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Expression of Gill Vacuolar-Type H⁺-ATPase B Subunit, and Na⁺, K⁺-ATPase α_1 and β_1 Subunit Messenger RNAs in Smolting *Salmo salar*

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ABSTRACT—Changes in gill vacuolar-type H⁺-ATPase B subunit, and Na⁺,K⁺-ATPase α and β subunit mRNA expression were examined during the course of smoltification in *Salmo salar*. We cloned and sequenced cDNA fragments of *S. salar* gill i) vacuolar-type H⁺-ATPase (V-H⁺-ATPase) B subunit, ii) Na⁺,K⁺-ATPase α_1 subunit, and iii) Na⁺,K⁺-ATPase β_1 subunit, and used these as Northern blotting probes. During smoltification, the salmon showed a typical increase in gill Na⁺,K⁺-ATPase activity and improved hypo-osmoregulatory ability as judged by their ability to regulate plasma [Cl⁻] in a 24-hr seawater challenge test (35 ppt). Gill Na⁺,K⁺-ATPase α_1 and β_1 subunit mRNA levels were regulated at a constant ratio during smoltification. Both transcripts were elevated during the build-up of gill Na⁺,K⁺-ATPase activity, underlining the importance of increased mRNA levels for increased enzyme activity. Gill V-H⁺-ATPase B subunit mRNA levels were high during the early phase of smoltification. These results support our hypothesis that gill V-H⁺-ATPase expression may be elevated during the early stages of smoltification in order to counter the effects of increased ionic efflux when in FW. The peak smolt stage was, however, characterized by simultaneously elevated gill Na⁺,K⁺-ATPase expression and low V-H⁺-ATPase expression, and possibly ensures the complete transformation of the gill into a hypo-osmoregulatory organ and hence the development of optimal SW-tolerance of the smolt.

INTRODUCTION

In seawater (SW) fish, gill chloride cells (CCs) are believed to be responsible for the excretion of salts. In the baso-lateral tubular system of CCs, Na⁺,K⁺-ATPases may be packed at 200 million enzymes per cell (review by Karnaky, 1986), and constitute the basal driving force for trans- and paracellular extrusion of monovalent ions. By comparison, both pavement cells and CCs along the lamellae and filaments are suspected cellular candidates for monovalent ion uptake in the fresh water (FW) teleost gill (Lin *et al.*, 1994; Sullivan *et al.*, 1995; Wilson *et al.*, 2000). According to the current model for active Na⁺ uptake across tight epithelia such as the frog skin and the FW teleost gill, the driving force is set up by two ion-motive membrane pumps acting in series: an apical vacuolar-type H⁺-ATPase (V-H⁺-ATPase) and a basolateral Na⁺,K⁺-ATPase (Ehrenfeld and Garcia-Romeu, 1977; Avella and

Bornancin, 1989). The V-H⁺-ATPase extrudes protons to the surrounding dilute medium thereby energizing the uptake of Na⁺ through apical Na⁺-channels. Basolaterally, Na⁺ is moved into the blood by the action of the Na⁺,K⁺-ATPase. This model is supported by both localization studies using heterologous antibodies (Lin *et al.*, 1994; Sullivan *et al.*, 1995; Wilson *et al.*, 2000) and inhibitor-studies using either the Na⁺-channel inhibitor amiloride (Avella and Bornancin, 1989) or the V-H⁺-ATPase inhibitor bafilomycin A (Fenwick *et al.*, 1999).

Na⁺,K⁺-ATPase is a P-type ATPase consisting of an ($\alpha\beta$)₂ protein complex. Four α and three β isoforms as well as a small γ subunit have been found in mammals and birds (Blanco and Mercer, 1998). In teleost fish, complete α_1 -like isoforms have been cloned from white sucker (*Catostomus commersoni*: Schönrock *et al.*, 1991) and European eel (*Anguilla anguilla*: Cutler *et al.*, 1995a), and full-length β_1 and β_3 isoforms have been cloned from European eel (Cutler *et al.*, 1995b) and zebrafish (*Danio rerio*: Appel *et al.*, 1996), respectively. The V-H⁺-ATPase is a multi-subunit protein complex with more than 10 different subunits (review by Forgac, 1998). It is an ubiquitous enzyme found in organisms ranging from protozoa (Karcz *et al.*, 1994), over plants (Manolson *et al.*, 1988) to mammals (Südhof *et al.*, 1989). Cloning of

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teleost V-H⁺-ATPase subunits has just recently been reported in *A. anguilla* (Niederstaetter and Pelster, 2000) and rainbow trout, *Oncorhynchus mykiss* (Perry *et al.*, 2000).

Atlantic salmon (*Salmo salar*), like other anadromous salmonids, has the capability of migrating from FW directly into full strength SW only at a certain developmental stage. In wild stocks of salmon, this capability gradually develops during the spring preceding SW entrance, a process termed smoltification. Among the most characteristic biochemical changes is the pronounced increase in gill Na⁺,K⁺-ATPase activity (e.g. Nielsen *et al.*, 1999; D'Cotta *et al.*, 2000), which is causally related to an increased SW-tolerance (e.g. Nielsen *et al.*, 1999). Whereas three studies have reported on elevated levels of the gill Na⁺,K⁺-ATPase α subunit transcript (D'Cotta *et al.*, 1996, 2000; Nielsen *et al.*, 1999), no studies have yet reported on simultaneous changes in gill Na⁺,K⁺-ATPase α and β subunit mRNA levels. As SW-tolerance develops during smoltification, the fish gradually becomes maladapted to the FW-environment, as indicated by decreasing plasma ion levels and negative ionic balance at the late stages of smoltification (e.g. Houston, 1959; Primmatt *et al.*, 1988; Madsen and Naamansen, 1989). Even though active ion excretory mechanisms are possibly held partially inactive when the fish is still in FW, the question remains whether the fish needs compensatory mechanisms to balance an increased ion efflux observed during the late stages of smoltification in FW. Mobilization of gill V-H⁺-ATPase may well be an important mechanism to counter increased ion loss during the development of a more leaky epithelium in the gills of smolting FW salmonids.

The aim of this study was to investigate simultaneous changes in gill V-H⁺-ATPase B, Na⁺,K⁺-ATPase α_1 and β_1 subunit mRNA's in smolting *S. salar* and hence to provide us with clues as to whether relative changes in expression of these two ion pumps may be important to ultimate smolt development.

MATERIALS AND METHODS

Fish

One hundred and eighty immature upper-mode Atlantic salmon,

Salmo salar, parr (1 year old, >13 cm in length, mixed sex, first generation hatchery fish of the Irish Burrishoole River stock) were obtained in January 1997 from the Foslaks Hatchery (Randers, Denmark) where they had been hatched and reared in in-door tanks under simulated natural photoperiod and water temperature (minimum temperatures during winter 4°C). The fish were brought to the Odense University Campus and held in outdoor 500-l flow-through freshwater (FW) tanks supplied with Odense tap water (1.4 mM Cl⁻, 1.5 mM SO₄²⁻, 1.5 mM Na⁺, 0.16 mM K⁺, 3 mM Ca²⁺, 0.6 mM Mg²⁺, pH 8.3 and total CO₂ content of 5.5 mM). They were fed a 2% (body weight)⁻¹ diet of commercial trout pellets three times a week.

Sampling

Smolt development was assessed by regular 2–3 week interval samplings of fish from February through June (Table 1). On each date, two groups of fish were sampled: a group of 10 fish from the FW tank and a group of 10 fish which had been exposed to SW for 24 hr in order to assess SW-tolerance. Feeding was stopped four days prior to each sampling. Twenty-four hours prior to sampling, 10 randomly selected fish were transferred from the FW stock tank to a 35 ppt SW tank (400-l; 10°C; 12 hr light :12 hr dark cycle). The fish was stunned by a blow to the head. Blood was drawn from the caudal vessels into heparinized syringes, and the plasma was immediately separated by centrifugation at 5000×g for 3 min. The length and weight of the fish were measured. The fish was then decapitated, and additional sampling occurred as outlined below.

From 4 FW-fish per group one 1st, two 3rd, and two 4th gill arches were pooled and immediately homogenized in 2.5 ml ice-cold denaturing solution for subsequent mRNA analyses (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M β -mercaptoethanol, and 0.3% antifoam, pH 7.0).

From all FW- and SW-challenged fish one 2nd gill arch was dissected and placed in SEI-buffer for subsequent Na⁺,K⁺-ATPase activity analysis (300 mM sucrose, 20 mM EDTA, 50 mM imidazole, pH 7.3). All samples were immediately frozen in liquid nitrogen and stored at –80°C until analyzed. A piece of paraxial muscle was dissected and immediately weighed and dried to constant weight to determine total water content.

Cloning of gill ion transporters

The overall cloning procedure followed the description by Cutler *et al.* (1995b) as modified by Cutler *et al.* (1997). In short, total RNA was extracted from gills of FW-acclimated *S. salar*. First-strand cDNA synthesis from 5 μ g total RNA was done using Superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA) for 5 h at 45°C. Using degenerate primer pairs (Table 2), cDNA fragments of the gill Na⁺,K⁺-ATPase α and β subunits and the vacuolar-type H⁺-ATPase B subunit were amplified by Polymerase Chain Reaction (PCR). Forty

Table 1. Mean daily water temperature \pm SD since the previous sampling, mean fork length \pm SD of FW and SW-challenged salmon, mean body weight \pm SD of FW salmon, and condition factor ($100 \times \text{weight}^* \text{length}^{-3}$) \pm SD of FW salmon.

Date	5 Feb	26 Feb	12 Mar	25 Mar	9 Apr	22 Apr	5 May	25 May	9 Jun
Temp. (°C)	5.9 *	7.5	8.4	4.7	6.2	5.7	9.8	11.1	12.8
SD	0.95	1.24	0.92	1.43	1.54	1.45	1.38	1.30	1.50
Length (cm)	13.3	13.8	13.9	13.7	14.1	14.3	14.3	14.7	15.5
SD	0.72	0.61	0.70	0.70	0.78	0.83	0.57	0.56	0.74
Weight (g)	25.4	28.7	28.9	27.7	28.7	33.3	27.0	30.8	35.2
SD	4.44	1.84	5.94	3.25	3.82	7.53	3.64	3.17	5.75
Cond.-factor	1.01	1.05	1.06	1.03	1.00	1.07	0.96	1.01	0.93
SD	0.046	0.048	0.044	0.057	0.034	0.059	0.035	0.038	0.068

*) mean daily water temperature from start of experiment at January 17 until February 5. N equals 20 for length, 10 for weight measurements, and 10 for condition factor numbers.

Table 2. The three pairs of degenerate primers used during the PCR amplification of the Atlantic salmon Na⁺,K⁺-ATPase α subunit, Na⁺,K⁺-ATPase β subunit, and the vacuolar-type H⁺-ATPase B subunit cDNA fragments.

Na⁺,K⁺-ATPase α subunit	
Sense (aa: 85–92)	5' AC ^I /cCC ^I /cGA ^A /gTGG ^A /gT ^I /cAA ^A /gTT ^I /cTG 3'
Antisense (aa: 372–381)	5' GT ^I /cA ^A /g ^I /cGT ^I /cCC ^I /cGT ^I /cTT ^A /gTC ^I /cGA ^A /gCA ^I /cAT 3'
Na⁺,K⁺-ATPase β subunit	
Sense (aa: 173–182)	5' ^A /cA ^A /gCC ^I /cTG ^I /cIT ^I /c ^A /T ^I /c ^A /gT ^I /cAA ^A /g ^A /T ^I /cAA ^A /g ^A /cG 3'
Antisense (aa: 241–250)	5' AC ^I /c ^A /g ^I /c ^A /T ^I /T ^I /g ^I /c ^T /cCC ^A /gTA ^A /gTA ^I /gGG ^A /g ^A /T ^A /gTA 3'
V-type H⁺-ATPase B subunit	
Sense (aa: 195–203)	5' GC ^I /cGC ^I /cGG ^I /c ^T /cCC ^I /cCA ^T /cAA ^T /cGA ^A /gA 3'
Antisense (aa: 357–365)	5' GG ^A /gTG ^I /cGT ^I /cAT ^A /gTC ^A /gTC ^A /gTT ^I /cGGCA 3'

The amino acid (aa) number noted for each primer represents the position of the primer within the amino acid sequence of the European eel α , Na⁺,K⁺-ATPase (Cutler *et al.* 1995a), the eel β , Na⁺,K⁺-ATPase (Cutler *et al.* 1995b), and the eel vacuolar-type H⁺-ATPase B subunits (Niederstätter and Pelster, 2000). Inosine nucleotides (I) are marked in bold type.

PCR cycles were performed. Each PCR cycle consisted of 5 s denaturation at 92°C, 30 s annealing at 51–60°C, followed by 60 s primer-extension at 72°C. The primer-pairs used were designed as degenerate primers, the sequences of which were taken from two regions of amino acids which were identical between all published vertebrate sequences of the Na⁺,K⁺-ATPase α and β subunits, and the V-H⁺-ATPase B (56 kDa) subunit, respectively. All degenerate primers had inosine/cytosine wobbles incorporated at positions of nucleotide uncertainty as previously described by Cutler *et al.* (1995b). Positive fragments were purified from agarose gels with a GeneClean kit (Bio101, CA, USA), ligated into a pCR-Blunt II-TOPO vector using the Zero Blunt TOPO PCR Cloning kit (Invitrogen, CA, USA), and sequenced by a dideoxy chain termination method using the Big Dye Terminator sequencing kit (Perkin Elmer, CA, USA). The sequences of 3 clones from each individual fragment were compared using GeneJockey II software (Premier Biosoft Int., CA, USA) to give the precise sequences shown in Figs. 1 and 2. Comparison to known DNA sequences were performed using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/blast/>).

Analyses

Plasma [Cl⁻] was measured by coulometric titration (Radiometer CMT 10, Copenhagen, Denmark). Specific Na⁺,K⁺-ATPase activity was analyzed at 25°C in crude homogenates of gill by the method of McCormick (1993) using a plate reader (Spectramax, Molecular Devices, Sunnyvale, CA, USA). Protein content was measured by the method of Lowry *et al.* (1951) modified for plate reader.

For Northern blotting, total RNA was isolated as described by Madsen *et al.* (1995). Total RNA (20 μ g) from the gills of 4 FW-salmon from each of the 9 sampling dates were analyzed by formaldehyde gel electrophoresis on the same gel and transferred by capillary blotting onto a nylon membrane (Zeta probe, Bio-Rad, Hercules, CA, USA). The Northern blot membrane was pre-hybridized for 4 hr at 47°C in 10 ml of pre-hybridization buffer containing 50% deionized formamide, 5 \times SSC (0.75 M NaCl, 75 mM Na₃citrate, pH 7.0), 1% SDS (sodium dodecyl sulphate), 5 \times Denhardt's (0.1% Ficoll, 0.1% polyvinyl pyrrolidone, 0.1% bovine serum albumin), 0.1% NaP₂P₄ (tetrasodium pyrophosphate), 1 mM EDTA (ethylenediamine tetraacetic acid), 5 mg denatured calf thymus DNA, and 2 mg denatured yeast transfer RNA. Complementary DNA probes were radio-labelled (α ³²P-dCTP) by random primer extension (Oligolabelling kit, Pharmacia, Uppsala, Sweden) and separated from the unincorporated nucleotides on a G-50 micro column (ProbeQuant™, Pharmacia), denatured, and added to the prehybridization-buffer, and hybridized for 16 hr. Radioactivity was detected by phosphor imaging (Storm, Molecular Dynamics, Sunnyvale, CA, USA), and relative band intensities were analyzed by the ImageQuaNT 4.1 software

(Molecular Dynamics). The membrane was stripped and reprobed using the specific cloned fragments amplified by PCR. To adjust for unequal loading, data are presented as the ratio of specific mRNA of interest to β -actin mRNA content. The approximate sizes of hybridization bands were evaluated by including a 0.24–9.5 kb RNA ladder (Gibco BRL).

Statistics

Statistical differences were analyzed using SYSTAT 5.03 (Systat, 1991, Evanston, IL, USA). When necessary, square root- or log-transformations of data were performed to meet the parametric ANOVA assumption of homogeneity of variances (evaluated by residual-plots). For each individual parameter, data were analyzed by one-way ANOVA. Differences among individual groups were analyzed by Tukey's Honestly Significant Difference Test. A significance level of $\alpha = 0.05$ was used.

RESULTS

Cloning and sequencing of gill ion transporter cDNA fragments

The PCR-amplifications on Atlantic salmon gill tissue cDNA using the degenerate primers shown in Table 2 resulted in single cDNA fragments with the predicted sizes: i) Na⁺,K⁺-ATPase α ₁ subunit app. 890 base pairs (bp), ii) Na⁺,K⁺-ATPase β ₁ subunit app. 233 bp, and iii) V-H⁺-ATPase B subunit app. 510 bp. Three clones of each cDNA fragment were completely sequenced on both strands and compared to obtain the Taq-polymerase error-free sequences provided in Figs. 1 and 2.

Size estimation of specific gill ion transporter mRNAs

The sizes of the prominent mRNAs of the three ion transporter subunits were: 3.8 kb (Na⁺,K⁺-ATPase α ₁ subunit, Fig. 3A), 2.4 kb (Na⁺,K⁺-ATPase β ₁ subunit, Fig. 3B), and 3.0 kb (V-H⁺-ATPase B subunit, Fig. 3C). The intensity of the hybridization signal of all mRNA species increased with increasing amount of total RNA loaded: 1, 5, or 20 μ g total gill RNA.

Smolt development

The salmon showed clear signs of smoltification during the course of this study. There was a markedly improved salinity tolerance during the spring as judged by decreased deflection in plasma [Cl⁻] and muscle water content when chal-

A: Na⁺,K⁺-ATPase α -subunit

1	Arg	Gln	Leu	Phe	Gly	Gly	Phe	Ser	Met	Leu	Leu	Trp	Ile	Gly	Ala	15
2	AGG	CAG	CTC	TTT	GGT	GGG	TTC	TCT	ATG	CTC	CTA	TGG	ATT	GGT	GCT	
	Met	Leu	Cys	Phe	Leu	Ala	Tyr	Gly	Ile	Gln	Ala	Ala	Ser	Glu	Asp	30
47	ATG	CTC	TGC	TTC	CTG	GCC	TAC	GGA	ATC	CAG	GCC	GCC	TCC	GAG	GAT	
	Glu	Pro	Ala	Asn	Asp	Asn	Leu	Tyr	Leu	Gly	Val	Val	Leu	Ser	Val	45
92	GAG	CCG	GCC	AAT	GAT	AAT	TTG	TAC	CTG	GGG	GTT	GTG	CTC	TCT	GTT	
	Val	Val	Ile	Val	Thr	Gly	Cys	Phe	Ser	Tyr	Tyr	Gln	Glu	Ala	Lys	60
137	GTT	GTC	ATF	GTT	ACT	GGC	TGT	TTC	TCC	TAC	TAC	CAA	GAG	GCC	AAG	
	Ser	Ser	Lys	Ile	Met	Asp	Ser	Phe	Lys	Asn	Leu	Val	Pro	Gln	Gln	75
182	AGC	TCA	AAG	ATC	ATG	GAC	TCC	TTC	AAG	AAC	CTG	GTC	CCA	CAG	CAA	
	Ala	Leu	Val	Val	Arg	Asp	Gly	Glu	Lys	Lys	Asn	Ile	Asn	Ala	Glu	90
227	GCC	CTT	GTT	GTC	CGT	GAT	GGT	GAG	AAG	AAG	AAC	ATC	AAC	GCT	GAA	
	Glu	Val	Val	Val	Gly	Asp	Leu	Val	Glu	Val	Lys	Gly	Gly	Asp	Arg	105
272	GAA	GTG	GTG	GTT	GGA	GAT	CTG	GTG	GAG	GTG	AAA	GGA	GAT	AGA		
	Ile	Pro	Ala	Asp	Leu	Arg	Ile	Val	Ser	Ala	Ser	Gly	Cys	Lys	Val	120
317	ATC	CCA	GCT	GAT	TTG	CGT	ATT	GTC	TCT	GCC	AGC	GGC	TGC	AAG	GTG	
	Asp	Asn	Ser	Ser	Leu	Thr	Gly	Glu	Ser	Glu	Pro	Gln	Thr	Arg	Thr	135
362	GAC	AAC	TCC	TCC	CTC	ACT	GGT	GAA	TCT	GAG	CCC	CAG	ACA	CGT	ACT	
	Pro	Asp	Phe	Ser	Asn	Asp	Asn	Pro	Leu	Glu	Thr	Arg	Asn	Ile	Ala	150
407	CCG	GAT	TTC	TCC	AAT	GAC	AAC	CCC	CTG	GAG	ACA	AGG	AAC	ATT	GCC	
	Phe	Phe	Ser	Thr	Asn	Cys	Val	Glu	Gly	Thr	Ala	Arg	Gly	Ile	Val	165
452	TTC	TTC	TCT	ACC	AAC	TGT	GTT	GAA	GGA	ACT	GCC	AGA	GGT	ATC	GTC	
	Ile	Asn	Thr	Gly	Asp	His	Thr	Val	Met	Gly	Arg	Ile	Ala	Thr	Leu	180
497	ATC	AAC	ACT	GGT	GAC	CAC	ACT	GTC	ATG	GGT	CGT	ATT	GCC	ACC	TTG	
	Ala	Thr	Ser	Leu	Glu	Gly	Gly	Lys	Thr	Pro	Ile	Ala	Lys	Glu	Ile	195
542	GCC	ACG	AGT	CTT	GAG	GGT	GGG	AAG	ACG	CCT	ATA	GCC	AAA	GAG	ATT	
	Glu	His	Phe	Ile	His	Ile	Ile	Thr	Gly	Val	Ala	Val	Phe	Leu	Gly	210
587	GAG	CAC	TTT	ATC	CAC	ATC	ATC	ACC	GGT	GTG	GCC	GTC	TTC	CTG	GGC	
	Val	Ser	Phe	Phe	Val	Leu	Ser	Leu	Ile	Leu	Gly	Tyr	Gly	Trp	Leu	225
632	GTG	TCT	TTC	TTC	GTC	CTC	TCC	CTC	ATT	CTG	GGA	TAT	GGT	TGG	CTA	
	Glu	Ala	Val	Ile	Phe	Leu	Ile	Gly	Ile	Ile	Val	Ala	Asn	Val	Pro	240
677	GAA	GCT	GTC	ATC	TTC	CTC	ATT	GGA	ATC	ATC	GTT	GCT	AAT	GTG	CCA	
	Glu	Gly	Leu	Leu	Ala	Thr	Val	Thr	Val	Cys	Leu	Thr	Leu	Thr	Ala	255
722	GAG	GGT	CTC	CTG	GCT	ACT	GTG	ACT	GTG	TGT	CTA	ACT	CTG	ACT	GCC	
	Lys	Arg	Met	Ala	Lys	Lys	Asn	Cys	Leu	Val	Lys	Asn	Leu	Glu	Ala	270
767	AAG	CGT	ATG	GCC	AAG	AAG	AAC	TGC	CTG	GTG	AAG	AAT	CTG	GAA	GCT	
	Val	Glu	Thr	Leu	Gly	Ser	Thr	Ser	Thr							279
812	GTT	GAG	ACC	CTG	GGG	TCC	ACC	TCC	ACC							

B: Na⁺,K⁺-ATPase β -subunit

1	Ile	Val	Asn	Phe	Arg	Pro	Arg	Pro	Pro	Ser	Ser	Asn	Glu	Ser	Ile	15
2	ATC	GTC	AAC	TTC	AGG	CCA	AGG	CCC	CCC	AGC	TCC	AAT	GAA	AGC	ATC	
	Pro	Glu	Gly	Ala	Gln	Thr	Lys	Val	Gln	Pro	Asn	Val	Met	Pro	Ile	30
47	CCT	GAG	GGA	GCG	CAG	ACC	AAG	GTC	CAG	CCC	AAC	GTC	ATG	CCC	ATC	
	Phe	Cys	Thr	Asn	Lys	Arg	Glu	Glu	Asp	Ala	Gly	Lys	Ile	Gly	Glu	45
92	TTT	TGC	ACC	AAC	AAG	AGA	GAG	GAG	GAC	GCC	GGT	AAG	ATC	GGG	GAG	
	Val	Lys	Tyr	Tyr	Gly	Ile	Gly	Glu	Gly	Phe	Pro	Leu	Gln			58
137	GTG	AAG	TAC	TAC	GGC	ATT	GGG	GAA	GGT	TTC	CCC	CTC	CAG			

Fig. 1. Nucleotide and deduced amino acid sequences of the Atlantic salmon gill Na⁺,K⁺-ATPase α subunit (A) and Na⁺,K⁺-ATPase β subunit (B) cDNA fragments. The cDNA fragments were amplified by PCR using the degenerate primers provided in Table 2. Nucleotides are numbered from 1 on the left hand side, amino acids from 1 on the right hand side. The cDNA sequences have been submitted to the EMBL Data Library under the accession numbers: Na⁺,K⁺-ATPase α subunit (AJ250809) and Na⁺,K⁺-ATPase β subunit (AJ250810).

lenged with SW from February through April (Fig. 4). Optimal performance in the SW-test was seen in May. The improved hypo-osmoregulatory ability coincided with a steady increase in gill Na⁺,K⁺-ATPase activity from February until May (Fig. 5). This was followed by an abrupt decrease in both enzyme

activity and salinity tolerance in the salmon sampled in June. The salmon thus showed typical physiological smolt development in April-May.

Vacuolar-type H⁺-ATPase B-subunit

1	Ala Ala Gln Ile Cys Arg Gln Ala Gly Leu Val Lys Lys Ser Lys	TT
3	GCT GCC CAG ATT TGT CGT CAG GCT GGC CTG GTG AAG AAA TCC AAG	15
	Asp Val Met Asp Tyr Ser Asp Asp Asn Phe Ala Ile Val Phe Ala	30
48	GAT GTG ATG GAC TAC AGC GAC GAT AAC TTT GCC ATT GTC TTT GCT	
	Ala Met Gly Val Asn Met Glu Thr Ala Arg Phe Phe Lys Ser Asp	45
93	GCC ATG GGG GTG AAC ATG GAA ACT GCT CGC TTC TTC AAG TCG GAC	
	Phe Glu Glu Asn Gly Ser Met Asp Asn Val Cys Leu Phe Leu Asn	60
138	TTT GAG GAG AAT GGA TCC ATG GAC AAT GTT TGC CTG TTC TTG AAC	
	Leu Ala Asn Asp Pro Thr Ile Glu Arg Ile Ile Thr Pro Arg Leu	75
183	CTA GCC AAC GAC CCC ACT ATT GAG CGC ATC ATC ACC CCT CGC CTG	
	Ala Leu Thr Ser Ala Glu Tyr Leu Ala Tyr Gln Cys Glu Lys His	90
228	GCT CTG ACC TCA GCT GAG TAC CTG GCC TAC CAG TGT GAG AAG CAT	
	Val Leu Val Ile Leu Thr Asp Met Ser Ser Tyr Ala Glu Ala Leu	105
273	GTC CTG GTC ATC CTG ACT GAC ATG AGC TCC TAC GCC GAA GCT CTG	
	Arg Glu Val Ser Ala Ala Arg Glu Glu Val Pro Gly Arg Arg Gly	120
318	AGA GAG GTG TCT GCT GCC AGA GAG GAG GTG CCT GGT CGT CGT GGT	
	Phe Pro Gly Tyr Met Tyr Thr Asp Leu Ala Thr Ile Tyr Glu Arg	135
363	TTC CCC GGT TAC ATG TAC ACT GAT CTG GCC ACC ATC TAC GAG CGT	
	Ala Gly Arg Val Glu Gly Arg Asn Gly Ser Ile Thr Gln Ile Pro	150
408	GCC GGG AGA GTG GAG GGC AGG AAC GGC TCC ATC ACT CAG ATC CCC	
	Ile Leu Thr	153
453	ATC CTC ACC A	

Fig. 2. Nucleotide and deduced amino acid sequence of the Atlantic salmon gill vacuolar-type H⁺-ATPase B subunit cDNA fragment. The cDNA fragment were amplified by PCR using the degenerate primers provided in Table 2. Nucleotides are numbered from 1 on the left hand side, amino acids from 1 on the right hand side. The cDNA sequence has been submitted to the EMBL Data Library under the accession number: AJ250811.

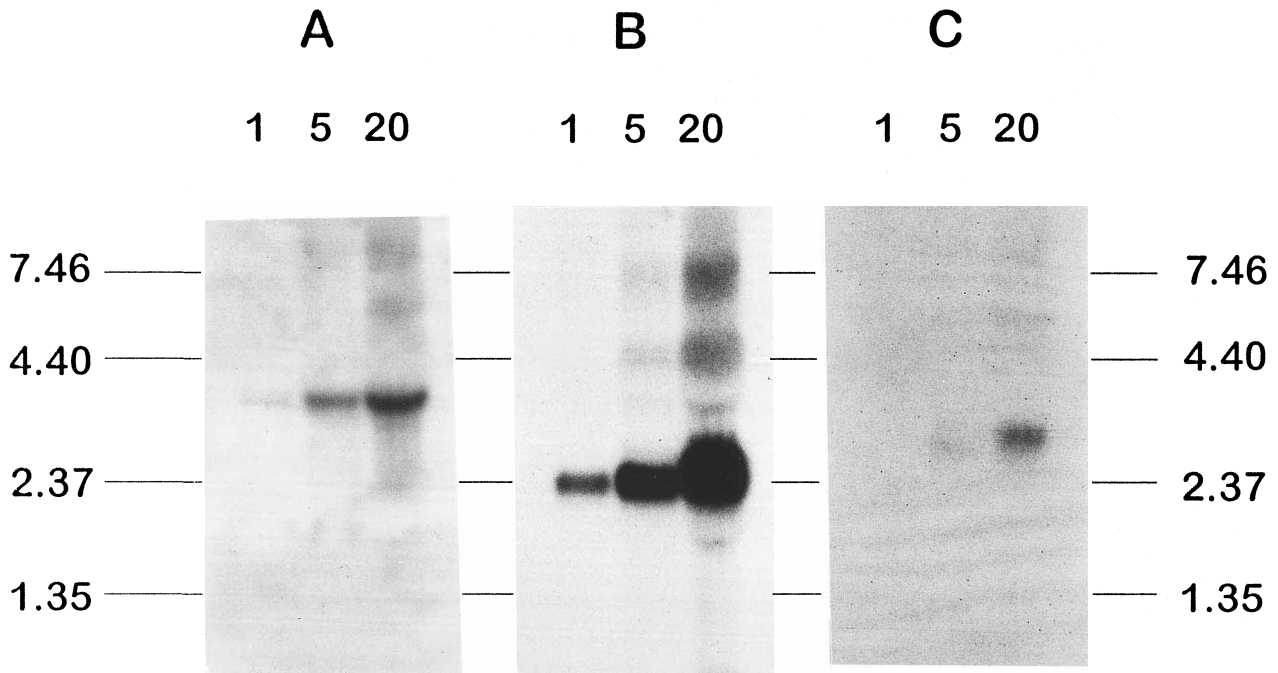


Fig. 3. Northern Blots of 1, 5, and 20 µg of total RNA extracted from the gills of a FW-acclimated Atlantic salmon pre-smolt. The same blot was hybridized with a radio-labelled cloned cDNA fragment of the a) Na⁺,K⁺-ATPase α subunit, b) Na⁺,K⁺-ATPase β subunit, or c) the Vacuolar-type H⁺-ATPase B subunit (see Figs. 1 and 2). Autoradiographic films were exposed for 4 days at -80°C. Molecular size markers (kb) are indicated. The estimated sizes of the specific mRNA's were: a) 3.8 kb, b) 2.4 kb, and c) 3.0 kb, respectively.

Gill ion transporter mRNA levels

Gill Na⁺,K⁺-ATPase α₁ and β₁ subunit mRNA levels both increased during the spring reaching their maximum values

by April (α₁ subunit 2.3-fold and β₁ subunit 1.8-fold the values in early February, Fig. 6A,B). In June, both mRNA species were back to the low levels observed in February. The α₁ and

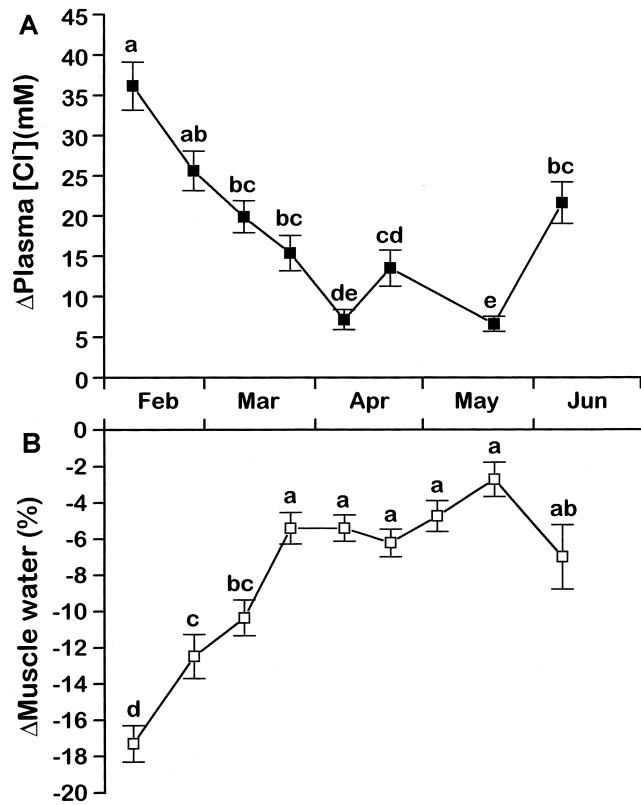


Fig. 4. Deflections in plasma $[Cl^-]$ (A) and muscle water (B) in 24-hr 35 ppt SW-challenged Atlantic salmon during smoltification. Data are shown as mean difference \pm SEM of 10 SW-challenged salmon to the mean of 10 FW-fish. Values with shared letters are not significantly different ($P > 0.05$).

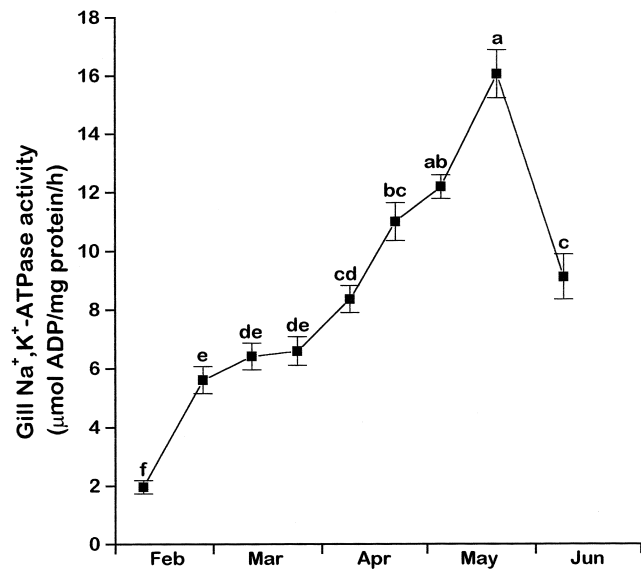


Fig. 5. Gill Na^+,K^+ -ATPase activity in Atlantic salmon during smoltification. Data are shown as mean \pm SEM of the pooled values of 10 FW- and 10 SW-challenged salmon. Values with shared letters are not significantly different ($P > 0.05$).

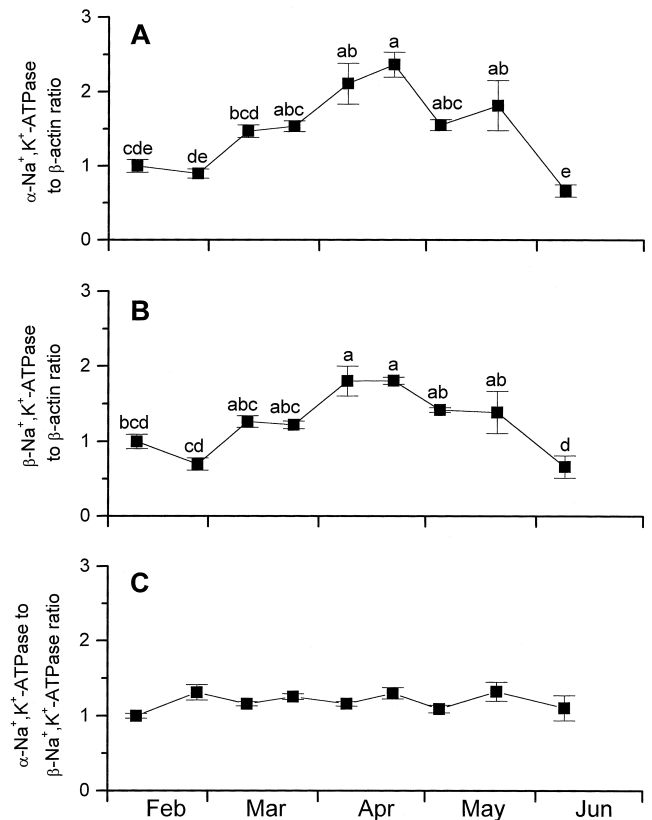


Fig. 6. Beta-actin normalized levels of gill Na^+,K^+ -ATPase α subunit (A) and Na^+,K^+ -ATPase β subunit (B) in Atlantic salmon during smoltification. In (C) the ratio of α : β subunit levels is shown. Data are shown as mean \pm SEM of 4 FW-salmon. Values with shared letters are not significantly different ($P > 0.05$). The Na^+,K^+ -ATPase α subunit to Na^+,K^+ -ATPase β subunit ratio did not change during smoltification (C, $P > 0.13$).

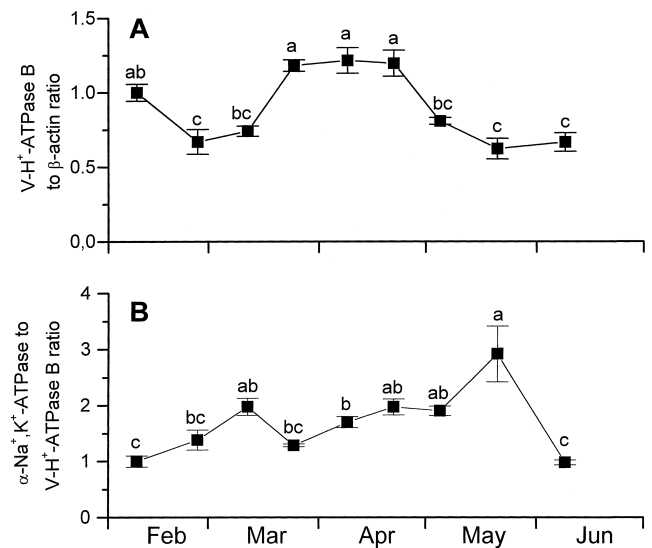


Fig. 7. Beta-actin normalized levels of gill vacuolar-type H^+ -ATPase B subunit mRNA (A) in Atlantic salmon through smoltification. In (B) the ratio of Na^+,K^+ -ATPase α subunit to vacuolar-type H^+ -ATPase B subunit mRNA ratio is shown. Data are shown as mean \pm SEM of 4 FW-salmon. Values with shared letters are not significantly different ($P > 0.05$).

β subunit mRNA levels changed similarly during smoltification, as indicated by the stable ratio between levels of the two mRNA species (Fig. 6C; linear correlation analysis using individual paired values: $R=0.92$).

Gill V-H⁺-ATPase B subunit mRNA levels changed significantly during smoltification (Fig. 7A). Transcript levels were high in the beginning of February followed by low levels in late February–early March. It then increased 2-fold to reach a plateau by late March–April. The level of B subunit mRNA then fell to low levels through May and June.

Gill Na⁺,K⁺-ATPase subunit mRNAs and V-H⁺-ATPase B subunit mRNA levels did not change in parallel through smoltification, i.e. the ratios varied (Fig. 7B; data for the Na⁺,K⁺-ATPase β_1 subunit : V-H⁺-ATPase B subunit mRNA ratio not shown). The ratio of Na⁺,K⁺-ATPase α_1 subunit and β_1 subunit, respectively, to V-H⁺-ATPase B subunit mRNA levels increased through April and May. This was followed by a sharp decline to pre-smolt values in June.

DISCUSSION

This is the first paper to report on simultaneous changes in gill Na⁺,K⁺-ATPase α and β subunits and V-type H⁺-ATPase B subunit mRNA expression during the complete cycle of salmonid smoltification.

Gill Na⁺,K⁺-ATPase α and β subunit cDNA fragments

The α Na⁺,K⁺-ATPase cDNA fragment cloned in this study is most likely an α_1 isoform based on the high identity of the deduced amino acid sequence with other cloned teleost α_1 -subunits: 81% with the α_1 Na⁺,K⁺-ATPase of white sucker (Schönrock *et al.*, 1991) and 85% with European eel (Cutler *et al.*, 1995a). The size of the prominent α subunit mRNA species in the Atlantic salmon gill (3.8 kb) is similar to those reported earlier in gills of Atlantic salmon using either rainbow trout (D'Cotta *et al.*, 1996, 2000) or *Xenopus* (Madsen *et al.*, 1997) α subunit cDNA probes. Apart from a major 3.7 kb α subunit transcript, D'Cotta *et al.* (1996) also reported a minor transcript of 1.8 kb. We did not observe a similar size transcript. The existence of such a minor, possibly truncated form of the same α subunit expressed in salmon gills may, however, still be possible as the present α subunit isoform could differ from the one cloned by D'Cotta *et al.* (1996). Alternatively, the α subunits may be the same, and the truncated mRNA species was just not detectable using our cDNA probe. This is possible as the 670 bp cDNA fragment that D'Cotta *et al.* (1996) cloned, corresponds to the eel α Na⁺,K⁺-ATPase amino acids 615-826 (Cutler *et al.*, 1995a) and thus is located further towards the 3' end of the transcript than the present cDNA. The present 3.8 kb α transcript is also comparable to α subunit transcripts reported from other teleosts (e.g. white sucker: 3.8–4.15 kb, Schönrock *et al.*, 1991; rainbow trout: 3.7 kb, Kisen *et al.*, 1994; eel: 3.5 kb, Cutler *et al.*, 1995a; brown trout: 3.8 kb, Madsen *et al.*, 1995). Even though highly stringent hybridization conditions were used, there is a slight possibility that additional (similar sized) isoforms were detected

by the present cDNA. In future studies it is crucial that multiple isoform expression is carefully investigated using isoform specific clones.

The cloned β Na⁺,K⁺-ATPase cDNA fragment is most likely a β_1 isoform as the deduced amino acid sequence shares 79% identity with European eel β_1 Na⁺,K⁺-ATPase sequence (Cutler *et al.*, 1995b) and because the last 5 amino acids (Gly, Phe, Pro, Leu, Gln) in the deduced sequence are conserved in β_1 isoforms from different species but not in other β Na⁺,K⁺-ATPase isoforms. Identities with β_1 isoforms of other vertebrate species are low though similar to that generally shared among β_1 orthologs (e.g. 43%: *Torpedo californica*: Noguchi *et al.*, 1986; 48% *Homo sapiens*: Kawakami *et al.*, 1986). The size of the prominent β_1 subunit mRNA species in the Atlantic salmon gill (2.4 kb) is similar to the single transcript sizes reported for both the β_1 isoform (eel gills: 2.35 kb; Cutler *et al.*, 1995b) and for the brain-specific β_3 isoform (zebrafish and eel: 2.4 kb; Appel *et al.*, 1996; Cutler *et al.*, 1997).

Gill V-H⁺-ATPase 56 kDa B subunit cDNA fragment

The cloned V-H⁺-ATPase B subunit cDNA fragment is from the middle region of the coding sequence which is the most conserved between species and subunit isoforms (Nelson *et al.*, 1992). The deduced 153 amino acid sequence is identical and at the nucleotide level has 97% identity with the recently cloned V-H⁺-ATPase B-subunit fragment from rainbow trout gill (Perry *et al.*, 2000). The deduced amino acid sequence also shows a very high degree of identity with V-H⁺-ATPase B subunits from other vertebrate species such as chicken (97%: Bartkiewicz *et al.*, 1995), human and bovine B2 isoform (96%: Bernasconi *et al.*, 1990; Nelson *et al.*, 1992), and human and bovine B1 isoform (94%: Sühof *et al.*, 1989; Nelson *et al.*, 1992).

The size of the B subunit mRNA species detected in the gills of Atlantic salmon is 3.0 kb which is in good accordance with the 3.0-3.2 kb found for rainbow trout gill (Perry *et al.*, 2000) and for both human and bovine B1 and B2 subunit transcripts (Sühof *et al.*, 1989; Bernasconi *et al.*, 1990; Nelson *et al.*, 1992). Interestingly, Niederstätter and Pelster (2000) recently cloned both a V-H⁺-ATPase B1 and a B2 isoform from *A. anguilla* swimbladder gas gland which shared 97 and 98% identity, respectively, with the fragment cloned from *S. Salar* in the present study. The eel gas gland B1 transcript was 2.9 kb and the B2 transcript was 3.5 kb in size. In the present study, all the isolated plasmid clones had identical inserts, and we detected only one size mRNA species. Although the sequence identity suggests that the salmonid gill V-H⁺-ATPase B-subunit is a B2 isoform (Perry *et al.*, 2000; this study), Niederstätter and Pelster (2000) based on transcript size, concluded that the B-subunit isoform isolated from the gills of *O. mykiss* most likely is a B1 isoform. Furthermore, 2 poly A signals were shown to exist in the *A. anguilla* B2 gene although only the second poly A site seemed to be in use. In contrast, using low-stringency hybridization, Puopolo *et al.* (1992) detected 2 transcripts of the sizes 2.0 and 3.2 kb in bovine tissues using a cDNA fragment of the B2 subunit

isoform. Similarly, two transcripts with sizes of 1.7 and 3.5 kb were observed in chicken tissues (Bartkiewicz *et al.*, 1995). The high-stringency hybridization procedure used in the present study prevented the cross-hybridization of the specific probe to any possible isoform gene product of different size or differentially processed product of the same gene, suggesting that only one V-H⁺-ATPase B-subunit gene is expressed in the gills of *S. salar*.

Gill Na⁺,K⁺-ATPase expression during smoltification

The most characteristic biochemical and physiological signs of salmonid smoltification are the spring surge in gill Na⁺,K⁺-ATPase activity and the associated increase in SW-tolerance (cf. introduction). In the present study, the smolt climax was reached in April-May, after which the process of de-smoltification was abruptly initiated (Figs. 4A,B and 5). The data suggest that the increased Na⁺,K⁺-ATPase activity (i.e. pump abundance when measured under the present conditions of V_{max}) at least in part is caused by the simultaneous increase of both α and β subunit transcripts in the gill through the course of smoltification (Fig. 6A, B). There is an overall picture that mRNA expression and enzyme activity change in parallel. However, there is one exception. The almost 3-fold increase in Na⁺,K⁺-ATPase activity in late February occurs without any detectable change in mRNA levels. Thus, there is not a consistent 1:1 relationship between changes in subunit mRNA expression and enzyme activity. Uncoupling between α subunit mRNA levels and overall Na⁺,K⁺-ATPase activity during smoltification is also evident in previous studies of smolting Atlantic salmon (D'Cotta *et al.*, 1996, 2000) and brown trout (Nielsen *et al.*, 1999). This is not surprising, since additional factors such as translational efficiency/rate, and post-translational processing and protein stability may affect functional enzyme assembly maturation or degradation. Further, there is a possibility that additional isoforms which are not detected by the Northern cDNA probes may contribute to enzymatic activity. The very abrupt decrease in enzyme mRNA and activity at the onset of de-smoltification is, however, characterized by a strong parallelism indicating that mRNA expression is one tool of regulating synthesis and abundance of protein. During FW- to SW-acclimation of euryhaline teleosts, similar increases to the ones seen during salmon smoltification have been observed at the level of both Na⁺,K⁺-ATPase α (Cutler *et al.*, 1995a; Kisen *et al.*, 1994; Madsen *et al.*, 1995) and β subunit (Cutler *et al.*, 1995b) mRNA. Increased transcription of the subunit genes and/or stabilization of both subunit mRNAs thus appear to be a common part of the molecular mechanism of Na⁺,K⁺-ATPase mobilization during smoltification and FW- to SW-acclimation.

Nielsen *et al.* (1999) speculated that one mechanism of controlling functional enzyme synthesis during smoltification could occur at the level of differential synthesis of α and β subunits. The present study provides evidence that such regulation does not occur in Atlantic salmon at least at the level of mRNA expression, as these were regulated similarly during smoltification (Fig. 6C). Such highly coordinated synthesis

seems to be the typical case in mammalian systems also (review by Geering, 1990), even though cases of non-coordinated changes in the expression ratio have also been documented (Lavoie *et al.*, 1997).

Gill V-type H⁺-ATPase expression during smoltification

Interestingly, V-H⁺-ATPase B subunit mRNA levels were increased at intermediate stages of smoltification (March-April), concurrently with increased levels of α and β subunit mRNAs. However, at the peak of smoltification (in May), when both α and β subunit mRNAs, Na⁺,K⁺-ATPase activity, and SW-tolerance reached their maximal levels, there was a simultaneous drop in H⁺-ATPase B subunit expression and hence an increase in the Na⁺,K⁺-ATPase to V-H⁺-ATPase B-subunit mRNA ratio (Fig. 7A,B). There is increasing evidence that V-H⁺-ATPase plays an important role for branchial Na⁺ uptake in FW-teleosts (cf. Introduction). Recent *in vivo* experiments using the specific V-H⁺-ATPase activity inhibitor Bafilomycin A (Fenwick *et al.*, 1999) strongly suggests that the original model by Krogh (1938), including an apical entry of Na⁺ via Na⁺/H⁺ (NH₄⁺) should be replaced by the model proposed by Avella and Bornancin (1989) in which apical Na⁺ entry occurs through Na⁺ channels, driven by active pumping of protons from the epithelial cell cytosol into the surrounding medium. Since its first purification from vacuolar membranes of yeast (Kakinuma *et al.*, 1981), the vacuolar-type H⁺-ATPase has been shown also to be the proton pump located in the apical membrane of many absorptive epithelia (e.g. mammalian kidney: Brown *et al.*, 1988; osteoclast bone resorptive cells: Blair *et al.*, 1989; frog skin: Klein *et al.*, 1997) including the fish gill epithelium (Lin *et al.*, 1994). In these tight epithelia, the V-H⁺-ATPase is targeted to the apical plasma membrane (Nelson *et al.*, 1992; Lin *et al.*, 1994). Thus the present changes in B subunit mRNA levels are likely to translate into changes in expression of the B subunit protein, which makes up part of the V₁ cytosolic domain of the plasma membrane V-H⁺-ATPase (review by Forgac, 1998). Mature proton pumps hence are inserted directly into the gill apical plasma membrane or in cytoplasmic vesicles which subsequently may fuse with the apical plasma membrane, as seen in catfish (*Ictalurus nebulosus*) experiencing a hypercapnic acidosis (Laurent *et al.*, 1994).

The gradual build-up of gill Na⁺,K⁺-ATPase activity and hypo-osmoregulatory ability may be viewed as a preparation to encounter the marine environment. Thus, the developing smolt should be considered "a SW-fish residing in FW", and there is evidence that this is indeed a functional implication of the physiological transformation. Concurrent with the development of SW-type chloride cell-accessory cell complexes, the gill epithelium gradually becomes more leaky, and the developing smolt enters a state of negative sodium balance (Primmitt *et al.*, 1988) with an associated decline in plasma ion levels (e.g. Houston, 1959; Madsen and Naamansen, 1989). This indicates that a gradual mal-adaptation to the FW environment may take place as a consequence of smolt development. Providing that the messenger is translated into

functional protein, one plausible explanation for the present high expression of V-H⁺-ATPase during smoltification is, that it is needed in order to counteract excess salt loss while in FW. This hypothesis is in accordance with the elevated unidirectional branchial Na⁺ influx observed during smoltification in Atlantic salmon (Primmitt *et al.*, 1988). The H⁺-ATPase expression restores to pre-smolt values around the time where the peak smolt stage is reached. This is the time where the sea is normally encountered during migration, and in this way, the drop in H⁺-ATPase expression may be seen as the last in a series of preparative changes minimizing Na⁺ uptake and thus facilitating the “anticipated” SW-encounter.

Conclusion and perspectives

This study shows that the expression of gill Na⁺,K⁺-ATPase α and β subunit genes are increased to a similar extent during *S. salar* smoltification, which at least partly causes the well-known build-up of gill Na⁺,K⁺-ATPase activity levels. The expression of the gill V-H⁺-ATPase B-subunit also changes during smoltification in a manner which suggests that the V-H⁺-ATPase enzyme may be an important mechanism to secure ion-uptake during smoltification in FW until the time where the V-H⁺-ATPase expression drops and the fish becomes a “fully prepared” smolt. Alternatively, changes in V-H⁺-ATPase may be associated with changes in acid-base regulation occurring during smoltification. It has been demonstrated that V-H⁺-ATPase subunits (mRNA and protein) are expressed in teleost osmo- and acid-base regulatory epithelia such as the gill (Lin *et al.*, 1994; S *et al.*, 1995; Perry *et al.*, 2000; Wilson *et al.*, 2000; this study), kidney (Perry and Fryer, 1997; Perry *et al.*, 2000), and intestine (Perry *et al.*, 2000; M. Seidelin, unpublished), but further studies are needed to elucidate subunit expression patterns and regulation as well as functional maturation of V-H⁺-ATPase enzyme at the cellular level in fish.

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