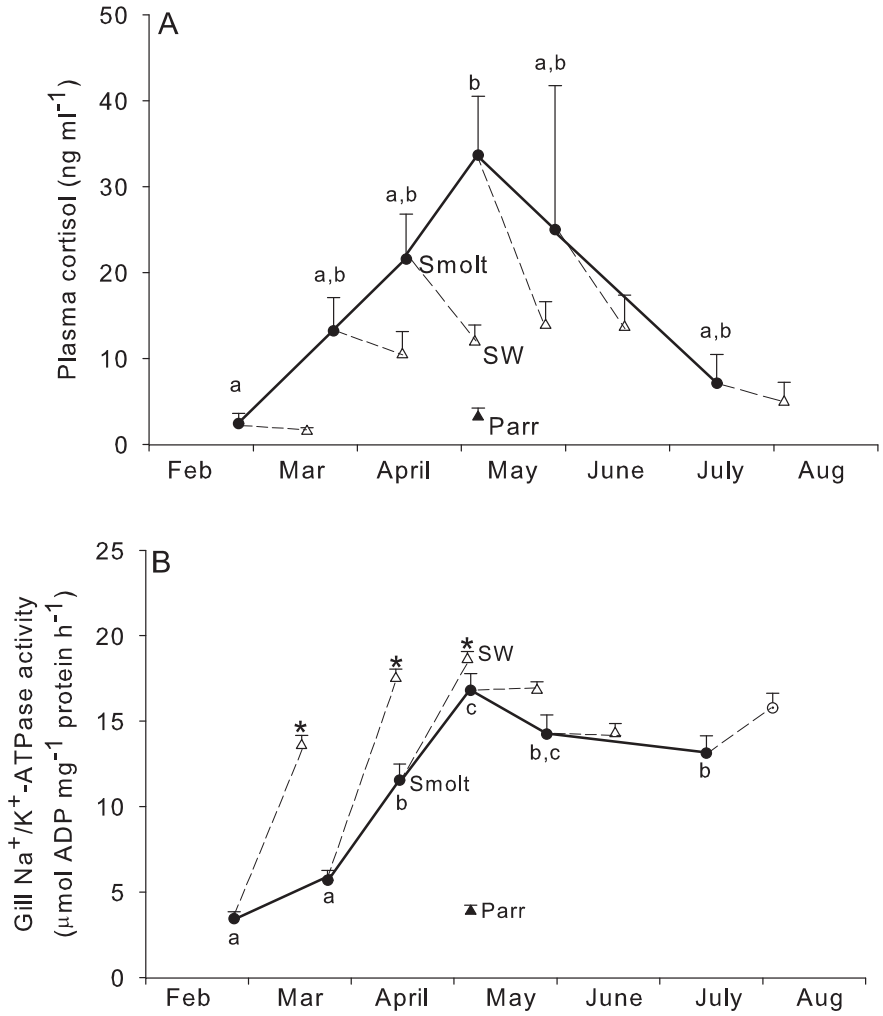

Fig. 2. Plasma cortisol concentration (A) and gill Na^+/K^+-ATPase activity (B) of Atlantic salmon smolts (circles) and parr (triangle) during freshwater rearing (filled shapes) and 20 days after transfer from freshwater to seawater (SW, open shapes). Significant differences from the initial February sampling date in plasma cortisol in smolts in freshwater are indicated by letter (two-way ANOVA, SNK *post hoc* test, $P < 0.05$). Values are means ± s.e.m. of 10 to 32 fish per group (parr $N = 12$; smolts in FW $N = 82$; smolts in SW $N = 156$). Significant differences from the initial February sampling date in gill Na^+/K^+-ATPase activity in smolts in freshwater are indicated by letter and differences after seawater transfer are indicated by an asterisk (two-way ANOVA, SNK *post hoc* test, $P < 0.05$). Values are means ± s.e.m. of 12 to 28 fish per group (parr $N = 12$; smolts in FW $N = 70$; smolts in SW $N = 143$).

3.2. Plasma cortisol and gill NKA activity

Plasma cortisol in freshwater reared smolts increased significantly during spring, from 2.4 ng ml^{-1} in February to 33.6 ng ml^{-1} in early May, then declined in July (7.1 ng ml^{-1}; Fig. 2A). Plasma cortisol decreased in smolts after each 20 day seawater growth trial. There were significant two-way ANOVA effects of time ($P < 0.0001$) and salinity ($P = 0.0299$). Plasma cortisol concentration of smolts in early May was 10-fold higher than that of parr.

Gill NKA activity in freshwater reared smolts exhibited a significant fivefold increase from February to early May (3.4–17.1 µmoles ADP mg protein^{-1} h^{-1}), then decreased significantly through July (13.1 µmoles ADP mg protein^{-1} h^{-1}; Fig. 2B). Gill NKA activity increased after each seawater growth trial, with statistically significant increases observed before May. There were significant two-way ANOVA effects of time ($P < 0.0001$), and salinity ($P < 0.0001$), and a significant interaction of the two independent variables ($P < 0.0001$). Gill NKA activity of smolts in early May was fourfold higher than that of parr.

3.3. Gill ion-transporter isoform mRNA and protein abundance

Gill NKAα1b mRNA abundance in freshwater remained relatively constant from February through July (Fig. 3A). Values increased after each seawater growth trial, the most dramatic of which was a significant fourfold increase in late May. There were significant two-way ANOVA effects of time ($P = 0.0140$) and salinity ($P < 0.0001$). Gill NKAα1b mRNA abundance in parr in early May was 10% less than that of smolts.

Gill NKAα1b protein abundance in freshwater reared smolts increased 30-fold from February through early May, then remained relatively stable through July (Fig. 3B). Gill NKAα1b protein abundance was also measured after the first (February) and fourth (early May) seawater growth trial, and increased in both cases but only significantly in February. Gill NKAα1b protein abundance in smolts in early May was 45-fold higher than that of parr.

Gill NKAα1a mRNA abundance in freshwater reared smolts decreased significantly from February to March and then remained relatively constant (Fig. 3C). Values decreased significantly after each seawater growth trial, the most dramatic of which was a 95% decrease in February. There were significant two-way ANOVA effects of time ($P < 0.0001$), and salinity ($P < 0.0001$), and significant interaction of the two independent variables ($P = 0.0011$). Gill NKAα1a mRNA abundance in parr in early May was sevenfold that of smolts.

Gill NKAα1a protein abundance in freshwater reared smolts decreased slightly though the early spring with a significant 60% reduction by early May (Fig. 3D). Values then increased through late spring into summer nearly returning to February levels. Gill NKAα1a protein abundance was significantly lower in after both seawater growth trials. Gill NKAα1a protein abundance in parr in early May was fourfold higher than that of smolts.

Gill NKCC1 mRNA abundance in freshwater reared smolts increased fivefold from February through the spring and were significantly after February (Fig. 3E). Values increased after each seawater growth trial and increase was significant in April. There were significant two-way

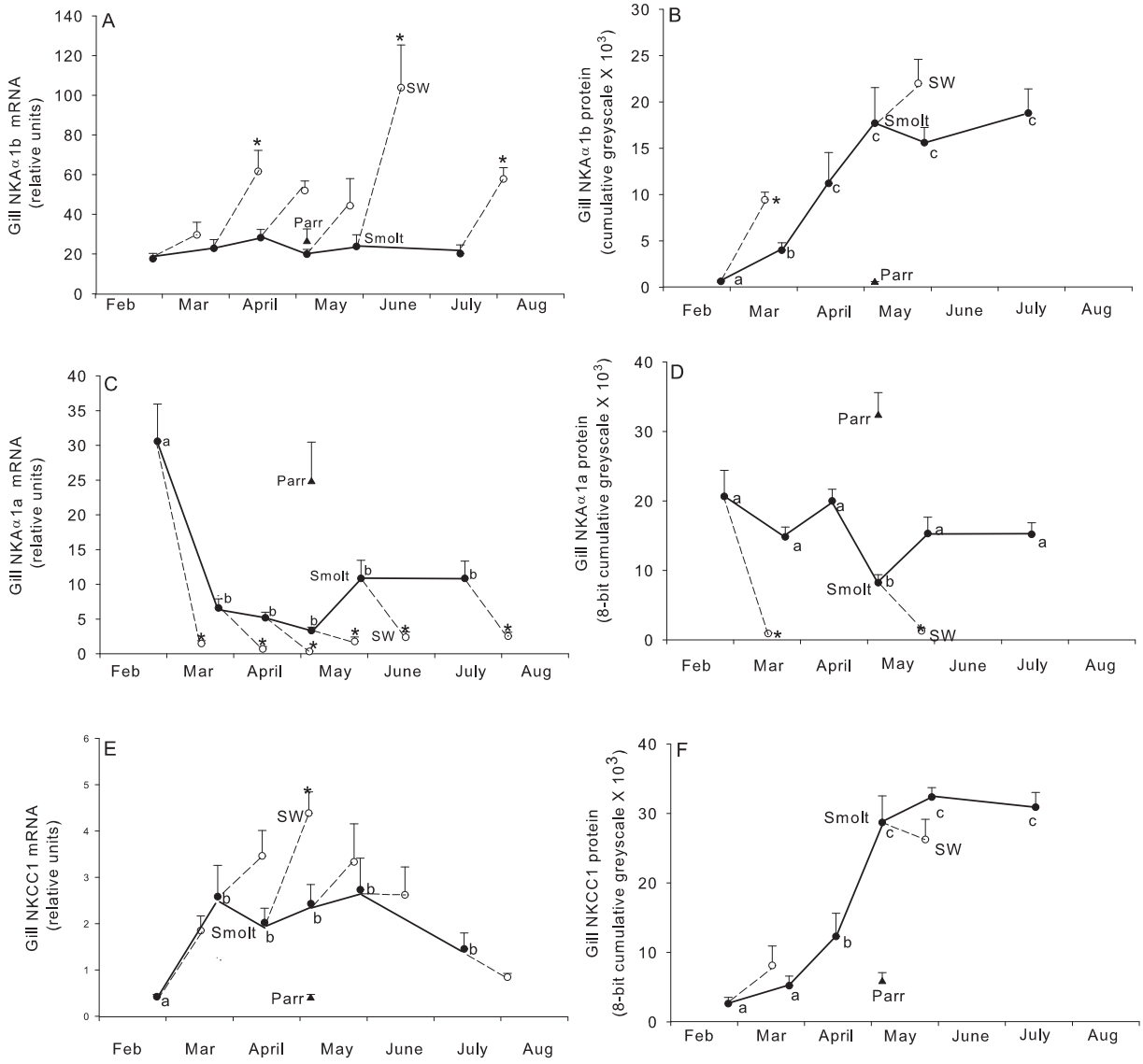

Fig. 3. Abundance of gill NKAα1b mRNA (A) and protein (B), NKAα1a mRNA (C) and protein (D), NKCC1 mRNA (E) and protein (F) in Atlantic salmon smolts (circles) and parr (triangle) during freshwater rearing (filled shapes) and 20 days after transfer from freshwater to seawater (SW, open shapes). Significant differences from the initial February sampling date of gill ion-transporter mRNA in freshwater are indicated by letter and differences in mRNA after seawater transfer are indicated by an asterisk (two-way ANOVA, SNK *post hoc* test, $P < 0.05$). Significant differences from the initial February sampling date of gill ion-transporter protein in freshwater are indicated by letter (one-way ANOVA, SNK *post hoc* test, $P < 0.05$) and differences in protein after seawater transfer are indicated by an asterisk (Student's *t*-test, $P < 0.05$). Values are means ± s.e.m. of 7 to 10, and 6 to 7, fish per group for mRNA and protein, respectively. The sample size for NKAα1b mRNA parr is 8, smolts in FW $N = 52$, smolts in SW $N = 58$; NKAα1a mRNA parr is 7, smolts in FW $N = 51$, smolts in SW $N = 58$; NKCC1 mRNA parr is 8, smolts in FW $N = 51$, smolts in SW $N = 56$. For all proteins the sample size for parr is 7, smolts in FW $N = 40$, smolts in SW $N = 13$.

ANOVA effects of time ($P < 0.0001$), and salinity ($P = 0.0011$), and significant interaction between the independent variables ($P = 0.0008$). Gill NKCC1 mRNA abundance in smolts in early May was sixfold higher than that of parr.

Gill NKCC1 protein abundance in freshwater reared smolts increased 10-fold from February through early May, then remained relatively stable through July (Fig. 3F). Gill NKCC1 protein abundance in smolts in early May was fivefold higher than that of parr.

3.4. Correlation of ion-transporter isoform mRNA to protein abundance

The abundance of NKAα1b mRNA did not significantly correlate to levels of NKAα1b protein in freshwater reared smolts and parr, and smolts after the first and fourth seawater growth trial ($r = 0.40$, $P = 0.286$; Fig. 4A). Levels of NKAα1a and NKCC1 mRNA were significantly correlated to levels of their protein product (NKAα1a, $r = 0.768$, $P = 0.016$; NKCC1, $r = 0.898$, $P = 0.001$) in freshwater reared smolts and parr, and smolts after the first and fourth seawater growth trial (Fig. 4B,C).

3.5. CFTR isoform mRNA abundance

Gill CFTRI mRNA abundance in freshwater reared smolts increased through the spring (Fig. 5A). There was a significant effect of time ($P = 0.0015$) and a significant effect of salinity ($P = 0.0006$). Gill CFTRI mRNA abundance in parr in early May was about 40% that of smolts. Gill CFTRII mRNA abundance varied over the course of the study, but there were no statistically significant changes or clear trends in the values (Fig. 5B). Gill CFTRII mRNA levels were an order of magnitude less than CFTRI.

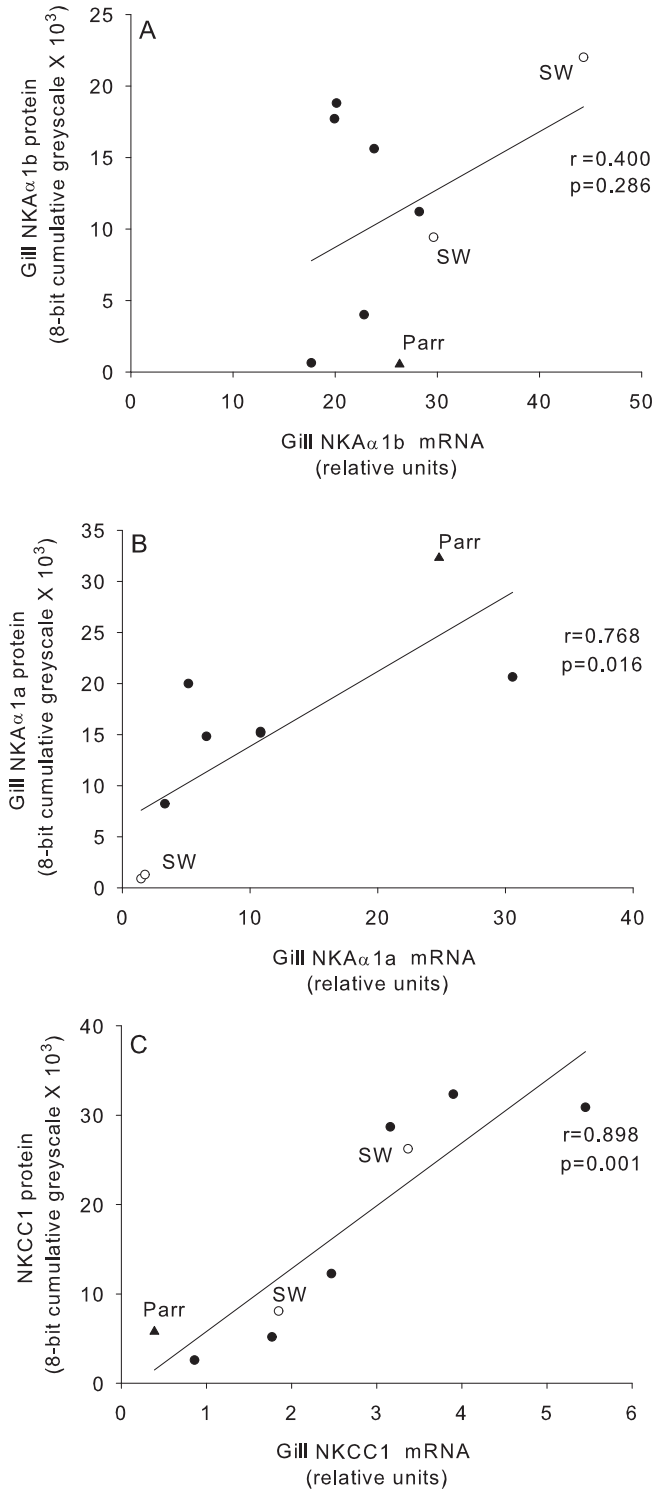

Fig. 4. Correlation of gill NKAα1b mRNA to protein abundance (A), NKAα1a mRNA to protein abundance (B), and NKCC1 mRNA to NKCC1 protein abundance (C). Values are from smolts (circles) and parr (triangle) during freshwater rearing (filled shapes) and 20 days after transfer from freshwater in February or early May to seawater (SW, open shapes).

3.6. Correlation of measured variables to seawater tolerance and SGR in seawater

We next determined which of the variables measured prior to seawater transfer best correlated to plasma chloride concentration 24 h after transfer and SGR after the first growth interval (Table 1). Both plasma chloride and SGR correlated well with NKA activity ($r = -0.794$, $r = 0.868$), gill NKAα1b protein abundance ($r = -0.763$, $r = 0.931$) and the ratio of gill NKAα1b to NKAα1a protein abundance ($r = 0.910$, $r = -0.886$). Plasma chloride also correlated well with plasma cortisol concentration ($r = -0.768$), gill NKAα1a mRNA ($r = 0.955$) and CFTRI mRNA abundance ($r = -0.874$), and the ratio of gill NKAα1b to NKAα1a mRNA abundance ($r = 0.953$). SGR also correlated well with gill NKCC1 mRNA ($r = 0.864$) and NKCC1 protein abundance ($r = 0.799$). Smolts from the early May seawater growth trial were identified as those most tolerant of seawater (peak smolt) based on plasma chloride after seawater challenge, SGR, plasma cortisol, and gill NKA activity. In order to identify those dependent variables that exhibited a pronounced change with low variability at this point, we reported the fold-change from February as well as the coefficient of variation (CV). Two dependent variables that correlated well with performance metrics and exhibited a high fold change were gill NKAα1b protein, and the ratio of gill NKAα1b to NKAα1a, protein abundance, and each of these variables had a moderate CV.

4. Discussion

Smolt development entails morphological, behavioral, and physiological adaptations that prepare salmonids for life in a marine environment (McCormick and Saunders, 1987; Hoar, 1988). A critical component of these changes is a transition of the gill epithelium from a tissue of net ion absorption to one capable of ion secretion, which is driven in large part by altered transcription of gill ion transport genes (Tipsmark et al., 2002; Nilsen et al., 2007) and subsequent expression of their protein products (Pelis et al., 2001; McCormick et al., 2013). In the present study we investigate 1) the relationship between the mRNA and protein of important ion-transporters at discrete time points during smolt development, upon transfer from freshwater to seawater throughout development, and 2) how these changes correlate to seawater tolerance and growth performance after seawater transfer. Our results highlight that the abundance of an ion-transporter mRNA is not always reflected in the abundance of the protein product during smolt development and seawater acclimation. Moreover, of the variables we measured those that best correlated to seawater tolerance and growth performance after seawater transfer were decreasing abundance of NKAα1a mRNA and increasing abundance of NKAα1b protein.

4.1. Correlation of gill ion-transporter isoform mRNA to protein abundance

There were significant differences in the correlation of mRNA and protein levels for each of the ion-transporters (Fig. 4); the correlation was low for gill NKAα1b ($r = 0.400$), intermediate for NKAαla ($r = 0.768$), and high for NKCC1 ($r = 0.898$). Inconsistency in these values is in agreement with recent systems biology studies in mammalian tissues and tumors, which have demonstrated that corresponding mRNA and protein pairs are not always well-correlated ($r^2 < 0.4$)(Ghazalpour et al., 2011; Kristensen et al., 2013; Li and Biggin, 2015), which could be taken to suggest that < 40% of the variation observed in the abundance of a protein can be explained by the abundance of its corresponding mRNA (Maier et al., 2009; Vogel and Marcotte, 2012). Thus, 60% of the variation in protein levels should be explained by regulation of protein translation and degradation. Indeed, comparative analyses across taxa have suggested that there is a greater selective pressure to tightly regulate protein rather than mRNA levels. Khan and colleagues identified dozens of genes in rhesus macaque, chimpanzee, and human lymphoblastoid cell lines that differed significantly in the abundance of mRNA but not protein, suggesting rates of mRNA turnover can be compensated for by rates of mRNA translation and/or protein turnover (Khan et al., 2013). Similar results have come from studies using largescale transcriptome (DNA microarray) and proteome (mass spectrometry) data sets to compare orthologues of taxa as diverse as bacteria, fungi, plants, insects,

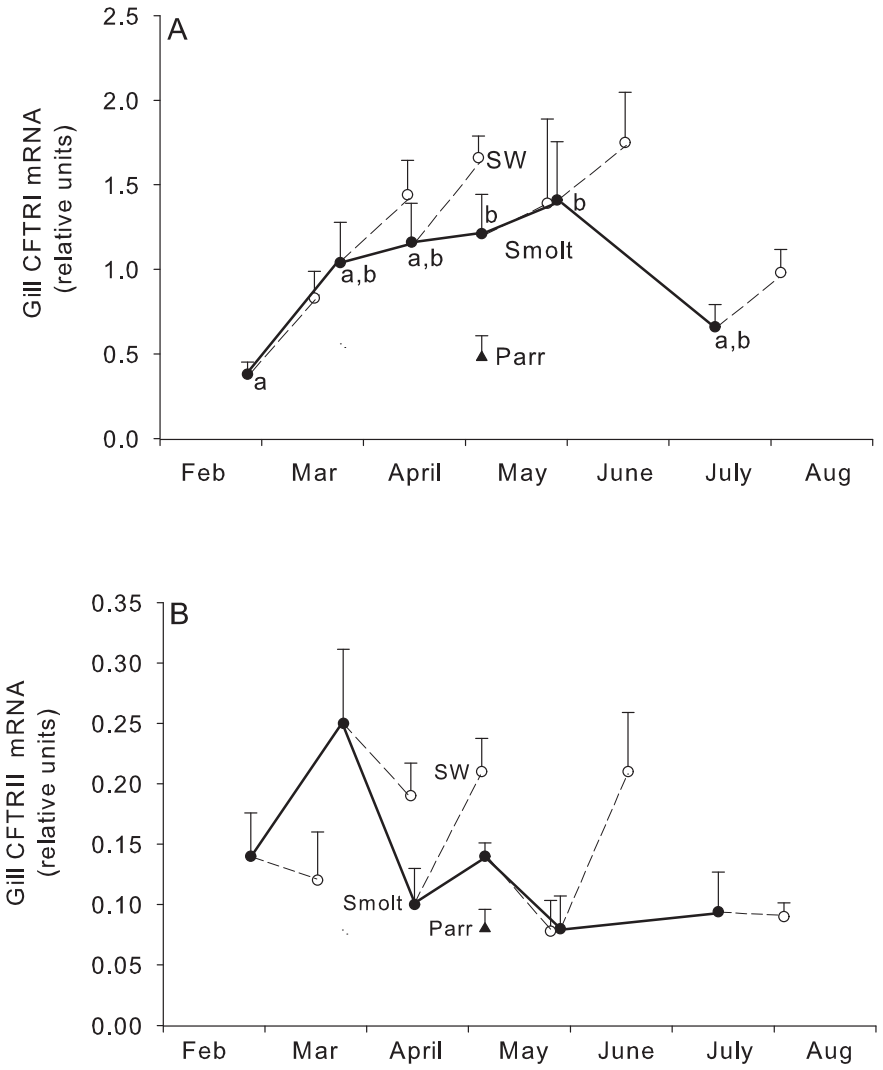

Fig. 5. Abundance of gill CFTRI (A) and CFTRII (B) mRNA during freshwater rearing (FW; solid shapes) and 20 days after transfer to seawater (SW; open circles) in Atlantic salmon smolts (circles) and parr (triangle). Significant differences from the initial February sampling date in smolts in freshwater are indicated by letter (two-way ANOVA, Student-Newman-Keuls (SNK) *post hoc* test, $P < 0.05$) Values are means ± s.e.m. of 7 to 10 fish per group. The sample size for CFTRI parr is 8, smolts in FW $N = 57$, smolts in SW $N = 58$; for CFTRII parr is 8, smolts in FW $N = 51$, smolts in SW $N = 56$.

nematodes, and humans (Schrimpf et al., 2009; Laurent et al., 2010). It should be noted that a few studies have found a stronger relationship between mRNA and protein abundance after employing statistical analyses that estimate or reduce biases associated with technical artifacts (Lackner et al., 2012; Li et al., 2014; Battle et al., 2015). A lack of consensus highlights the importance of determining how mRNA levels actually translate to protein levels for a given gene before depending on mRNA abundance as an adequate proxy for protein levels, which ultimately determine the true phenotype of a cell or tissue.

4.2. Changes in NKAα1a mRNA with stable protein levels

Gill NKAα1a is thought to be important for hyperosmoregulation in salmonids and other species that possess salinity dependent isoform switching. During smolt development we observed a strong down regulation in the abundance of gill NKAα1a mRNA whereas gill NKAα1a protein remained relatively constant. In contrast, after transfer to seawater both NKAα1a mRNA and protein abundance substantially decreased (Fig. 3C, D). Previous studies in salmonids have suggested that NKAα1a is a freshwater type ion-transporter based on observations that gill mRNA (Richards et al., 2003; Nilsen et al., 2007; Madsen et al., 2009) and protein (McCormick et al., 2009; McCormick et al., 2013) decrease during the parr-smolt transformation and after transfer to seawater. The present study reveals a distinct difference in how NKAα1a expression is regulated during smolt development compared to seawater acclimation. During smolt development, gill NKAα1a mRNA decreased despite the requirement of steady state protein levels to maintain ion homeostasis. The maintenance of protein level may result from increases in translation and/or protein stability. The down-regulation of NKAα1a mRNA during development may be a trade-off resulting from a shift in the emphasis of the tissue to proteins important for ion-secretion, such as NKAα1b, as the animal prepares for seawater entry (McCormick et al., 2013). Subsequent to transfer to seawater, gill NKAα1a mRNA and protein levels were in better agreement and decreased significantly at the end of each 20 day growth trial. The actual decreases probably occurred early in the growth trial, as we observed a substantial reduction in gill NKAα1a protein levels in smolts within two days of seawater transfer (McCormick et al., 2009).

4.3. Changes in NKAα1b and NKCC1 mRNA and protein abundance

Gill NKAα1b and NKCC1 are thought to be important for hypoosmoregulation. We observed an increase in both mRNA and protein abundance of gill NKAα1b and NKCC1 through smolt development and

Table 1
Correlation of dependent variables to plasma chloride concentration and Specific Growth Rate (SGR), and fold change and coefficient of variance (CV) of gill variables at peak smolt in early May. Plasma chloride values are correlated to measured variables in smolts at the beginning each growth trial and parr in early May. Specific growth rate values of smolts during the first growth interval in seawater are correlated to measured variables in smolts at the beginning each growth trial. $P < 0.05$ is indicated with an asterisk, $P < 0.005$ is indicated by two asterisks.

Dependent variable	Plasma chloride		Specific growth rate		Peak smolt (early May)	
	r	P	r	P	Fold change	CV
NKA activity	−0.794	0.033*	0.868	0.025*	4.9	18.5
Plasma cortisol	−0.768	0.044*	0.512	0.299	13.8	70.8
NKAα1b mRNA	−0.374	0.408	0.303	0.560	1.1	31.8
NKAα1b protein	−0.763	0.046*	0.931	0.007*	29.5	34.8
NKAα1a mRNA	0.955	0.001**	−0.785	0.064	−9.3	39.5
NKAα1a protein	0.591	0.162	−0.570	0.237	−2.5	3.7
NKCC1 mRNA	−0.665	0.103	0.864	0.026*	3.6	44.9
NKCC1 protein	−0.661	0.106	0.799	0.056	11.0	35.4
CFTRI mRNA	−0.874	0.010*	0.479	0.336	3.0	5.9
CFTRII mRNA	−0.016	0.973	−0.388	0.447	1.0	20.0
NKAα1b/ NKAα1a mRNA	0.953	0.001**	−0.763	0.078	10.0	0.3
NKAα1b/ NKAα1a protein	0.910	0.004**	−0.886	0.019*	104.0	36.8

after seawater transfer, although the increase in NKAα1b was not significant (Fig. 3A, B, E, F) These results are generally consistent with previous studies in Atlantic salmon (Pelis et al., 2001; Nilsen et al., 2007; Madsen et al., 2009; McCormick et al., 2009; McCormick et al., 2013) that have associated these two genes with smolt development and ion-secretion in seawater. Peak levels of gill NKAα1b and NKCC1 mRNA preceded peak protein levels by at least a month. This is similar to previous observations of gill NKA α-subunit mRNA and protein levels during smolting, although the probes used for detection in this report were not isoform specific (D'Cotta et al., 2000). By comparing the gill NKAα1b and NKCC1 mRNA and protein profiles through smolt development, we show a similar trend to that of gill NKAα1a, in that protein levels increase or are maintained at higher levels than would be expected from mRNA levels. Unlike NKAα1a where protein increases are compensatory, increases in gill NKAα1b and NKCC1 protein levels appear to be driven at least in part by increases in mRNA. The abundance of gill NKAα1b and NKCC1 protein are highest in May, coincident with maximal hypoosmoregulatory capacity. After seawater transfer, the abundance of NKAα1b and NKCC1 mRNA increased but protein levels remained relatively stable. This may be a consequence of decreased protein stability in the face of increased oxidative stress due to increased metabolism that would occur in active ionocytes. Such a scenario is supported by greater turnover of ionocytes in seawater than in freshwater (Uchida and Kaneko, 1996).

In the present, study relatively small increases (50%) in gill NKAα1b mRNA during smolt development were coupled to large increases in protein abundance. We hypothesize that this relationship can come about by the slow but steady accumulation of protein over many weeks that has a relatively low turnover because it is itself inactive and/or present in largely inactive cells. Changes in gill NKAα1b mRNA levels during smolting in the present study were less dramatic than those reported by Nilsen et al. (2007), which may be attributable to strain differences or differences in rearing temperatures. Since gill NKA activity in the two studies are similar, presumably the levels of gill NKAα1b protein are also similar. Accumulation of inactive preparatory gill proteins, in this case NKAα1b and NKCC1, in functionally transitioning ionocytes as well as in *de novo* dormant seawater type-ionocytes, may be rapidly activated upon seawater entry by a post-translational modification. Reversible phosphorylation of the NKA α-subunit and NKCC1 have been shown too quickly to activate or inactivate these enzymes in other species (Lopina, 2001; Flemmer et al., 2010). Proteins required for ion homeostasis in freshwater may be targeted for degradation after seawater entry, possibly by way of the ubiquitin-mediated degradation pathway. Previous studies in mammalian cell lines have shown that the NKA α-subunit can be poly-ubiquitinated and further demonstrated the targeted degradation of NKA in response to various stressors (Helenius et al., 2010). We hypothesize that the apparent disconnect between transcript and protein levels for the different transporters at different life stages is due to their expression in distinct cells with different translational and protein stability characteristics.

4.4. Changes in gill CFTRI and CFTRII mRNA abundance

We observed a significant increase in gill CFTRI mRNA abundance throughout smolt development, peaking in May and returning to winter (presmolt) levels in July. We did not observe any significant changes in gill CFTRII mRNA abundance throughout smolt development, and neither isoform increased significantly after transfer to seawater. These results are consistent with previous reports of CFTR isoform expressed in Atlantic salmon gill during smolting (Nilsen et al., 2007). We were unable to profile the expression of the CFTR isoforms at the protein level because no antibody currently exists that reacts with the protein, in spite of our repeated attempts to create such an antibody. To date, protein abundance of CFTR has been measured only in three teleost species, the killifish (*Fundulus heteroclitus*) (Marshall et al., 2002), sea bass (*Dicentrarchus labrax*)(Bodinier et al., 2009) and mudskipper (latin name)(Wilson et al., 2000).

4.5. Gill NKA activity

We measured the activity of gill NKA throughout the study as a metric of catalytically competent NKA in the gill epithelium (Fig. 2B). Because gill NKA has long been recognized as a critical constituent of the machinery that drives gill ion transport, it is widely used as an indicator of salt secretory capacity. Gill NKA activity had a similar pattern to the expression of gill NKAα1b protein, with levels increasing throughout the winter and spring and peaking in May (Fig. 3B). At this point, transferring the animals to seawater for 20 days failed to stimulate an increase in NKAα1b expression or NKA activity, suggesting the gill epithelium was optimized for salt secretion prior to seawater transfer. This is supported by relatively low levels of plasma chloride after 24 h in seawater (Fig. 1A) which are indicative of high seawater tolerance. The levels of gill NKA activity and plasma chloride reported in the present study suggest peak smolt development in mid-April through early May.

4.6. Hormones, smolting, and osmoregulation

The physiological changes associated with smolting are orchestrated by a developmental program that responds to changes in circulating hormones. Development of smolt characteristics and hypoosmoregulatory capacity are driven by cortisol, growth hormone (GH), and the insulin-like growth factor I (IGF-I)(McCormick and Saunders, 1987; Hoar, 1988). Conversely, prolactin has been linked to hyperosmoregulatory capacity and genes important for ion-uptake in teleost fish (Hirano, 1986; Sakamoto and McCormick, 2006; Breves et al., 2014). We observed an increase in cortisol throughout smolting with significant increases through the spring and summer, and a return to levels seen in early spring during the winter (Fig. 2A), which is consistent with role for cortisol in smolt development and stimulation of osmoregulatory ability (Bisbal and Specker, 1991; McCormick et al., 1991; McCormick et al., 2009). We also observed a trend for plasma cortisol

to decrease after 20 days in seawater, which may be a consequence of increased clearance of cortisol from the plasma by osmoregulatory target tissues, which may in turn be related to increased metabolic clearance rate of cortisol (Nichols and Weisbart, 1985).

4.7. Specific growth rate

The SGR of Atlantic salmon transferred from freshwater to seawater increased throughout smolt development for the first growth interval of each growth trial (Fig. 1B). Interestingly, the improvements were significant during the first growth interval (days 0–9) but not the second (days 10–20) suggesting that changes characteristic of smolting are critical for an acute (initial) response to changes in salinity. A reduced SGR soon after transfer to increased salinity is consistent with previous reports (McCormick and Saunders, 1987; Handeland et al., 1998; Handeland and Stefansson, 2002). During the second growth interval, differences across freshwater or seawater groups were not significant and differences between freshwater and seawater groups were attenuated, which is consistent with earlier studies demonstrating that salinity bears little impact on growth in smolts after an initial period of osmoregulatory adjustment (Usher et al., 1991; Duston, 1994). Since we observed no difference in growth rate in the second interval, compensatory increases do not appear to take place. Thus, the negative effect of growth of fish transferred to seawater early in smolt development may have long term negative impacts on fish size. The previous studies together with data from the present study suggest that the parr-smolt transformation is in large part a proactive alteration of gill ion-transporter proteins, including NKAα1b, NKCC1 and CFTR1, to minimize the crisis period after seawater entry. By the end of each of the growth trials NKA activity increased to levels roughly equivalent to those during peak smolting, which may have contributed to the insignificant differences between groups during the second growth interval. Over the course of smolting, the SGR of smolts transferred to seawater increased until it nearly matched those of the freshwater group, suggesting that changes relating to smolting had remediated the challenge of seawater transfer.

4.8. Salinity tolerance

Of the variables that we measured, the three that had the highest correlation to salinity tolerance were the abundance of gill NKAα1a mRNA, ratio of NKAα1b to NKAα1a mRNA, and ratio of NKAα1b to NKAα1a protein. That a decrease in gill NKAα1a mRNA correlates highly to salinity tolerance suggests that it may be a good predictor of seawater tolerance. The high correlation of the ratio of NKAα1b and NKAα1a relative abundance at the mRNA and protein level suggests that a shift in their relative abundances is critical for movement into, and acclimation to, seawater. If the β- or γ-subunit of the NKA holoenzyme are not tightly regulated, as suggested by Nilsen et al. (2007), the activity of an existing NKA pool may be highly dependent on α-subunit stoichiometry. The seasonal profiles of gill ion-transporter mRNA tend to exhibit trends that are not reflected by protein levels for several weeks or months; moreover, the proteins we measured reached their peak (or trough) expression levels coincident with maximum salinity tolerance. When considering which variables could best serve as predictors of smolt development, variables with a high fold change, low coefficient of variance, and peak in early May (peak smolt development) would have the greatest practical utility. Taken together, the abundance of NKA α-subunit proteins may be more functionally relevant, and thus, the best proxy for smolt development may be the relative abundance of NKAα1b to NKAα1a protein, or the abundance of the NKAα1b protein alone.

The variables that best correlated to SGR during the first interval after transfer to seawater were the ratio of NKAα1b to NKAα1a protein and to a greater extent the abundance of NKAα1b alone. There was a large relative change in the abundance of these variables through the winter and spring, while their variation was rather low, and their peak/trough was coincident with peak smolt development, suggesting that they could be useful predictors of preparedness for, and growth performance after, transfer to seawater. That NKAα1b isoform had a higher correlation at the protein rather than mRNA level highlights the value of measuring protein abundance when identifying functional shifts in cells and tissues. NKAα1a mRNA exhibited a decrease in abundance during development while protein levels remained constant. A possible explanation for this disparity may be that the gill tissue experiences overall increases in translation during smolt development; as a result, some down-regulated genes would maintain constant protein levels, and genes that are up-regulated would result in dramatic increases in their protein product. This paradigm is supported by previous evidence that gill protein synthesis in Atlantic salmon is elevated during smolting when compared to parr or post-smolts in seawater (Fauconneau et al., 1989; Noble, 1989). This mechanism could facilitate transitions in tissues preparing for, but not yet subject to, environmental changes. It is unclear if this mechanism influences other genes important for smolt development, or has broader application to other species preparing for, or responding to, changes in the environment. Development of new antibodies and application of proteomic tools may make the relationship between gene transcript and protein product abundance during periods of transition clearer. Nonetheless, our results indicate that there are substantial differences in the relationship between mRNA and protein abundances when comparing preparatory developmental changes and response to salinity, and the ratio of NKAα1b to NKAα1a protein, or the abundance of NKAα1b protein alone could serve as a strong predictors of salinity tolerance and growth performance after transfer to seawater.

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Competing interests

The authors declare no competing or financial interests.

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References

Battle, A., Khan, Z., Wang, S.H., Mitrano, A., Ford, M.J., Pritchard, J.K., Gilad, Y., 2015. Genomic variation. Impact of regulatory variation from RNA to protein. Science 347, 664–667.

Berge, Å.I., Berg, A., Barnung, T., Hansen, T., Fyhn, H.J., Stefansson, S.O., 1995. Development of salinity tolerance in underyearling smolts of Atlantic salmon (*Salmo salar*) reared under different photoperiods. Can. J. Fish. Aquat. Sci. 52, 243–251.

Bisbal, G.A., Specker, J.L., 1991. Cortisol stimulates hypo-osmoregulatory ability in Atlantic salmon, *Salmo salar* L. J. Fish Biol. 39, 421–432.

Björnsson, B.T., Ogasawara, T., Hirano, T., Bolton, J.P., Bern, H.A., 1988. Elevated growth hormone levels in stunted Atlantic salmon, *Salmo salar*. Aquaculture 73, 275–281.

Blanco, G., Mercer, R.W., 1998. Isozymes of the Na-K-ATPase: heterogeneity in structure,

diversity in function. Am. J. Phys. 275, F633–650.
Bodinier, C., Boulo, V., Lorin-Nebel, C., Charmantier, G., 2009. Influence of salinity on the localization and expression of the CFTR chloride channel in the ionocytes of *Dicentrarchus labrax* during ontogeny. J. Anat. 214, 318–329.
Breves, J.P., McCormick, S.D., Karlstrom, R.O., 2014. Prolactin and teleost ionocytes: new insights into cellular and molecular targets of prolactin in vertebrate epithelia. Gen. Comp. Endocrinol. 203, 21–28.
Carey, J.B., McCormick, S.D., 1998. Atlantic salmon smolts are more responsive to an acute handling and confinement stress than parr. Aquaculture 168, 237–253.
Chomczynski, P., 1993. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. BioTechniques 15, 532–534.
D'Cotta, H., Valotaire, C., le Gac, F., Prunet, P., 2000. Synthesis of gill Na(+)-K(+)-ATPase in Atlantic salmon smolts: differences in alpha-mRNA and alpha-protein levels. Am. J. Phys. Regul. Integr. Comp. Phys. 278, R101–110.
Duston, J., 1994. Effect of salinity on survival and growth of Atlantic salmon (*Salmo salar*) parr and smolts. Aquaculture 121, 115–124.
Edwards, S.L., Marshall, W.S., 2012. Principles and patterns of osmoregulation and euryhalinity in fishes. In: McCormick, S.D., Farrell, A.P., Brauner, C.J. (Eds.), Fish Physiology, Euryhaline Fishes. Academic Press, New York, pp. 1–44.
Evans, D., 2011. Freshwater fish gill ion transport: August Krogh to morpholinos and microprobes. Acta Physiol. 202, 349–359.
Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85, 97–177.
Fauconneau, B., Breque, J., Bielle, C., 1989. Influence of feeding on protein metabolism in Atlantic salmon (*Salmo salar*). Aquaculture 79, 29–36.
Flemmer, A.W., Monette, M.Y., Djurisic, M., Dowd, B., Darman, R., Gimenez, I., Forbush, B., 2010. Phosphorylation state of the Na^+-K^+-Cl^- cotransporter (NKCC1) in the gills of Atlantic killifish (*Fundulus heteroclitus*) during acclimation to water of varying salinity. J. Exp. Biol. 213, 1558–1566.
Ghazalpour, A., Bennett, B., Petyuk, V.A., Orozco, L., Hagopian, R., Mungrue, I.N., Farber, C.R., Sinsheimer, J., Kang, H.M., Furlotte, N., Park, C.C., Wen, P.Z., Brewer, H., Weitz, K., Camp II, D.G., Pan, C., Yordanova, R., Neuhaus, I., Tilford, C., Siemers, N., Gargalovic, P., Eskin, E., Kirchgessner, T., Smith, D.J., Smith, R.D., Lusis, A.J., 2011. Comparative analysis of proteome and transcriptome variation in mouse. PLoS Genet. 7, e1001393.
Handeland, S.O., Stefansson, S.O., 2002. Effects of salinity acclimation on pre-smolt growth, smolting and post-smolt performance in off-season Atlantic salmon smolts (*Salmo salar* L.). Aquaculture 209, 125–137.
Handeland, S.O., Berge, Å., Björnsson, B.T., Stefansson, S.O., 1998. Effects of temperature and salinity on osmoregulation and growth of Atlantic salmon (*Salmo salar* L.) smolts in seawater. Aquaculture 168, 289–302.
Helenius, I.T., Dada, L.A., Sznajder, J.I., 2010. Role of ubiquitination in Na,K-ATPase regulation during lung injury. Proc. Am. Thorac. Soc. 7, 65–70.
Hirano, T., 1986. The spectrum of prolactin action in teleosts. Prog. Clin. Biol. Res. 205, 53–74.
Hoar, W.C., 1988. The physiology of smolting salmonids. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology. Academic Press, New York, pp. 275–343.
Hwang, P.P., Lee, T.H., 2007. New insights into fish ion regulation and mitochondrion-rich cells. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 148, 479–497.
Jorgensen, P.L., 2008. Importance for absorption of Na^+ from freshwater of lysine, valine and serine substitutions in the α1a-isoform of Na, K-ATPase in the gills of rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). J. Membr. Biol. 223, 37–47.
Khan, Z., Ford, M.J., Cusanovich, D.A., Mitrano, A., Pritchard, J.K., Gilad, Y., 2013. Primate transcript and protein expression levels evolve under compensatory selection pressures. Science 342, 1100–1104.
Kristensen, A.R., Gsponer, J., Foster, L.J., 2013. Protein synthesis rate is the predominant regulator of protein expression during differentiation. Mol. Syst. Biol. 9, 689.
Lackner, D.H., Schmidt, M.W., Wu, S., Wolf, D.A., Bahler, J., 2012. Regulation of transcriptome, translation, and proteome in response to environmental stress in fission yeast. Genome Biol. 13, R25.
Laurent, J.M., Vogel, C., Kwon, T., Craig, S.A., Boutz, D.R., Huse, H.K., Nozue, K., Walia, H., Whiteley, M., Ronald, P.C., Marcotte, E.M., 2010. Protein abundances are more conserved than mRNA abundances across diverse taxa. Proteomics 10, 4209–4212.
Li, J.J., Biggin, M.D., 2015. Gene expression. Statistics requantitates the central dogma. Science 347, 1066–1067.
Li, J.J., Bickel, P.J., Biggin, M.D., 2014. System wide analyses have underestimated protein abundances and the importance of transcription in mammals. Peer J. 2, e270.
Lopina, O.D., 2001. Interaction of Na,K-ATPase catalytic subunit with cellular proteins and other endogenous regulators. Biochem. Mosc. 66, 1122–1131.
Madsen, S.S., Kiilerich, P., Tipsmark, C.K., 2009. Multiplicity of expression of Na^+,K^+-ATPase {alpha}-subunit isoforms in the gill of Atlantic salmon (*Salmo salar*): cellular localisation and absolute quantification in response to salinity change. J. Exp. Biol. 212, 78–88.
Maier, T., Guell, M., Serrano, L., 2009. Correlation of mRNA and protein in complex biological samples. FEBS Lett. 583, 3966–3973.
Marshall, W., Grosell, M., 2006. Ion transport, osmoregulation, and acid-base balance. In: Evans, D., Claiborne, J.B. (Eds.), The Physiology of Fishes, 3rd edn. CRC Press, Boca Raton, pp. 177–230.
Marshall, W.S., Howard, J.A., Cozzi, R.R.F., Lynch, E.M., 2002. NaCl and fluid secretion by the intestine of the teleost *Fundulus heteroclitus*: involvement of CFTR. J. Exp. Biol. 2002 (205), 745–758.
McCormick, S.D., 1993. Methods for non-lethal gill biopsy and measurement of Na^+, K^+-ATPase activity. Can. J. Fish. Aquat. Sci. 50, 656–658.
McCormick, S.D., 1995. Hormonal control of gill Na^+, K^+-ATPase and chloride cell function. Fish Physiol. 14, 285–315.
McCormick, S.D., 2013. Smolt physiology and endocrinology. In: McCormick, S.D., Farrell, A.P., Brauner, C.J. (Eds.), Euryhaline Fishes. Academic Press, Amsterdam, pp. 199–251.
McCormick, S.D., Saunders, R.L., 1987. Preparatory physiological adaptations for marine life of salmonids: osmoregulation, growth, and metabolism. Am. Fish. Soc. Symp. 1, 211–229.
McCormick, S.D., Dickhoff, W.W., Duston, J., Nishioka, R.S., Bern, H.A., 1991. Developmental differences in the responsiveness of gill Na^+,K^+-ATPase to cortisol in salmonids. Gen. Comp. Endocrinol. 84, 308–317.
McCormick, S.D., Shrimpton, J.M., Moriyama, S., Björnsson, B.T., 2007. Differential hormonal responses of Atlantic salmon parr and smolt to increased daylength: a possible developmental basis for smolting. Aquaculture 273, 337–344.
McCormick, S.D., Regish, A.M., Christensen, A.K., 2009. Distinct freshwater and seawater isoforms of Na^+/K^+-ATPase in gill chloride cells of Atlantic salmon. J. Exp. Biol. 212, 3994–4001.
McCormick, S.D., Regish, A.M., Christensen, A.K., Björnsson, B.T., 2013. Differential regulation of sodium–potassium pump isoforms during smolt development and seawater exposure of Atlantic salmon. J. Exp. Biol. 216, 1142–1151.
Nichols, D.J., Weisbart, M., 1985. Cortisol dynamics during seawater adaptation of Atlantic salmon *Salmo salar*. Am. J. Phys. 248, R651–659.
Nilsen, T.O., Ebbesson, L.O., Madsen, S.S., McCormick, S.D., Andersson, E., Bjornsson, B.T., Prunet, P., Stefansson, S.O., 2007. Differential expression of gill Na^+,K^+-ATPase alpha- and beta-subunits, Na^+,K^+,$2Cl^-$ cotransporter and CFTR anion channel in juvenile anadromous and landlocked Atlantic salmon *Salmo salar*. J. Exp. Biol. 210, 2885–2896.
Noble, B.S., 1989. Protein Turnover in the Tissues of Fish: The Effect of Nutrition, Exercise and Salinity. Ph.D. Thesis. University of Aberdeen, United Kingdom.
Olsvik, P.A., Lie, K.K., Jordal, A.E., Nilsen, T.O., Hordvik, I., 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. BMC Mol. Biol. 6, 21.
Pelis, R.M., Zydlewski, J., McCormick, S.D., 2001. Gill Na(+)-K(+)-2Cl(−) cotransporter abundance and location in Atlantic salmon: effects of seawater and smolting. Am. J. Phys. Regul. Integr. Comp. Phys. 280, R1844–1852.
Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper — excel-based tool using pair-wise correlations. Biotechnol. Lett. 26, 509–515.
Richards, J.G., Semple, J.W., Bystriansky, J.S., Schulte, P.M., 2003. Na^+/K^+-ATPase alpha-isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. J. Exp. Biol. 206, 4475–4486.
Sakamoto, T., McCormick, S.D., 2006. Prolactin and growth hormone in fish osmoregulation. Gen. Comp. Endocrinol. 147, 24–30.
Sardet, C., Pisam, M., Maetz, J., 1979. The surface epithelium of teleostean fish gills. Cellular and junctional adaptations of the chloride cell in relation to salt adaptation. J. Cell Biol. 80, 96–117.
Schrimpf, S.P., Weiss, M., Reiter, L., Ahrens, C.H., Jovanovic, M., Malmstrom, J., Brunner, E., Mohanty, S., Lercher, M.J., Hunziker, P.E., Aebersold, R., von Mering, C., Hengartner, M.O., 2009. Comparative functional analysis of the *Caenorhabditis elegans* and *Drosophila melanogaster* proteomes. PLoS Biol. 7, e48.
Stagg, R.M., Talbot, C., Eddy, F.B., Williams, M., 1989. Seasonal variations in osmoregulatory and respiratory responses to seawater exposure of juvenile Atlantic salmon (*Salmo salar*) maintained in freshwater. Aquaculture 82, 219–228.
Tipsmark, C.K., Madsen, S.S., Seidelin, M., Christensen, A.S., Cutler, C.P., Cramb, G., 2002. Dynamics of Na(+),K(+),2Cl(−) cotransporter and Na(+),K(+)-ATPase expression in the branchial epithelium of brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*). J. Exp. Zool. 293, 106–118.
Uchida, K., Kaneko, T., 1996. Enhanced chloride cell turnover in the gills of chum salmon fry in seawater. Zool. Sci. 13, 655–660.
Usher, M.L., Talbot, C., Eddy, F.B., 1991. Effects of transfer to seawater on growth and feeding in Atlantic salmon smolts (*Salmo salar* L.). Aquaculture 94, 309–326.
Vogel, C., Marcotte, E.M., 2012. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat. Rev. Genet. 13, 227–232.
Wilson, J.M., Randall, D.J., Donowitz, M., Vogl, A.W., Ip, A.K., 2000. Immunolocalization of ion-transport proteins to branchial epithelium mitochondria-rich cells in the mudskipper (*Periophthalmodon schlosseri*). J. Exp. Biol. 203, 2297–2310.