

## Reciprocal expression of gill Na<sup>+</sup>/K<sup>+</sup>-ATPase $\alpha$ -subunit isoforms $\alpha$ 1a and $\alpha$ 1b during seawater acclimation of three salmonid fishes that vary in their salinity tolerance

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### Summary

The upregulation of gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity is considered critical for the successful acclimation of salmonid fishes to seawater. The present study examines the mRNA expression of two recently discovered  $\alpha$ -subunit isoforms of Na<sup>+</sup>/K<sup>+</sup>-ATPase ( $\alpha$ 1a and  $\alpha$ 1b) in gill during the seawater acclimation of three species of anadromous salmonids, which vary in their salinity tolerance. Levels of these Na<sup>+</sup>/K<sup>+</sup>-ATPase isoforms were compared with Na<sup>+</sup>/K<sup>+</sup>-ATPase activity and protein abundance and related to the seawater tolerance of each species. Atlantic salmon (*Salmo salar*) quickly regulated plasma Na<sup>+</sup>, Cl<sup>-</sup> and osmolality levels within 10 days of seawater exposure, whereas rainbow trout (*Oncorhynchus mykiss*) and Arctic char (*Salvelinus alpinus*) struggled to ionoregulate, and experienced greater perturbations in plasma ion levels for a longer period of time. In all three species, mRNA levels for the  $\alpha$ 1a isoform quickly

decreased following seawater exposure whereas  $\alpha$ 1b levels increased significantly. All three species displayed similar increases in gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity during seawater acclimation, with levels rising after 10 and 30 days. Freshwater Atlantic salmon gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity and protein content was threefold higher than those of Arctic char and rainbow trout, which may explain their superior seawater tolerance. The role of the  $\alpha$ 1b isoform may be of particular importance during seawater acclimation of salmonid fishes. The reciprocal expression of Na<sup>+</sup>/K<sup>+</sup>-ATPase isoforms  $\alpha$ 1a and  $\alpha$ 1b during seawater acclimation suggests they may have different roles in the gills of freshwater and marine fishes; ion uptake in freshwater fish and ion secretion in marine fishes.

Key words: Na<sup>+</sup>/K<sup>+</sup>-ATPase, salmonid fish, seawater acclimation, ion regulation, gill.

### Introduction

Many species of salmonid fishes are euryhaline, able to migrate between freshwater and marine habitats. It is generally accepted that salmonid species of the genera *Oncorhynchus* and *Salmo* have a greater capacity for seawater acclimation than members of the genus *Salvelinus* (Hoar, 1976; Hoar, 1988). This is based on observations by Rounsefell (described in Hoar, 1988) of several criteria, including the size and age of first seaward migration and the relative time each species spends in the marine environment. Studies that have determined a time course for salinity acclimation also suggest some species do acclimate more quickly to seawater. The time required for seawater acclimation ranges from as little as 12 h for chum salmon (*Oncorhynchus gorbusha*) (Black, 1951), up to 36 h for coho salmon (*Oncorhynchus kisutch*) (Miles and Smith, 1968), 96 h for Atlantic salmon (*Salmo salar*) (Prunet and Boeuf, 1985) and 4–5 days for rainbow trout (*Oncorhynchus mykiss*) (Leray et al., 1981). The total time

required for adjustment of *Salvelinus* spp. to seawater appears to be much longer (Besner and Pelletier, 1991; Arnesen et al., 1992) with several studies reporting mortalities (Roberts, 1971; Gjedrem, 1975; Staurnes et al., 1992). What determines the difference in seawater tolerance between species (or individuals) is not well understood; but it is probably related to physiological adjustments made to the gill during salinity acclimation, in particular the regulation of gill Na<sup>+</sup>/K<sup>+</sup>-ATPase.

The gill is the site of active ion uptake by freshwater teleosts and active Na<sup>+</sup> and Cl<sup>-</sup> secretion by marine teleosts (for a review, see Evans et al., 2005). Na<sup>+</sup>/K<sup>+</sup>-ATPase plays a central role in both models as it maintains Na<sup>+</sup> and K<sup>+</sup> gradients across the basolateral membrane. These gradients act as the energy source for the active movement of ions into or out of the fish. Na<sup>+</sup>/K<sup>+</sup>-ATPase is the sole energy provider in the gill active ion secretion model of marine species (Silva et al., 1977), whereas Na<sup>+</sup>/K<sup>+</sup>-ATPase appears to work in tandem with an

apical  $\text{H}^+$ -ATPase to move  $\text{Na}^+$  across the gill epithelium in the active ion uptake model of freshwater fishes (Avella and Bornancin, 1989). The route for  $\text{Na}^+$  uptake appears to be *via* an apical epithelial sodium channel (ENaC)-like channel in rainbow trout (Reid et al., 2003), but has not yet been identified in other fish species. The action of gill  $\text{Na}^+/\text{K}^+$ -ATPase is therefore critical for ion homeostasis for all teleost fishes, regardless of the environment they inhabit. The migration of euryhaline teleost fishes from freshwater to seawater requires that the gill change from an ion absorbing tissue to an ion secreting tissue. For most euryhaline fishes, this reversal of ion pumping is associated with an up regulation of gill  $\text{Na}^+/\text{K}^+$ -ATPase activity (McCormick and Saunders, 1987). We have recently discovered that four  $\alpha$ -subunit isoforms of  $\text{Na}^+/\text{K}^+$ -ATPase are expressed in rainbow trout gill (Richards et al., 2003). The  $\alpha$ -subunit of  $\text{Na}^+/\text{K}^+$ -ATPase is the catalytic portion of the pump that contains the ATP, cation and ouabain binding sites (Lingrel and Kuntzweiler, 1994). In rainbow trout gill, the  $\alpha 1a$  and  $\alpha 1b$  isoforms are found at much higher levels than the  $\alpha 1c$  and  $\alpha 3$  isoforms and are reciprocally expressed during seawater acclimation, as levels of isoform  $\alpha 1a$  quickly drop while  $\alpha 1b$  increases following seawater exposure (Richards et al., 2003). These different gill  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha$ -subunit isoforms may be independently regulated, may be localized to different types of mitochondria-rich cells within the gills, may have different kinetic properties and may be specialized to operate in environments with differing salinities. The differential regulation of these gill  $\text{Na}^+/\text{K}^+$ -ATPase isoforms may also explain why some species acclimate more quickly to seawater than others. This study examines gill  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha 1a$  and  $\alpha 1b$  isoform levels, gill  $\text{Na}^+/\text{K}^+$ -ATPase activity and protein levels during seawater acclimation of three species of salmonids that differ in their salinity tolerance. We chose to directly compare Atlantic salmon and rainbow trout, two relatively good osmoregulators, with a relatively poor osmoregulator, the Arctic char (*Salvelinus alpinus*). Our objectives were: (1) to determine if the reciprocal expression of isoforms  $\alpha 1a$  and  $\alpha 1b$  seen in rainbow trout gills following seawater exposure is also evident in other salmonid species; and (2) to determine if interspecies differences in osmoregulatory capacity can be attributed to isoform-specific expression of  $\text{Na}^+/\text{K}^+$ -ATPase. We predicted that Atlantic salmon and rainbow trout would acclimate more quickly to seawater than Arctic char and that this would be associated with more rapid and greater increases in gill  $\text{Na}^+/\text{K}^+$ -ATPase activity in salmon and trout compared with char. We also predicted that mRNA levels of the  $\alpha 1b$  isoform would increase significantly during seawater acclimation for all three species, with the increase more prominent in Atlantic salmon and rainbow trout.

## Materials and methods

### Experimental procedure

Rainbow trout (*Oncorhynchus mykiss* Walbaum) and Arctic char (*Salvelinus alpinus* L.) were obtained from the

Alma aquaculture research station (Alma, ON, Canada), and Atlantic salmon (*Salmo salar* L.) were acquired from the Ontario Ministry of Natural Resources Normandale station (Normandale, ON, Canada; a kind gift from Dr D. Bureau, University of Guelph, ON, Canada). All fish were maintained at the University of Guelph Hagen Aqualab in 2000 l circular tanks containing freshwater at 10°C for at least 8 months before the start of the experiment under a simulated natural photoperiod that mimicked light conditions at 45° latitude. Fish were fed to satiation daily during this period with trout chow pellets (Martin Feeds, Elmira, ON, Canada). Arctic char (40.0±0.8 cm, 579.8±38.5 g; means ± s.e.m.) were significantly larger than Atlantic salmon (33.2±0.4 cm, 349.4±13.2 g) and rainbow trout (34.2±0.8 cm, 379.7±18.4 g), but trout and salmon sizes were not significantly different. There were no significant differences in fish size (weight or length) between experimental groups within each species. All seawater transfers were conducted during the month of June so the acclimation period coincided with the period when all three species should be well prepared for a seaward migration. For each control and seawater-acclimated group, eight individuals were transferred to a tank either containing full strength seawater (32‰, 10°C) or to a different freshwater tank with identical lighting conditions. For each species, control groups (freshwater acclimated) were sampled 1 and 30 days following transfer. Experimental groups were sampled 1, 2, 10 and 30 days following transfer for Arctic char, 2, 4, 10 and 30 days following transfer for rainbow trout and 4, 10 and 30 days following transfer for Atlantic salmon. The timing of the seawater transfer was coordinated to ensure groups from each species with similar acclimation periods were sampled within 2 days of one another. Owing to limited availability of seawater tank space sampling periods were slightly different between species. Care was taken to ensure minimum disturbance of each group prior to sampling. Feeding was discontinued 2 days prior to transfer and were food was withheld from all fish for the first 10 days of acclimation. This was because of the initial limited appetite of all three species following seawater exposure. Feeding resumed for the 30 day groups on the 11th day of acclimation. As recovery of appetite following seawater exposure varied between the three species it was necessary to limit the food ration of all groups to match that of the group with the lowest appetite, the seawater-acclimated Arctic char. A limited ration size was determined for all groups based on the appetite of the seawater-acclimated Arctic char in an attempt to eliminate feeding as a potential conflicting variable, which may influence seawater tolerance.

At the time of sampling for each group, blood was taken by caudal puncture using a heparinized (500 U ml<sup>-1</sup> heparin) syringe and 21G needle. The fish were then killed by a blow to the head and gill samples were quickly excised, frozen in liquid nitrogen and stored at -80°C. Blood was centrifuged at 3000 g for 5 min at 4°C and plasma was removed, frozen in liquid nitrogen and stored at -80°C for future analysis.

*Measurement of plasma osmolality and ion levels*

Plasma osmolality was measured using a vapour pressure osmometer (Model 5500, Wescor, Utah, USA).  $\text{Cl}^-$  levels were measured using a chloride titrator (Model CMT10, Radiometer, Copenhagen, Denmark).  $\text{Na}^+$  levels were measured using a flame photometer (Model FLM2, Radiometer, Copenhagen, Denmark).

*Measurement of  $\text{Na}^+/\text{K}^+$ -ATPase activity*

Gill tissue was scraped from filaments with a glass slide and homogenized on ice in SEI buffer (pH 7.5; 150 mmol  $\text{l}^{-1}$  sucrose, 10 mmol  $\text{l}^{-1}$  EDTA, 50 mmol  $\text{l}^{-1}$  imidazole) using a ground glass homogenizer. Homogenates were centrifuged for 1 min (4°C) at 5000 g to remove filaments and other insoluble material. The supernatant was used directly in the assay of enzyme activity.  $\text{Na}^+/\text{K}^+$ -ATPase activity was measured spectrophotometrically using a NADH-linked assay modified from the method of Gibbs and Somero (Gibbs and Somero, 1990). ADP, formed from the hydrolysis of ATP by ATPases, was enzymatically coupled to the oxidation of reduced NADH using commercial preparations of pyruvate kinase (PK) and lactate dehydrogenase (LDH). Gill samples were assayed for ATPase activity in the presence and absence of the  $\text{Na}^+/\text{K}^+$ -ATPase-specific inhibitor ouabain (final concentration 1 mmol  $\text{l}^{-1}$ ). Samples were run in triplicate with and without ouabain and the difference in the rate of NADH oxidation (millimolar extinction coefficient  $\epsilon_{340}=6.22$ ) between the two conditions was used to calculate  $\text{Na}^+/\text{K}^+$ -ATPase activity. Optimal assay conditions to give maximal enzyme activity were: 100 mmol  $\text{l}^{-1}$  NaCl, 20 mmol  $\text{l}^{-1}$  KCl, 5 mmol  $\text{l}^{-1}$   $\text{MgCl}_2$ , 50 mmol  $\text{l}^{-1}$  imidazole, 3 mmol  $\text{l}^{-1}$  ATP, 2 mmol  $\text{l}^{-1}$  phosphoenol pyruvate, 0.2 mmol  $\text{l}^{-1}$  NADH, 4U LDH and 5 U PK, pH 7.5.  $\text{Na}^+/\text{K}^+$ -ATPase activity is expressed as  $\mu\text{mol ADP h}^{-1} \text{mg}^{-1}$  protein. Maximal  $\text{Na}^+/\text{K}^+$ -ATPase activity was measured using a Cary 50 bio diode array spectrophotometer (Varian Inc., Palo Alto, CA, USA), equipped with a thermostated cell changer maintained at 10°C with a Haake D8 circulating water bath (Haake Buchler Instruments Inc., Saddlebrook, NJ, USA).

*Measurement of  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha$  subunit mRNA levels*

Total RNA was extracted from gill samples using TriPure Isolation Reagent (Boehringer Mannheim, Laval, QC, Canada) following the guanidine thiocyanate method (Chomczynski and Sacchi, 1987). Isolated total RNA was quantified spectrophotometrically and run (2  $\mu\text{g}$ ) on an agarose gel (1%) to check for RNA integrity. First strand cDNA was synthesized from 2  $\mu\text{g}$  of total RNA using oligo(dT)<sub>15</sub> primer and RevertAid™ H Minus M-MuLV reverse transcriptase following the manufacturer's instructions (MBI Fermentas, Burlington, ON, Canada). Quantitative RT-PCR (qRT-PCR) was performed on an ABI Prism 7000 sequence analysis system (Applied Biosystems Inc., Foster City, CA, USA). PCR reactions contained 1  $\mu\text{l}$  of cDNA, 150 pmoles of each primer and Universal SYBR green master mix (Applied Biosystems

Inc., Foster City, CA, USA). Forward and reverse primers used were designed to be  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha$ -subunit specific for the  $\alpha 1a$  and  $\alpha 1b$  isoforms and for the control gene elongation factor 1 $\alpha$  (EF1 $\alpha$ ) (Richards et al., 2003). Primer sequences were as follows:  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha 1a$  forward 5' GGC CGG CGA GTC CAA T 3',  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha 1a$  reverse 5' GAG CAG CTG TCC AGG ATC CT 3' (product size 66);  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha 1b$  forward 5' CTG CTA CAT CTC AAC CAA CAA CAT T 3',  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha 1b$  reverse 5' CAC CAT CAC AGT GTT CAT TGG AT 3' (product size 81); EF1 $\alpha$  forward 5' GAG ACC CAT TGA AAA GTT CGA GAA G 3', EF1- $\alpha$  reverse 5' GCA CCC AGG CAT ACT TGA AAG 3' (product size 71). QRT-PCR reaction conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The presence of a single product was confirmed through a melt curve analysis and by running several representative samples on a 1.5% agarose gel to ensure only one band (of the appropriate size) was present. In addition, amplified product from two individuals for each species and each gene were cloned into a T-vector (pGEM T-easy; Promega; Fisher Scientific, Nepean, ON, Canada), transformed into heat-shock competent *Escherichia coli* (strain JM109; Promega; Fisher Scientific, Nepean, ON, Canada) and colonies grown on ampicillin LB-agar plates. Colonies containing the ligated PCR product were selected and grown overnight in liquid culture. Plasmids were harvested from liquid culture using GenElute Plasmid Miniprep kit (Sigma-Aldrich, Oakville, ON, Canada) and sequenced using an ABI Prism 377 DNA sequencer (Applied Biosystems Inc., Foster City, CA, USA) at the University of Guelph, Molecular Supercenter (Guelph, ON, Canada). Negative control reactions for qRT-PCR were performed with original total RNA from several representative samples to determine potential genomic DNA contamination. For all three genes genomic contamination was found to be negligible, consisting of a maximum of 1:4996 starting copies for  $\text{Na}^+/\text{K}^+$ -ATPase isoform  $\alpha 1a$ , 1:6656 starting copies for  $\text{Na}^+/\text{K}^+$ -ATPase isoform  $\alpha 1b$  and 1:4786 starting copies for EF1 $\alpha$ . Relative quantities of each target gene were determined using the comparative  $C_T$  method (Applied Biosystems Inc., 2001). The relative quantity of  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha 1a$  and  $\alpha 1b$  mRNA in gill samples was normalized to an endogenous reference (EF1 $\alpha$ ) and expressed relative to the mean value for Arctic char acclimated to freshwater (30 day control) according to the formula:

$$\text{Amount} = 2^{-\Delta\Delta\text{CT}}$$

where  $\Delta\Delta$  refers to the difference in levels between the target gene and EF1 $\alpha$  and the relative levels relative to the mean value for the freshwater (30 day control) Arctic char group, and  $C_T$  refers to the fractional cycle number at which the amplified target reaches a fixed threshold. The calculation method was validated by examining the efficiency of target and reference amplification by comparing the average threshold value for each gene at different cDNA amounts (ranging from 1  $\mu\text{l}$  to 1  $\mu\text{l}$  of a 625 $\times$  dilution)

(Applied Biosystems Inc., 2001). All samples were run in duplicate.

#### Measurement of $\text{Na}^+/\text{K}^+$ -ATPase protein levels

Gill  $\text{Na}^+/\text{K}^+$ -ATPase protein levels were measured by the method of Else and Wu (Else and Wu, 1999) by monitoring binding of [ $^3\text{H}$ ]ouabain ( $0.588 \text{ TBq mmol}^{-1}$ ; obtained from Perkin Elmer, Boston, MA, USA) to gill tissue homogenate. Briefly, gill homogenates were prepared as described for  $\text{Na}^+/\text{K}^+$ -ATPase activity measurement. Homogenates were diluted to a concentration of  $1 \text{ mg protein ml}^{-1}$  and  $15 \mu\text{l}$  added to  $250 \mu\text{l}$  of incubation medium containing ( $10 \text{ mmol l}^{-1} \text{ NaH}_2\text{PO}_4$ ,  $5 \text{ mmol l}^{-1} \text{ MgCl}_2$ ,  $5 \times 10^{-5} \text{ mol l}^{-1}$  unlabelled ouabain plus  $1.5 \times 10^{-7} \text{ mol l}^{-1}$  [ $^3\text{H}$ ]ouabain, pH 7.4) in a Millipore Ultrafree-MC 30,000 NMWL filter centrifuge tube. Parallel tubes containing the same amount of homogenate in  $250 \mu\text{l}$  of incubation medium containing ( $10 \text{ mmol l}^{-1} \text{ NaH}_2\text{PO}_4$ ,  $5 \text{ mmol l}^{-1} \text{ MgCl}_2$ ,  $10^{-2} \text{ mol l}^{-1}$  unlabelled ouabain plus  $1.5 \times 10^{-7} \text{ mol l}^{-1}$  [ $^3\text{H}$ ]ouabain, pH 7.4) were run for each sample to determine nonspecific binding (NSB). With the addition of high levels of unlabelled ouabain in the NSB tubes the specific binding of labelled ouabain (to  $\text{Na}^+/\text{K}^+$ -ATPase) would be negligible, and therefore indicates non-specific binding. Sample and NSB tubes were run in duplicate and incubated for 2.5 h at  $25^\circ\text{C}$ . Tubes were then centrifuged ( $4000 \text{ g}$  for 5 min) and  $\text{Na}^+/\text{K}^+$ -ATPase remained on the filters. Filters were washed five times with  $50 \mu\text{l}$  of wash solution ( $10 \text{ mmol l}^{-1} \text{ NaH}_2\text{PO}_4$ ,  $5 \text{ mmol l}^{-1} \text{ MgCl}_2$ , pH 7.4), allowed to dry, then removed from their tubes and placed in scintillation vials containing 15 ml of Scintisafe Econo F scintillation fluid (Fisher Scientific, Nepean, ON, Canada) and left in the dark overnight. Vials were counted using a Beckman LS 6500 multi-purpose scintillation counter (Beckman Instruments, Fullerton, CA, USA) with d.p.m. correction. Ouabain was assumed to bind to  $\text{Na}^+/\text{K}^+$ -ATPase in a 1:1 ratio and  $\text{Na}^+/\text{K}^+$ -ATPase density calculated from the radioactivity difference between sample and NSB preparations. The volume (and concentration) of homogenate used was validated by ensuring a linear relationship between amount of homogenate and calculated  $\text{Na}^+/\text{K}^+$ -ATPase concentration (pmol). The incubation conditions (time and temperature) used were tested to ensure maximal [ $^3\text{H}$ ]ouabain binding and the number of washes (and volume) performed was found not to change results when between four and six washes (at  $50 \mu\text{l}$ ) were used and returned consistent values for NSB tubes.  $\text{Na}^+/\text{K}^+$ -ATPase levels are expressed as pmol  $\text{Na}^+/\text{K}^+$ -ATPase  $\text{mg}^{-1}$  protein.  $\text{Na}^+/\text{K}^+$ -ATPase levels were measured for five individuals (at random) from each group. Molecular activities (calculated as  $\text{Na}^+/\text{K}^+$ -ATPase activity divided by  $\text{Na}^+/\text{K}^+$ -ATPase protein levels;  $\text{ATP min}^{-1}$ ) were calculated using  $\text{Na}^+/\text{K}^+$ -ATPase activities measured at  $10^\circ\text{C}$ .  $\text{Na}^+/\text{K}^+$ -ATPase molecular activity may indicate a change in the abundance of different  $\text{Na}^+/\text{K}^+$ -ATPase isoforms. Protein content of tissue homogenates for  $\text{Na}^+/\text{K}^+$ -ATPase activity and  $\text{Na}^+/\text{K}^+$ -ATPase protein levels were measured using the Bio-Rad

standard protein assay (Bio-Rad Laboratories, Hercules, CA, USA), standardized with bovine serum albumin (BSA).

#### Chemicals

Chemicals not mentioned previously were purchased from Sigma Chemical Co. (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) with the exception of the BSA (purchased from BioShop, Burlington, ON, Canada). All chemicals used were of the highest available purity.

#### Statistical analysis

All data are presented as means  $\pm$  s.e.m. Comparisons of  $\text{Na}^+/\text{K}^+$ -ATPase activity, isoform mRNA levels and protein levels and molecular activity over time and between species were performed using a two-way analysis of variance (ANOVA). When required, a Tukey multiple comparison test was used to determine significance. For all comparisons  $P < 0.05$  was considered significant.

#### Results

Several fish died following exposure to seawater. Mortalities reduced the Arctic char sample sizes to seven for the 1 day, seven for the 2 day, six for the 10 day and five for the 30 day seawater-acclimated groups. One rainbow trout in the 10 day and two in the 30 day seawater groups also died, reducing the sample size to seven and six fish, respectively. No Atlantic salmon died during the experiment.

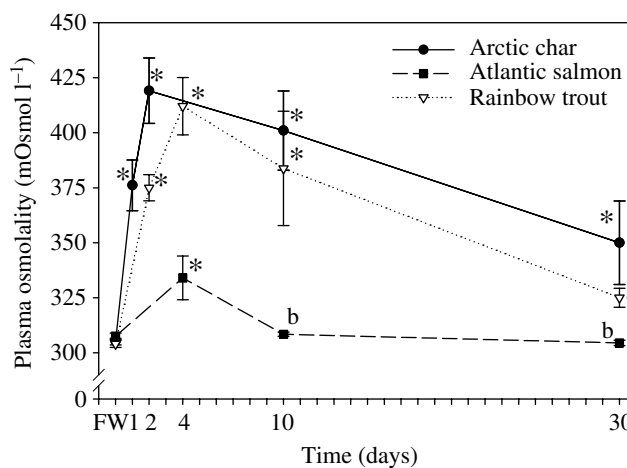


Fig. 1. Plasma osmolality ( $\text{mOsmol kg}^{-1}$ ) of Arctic char, Atlantic salmon and rainbow trout acclimated to freshwater (control) or seawater (32‰) for up to 30 days. For clarity only one control time point (1 day freshwater, FW) is included for each species as it was not significantly different from the corresponding 30 day freshwater control group. \*Significantly different ( $P < 0.05$ ) from both freshwater control groups; <sup>a</sup>significantly different ( $P < 0.05$ ) from corresponding 2, 4 and 10 day seawater groups, but not different from either freshwater control group; <sup>b</sup>significantly different ( $P < 0.05$ ) from corresponding 4 day seawater group, but not different from either freshwater control group. See text for interspecies comparisons. Values are means  $\pm$  s.e.m.; sample size ranged from 5 to 8, and is listed in Results.

Table 1. Plasma Na<sup>+</sup> and Cl<sup>-</sup> levels of Arctic char, rainbow trout and Atlantic salmon acclimated to freshwater or seawater for up to 30 days

Species	[Ion] (mEq l <sup>-1</sup> )	Days in freshwater (control)		Days in seawater (32‰)				
		1	30	1	2	4	10	30
Arctic char	Na <sup>+</sup>	150.3±2.8	152.1±2.5	208.6±7.6*	231.4±9.3*	ND	216.7±21.2*	181.2±13.6*.a
	Cl <sup>-</sup>	137.0±1.9	138.3±2.9	167.5±8.2*	174.5±7.4*	ND	168.5±17.0*	155.2±10.1*.a
Atlantic salmon	Na <sup>+</sup>	158.0±2.9	153.2±6.2	ND	ND	171.2±6.4*	156.2±1.5 <sup>b</sup>	158.9±2.0 <sup>b</sup>
	Cl <sup>-</sup>	134.0±1.7	130.5±3.2	ND	ND	136.9±3.5	124.0±1.9 <sup>b</sup>	121.1±0.7 <sup>b</sup>
Rainbow trout	Na <sup>+</sup>	157.4±1.3	159.7±3.5	ND	210.0±12.9*	218.3±4.6*	198.6±16.3*	165.8±5.2 <sup>a</sup>
	Cl <sup>-</sup>	131.4±2.2	136.2±2.8	ND	159.3±5.4*	172.7±4.9*	159.5±10.8*	139.8±4.2 <sup>a</sup>

Values are means ± s.e.m.; sample size ranged from 5 to 8, as listed in Results.

ND, not determined (species not sampled on that day).

\*Significantly different ( $P < 0.05$ ) from both freshwater control groups.

<sup>a</sup>Significantly different ( $P < 0.05$ ) from all other corresponding seawater groups.

<sup>b</sup>Significantly different ( $P < 0.05$ ) from corresponding 4 day seawater group.

Interspecies comparisons not included in table, see Results.

#### Plasma ions and osmolality

Plasma osmolality, Na<sup>+</sup> and Cl<sup>-</sup> levels were not different between the three species while in freshwater. Upon transfer to seawater, plasma osmolality, Na<sup>+</sup> and Cl<sup>-</sup> levels increased in all three species (Table 1, Fig. 1). For Atlantic salmon, plasma osmolality only increased at day 4 and returned to freshwater levels by day 10 of seawater exposure. The same was also true for plasma Na<sup>+</sup> levels, whereas plasma Cl<sup>-</sup> levels were never significantly different from those of freshwater salmon. Plasma osmolality and Na<sup>+</sup> and Cl<sup>-</sup> levels increased quite dramatically in both Arctic char and rainbow trout. Plasma osmolality increased to more than 400 mOsmol kg<sup>-1</sup> by day 2 in Arctic char and day 4 in rainbow trout. After 30 days seawater exposure, rainbow trout plasma osmolality decreased to levels not significantly different from freshwater trout, however, Arctic char plasma osmolality was still significantly elevated after 30 days in seawater. Plasma Na<sup>+</sup> and Cl<sup>-</sup> levels tracked the observed changes in plasma osmolality for both Arctic char and rainbow trout, also peaking at days 2 and 4, respectively. As a result of these differences, seawater-acclimated Atlantic salmon had plasma osmolality, Na<sup>+</sup> and Cl<sup>-</sup> levels that were significantly lower than both Arctic char and rainbow trout at all times measured.

#### Gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity

Freshwater Atlantic salmon (Fig. 2) had significantly (approx. threefold) higher gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity than both freshwater Arctic char and rainbow trout for both the 1 day and 30 day sampling times. Following seawater exposure, gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity remained similar to freshwater levels in all three species when measured after 1, 2 and 4 days. Na<sup>+</sup>/K<sup>+</sup>-ATPase activity increased significantly in all three species by day 10 of seawater exposure compared with freshwater controls and continued to rise significantly by day 30 (Fig. 2).

#### Gill Na<sup>+</sup>/K<sup>+</sup>-ATPase $\alpha$ -subunit isoform mRNA levels

Patterns of mRNA expression of gill Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha$ 1a and  $\alpha$ 1b isoforms were similar in Arctic char (Fig. 3), Atlantic salmon (Fig. 4) and rainbow trout (Fig. 5). Isoform  $\alpha$ 1a levels were highest in freshwater-acclimated fish. Upon exposure to seawater, the levels of  $\alpha$ 1a decreased rapidly in all three species.  $\alpha$ 1a was lower in seawater-acclimated Arctic char (Fig. 3A), but levels were only found to be significantly different from both freshwater controls in the 2 day seawater-

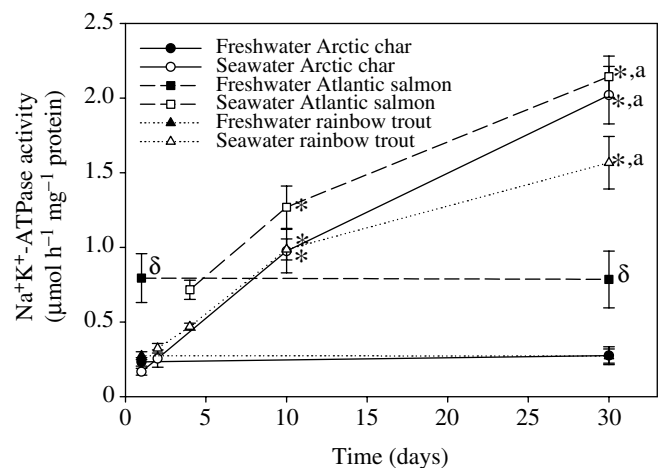


Fig. 2. Gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity of Arctic char, Atlantic salmon and rainbow trout acclimated to freshwater (control) or to seawater (32‰) for up to 30 days. \*Activity is significantly higher ( $P < 0.05$ ) than in both freshwater control groups and the corresponding 1, 2 and 4 day seawater-acclimated group (dependent on species); <sup>a</sup>activity is also significantly higher ( $P < 0.05$ ) than the corresponding 10 day seawater group; <sup>δ</sup>freshwater Atlantic salmon had significantly higher activity than both Arctic char and rainbow trout freshwater control groups. Sample size for each group ranged from 5 to 8 and is listed in Results.

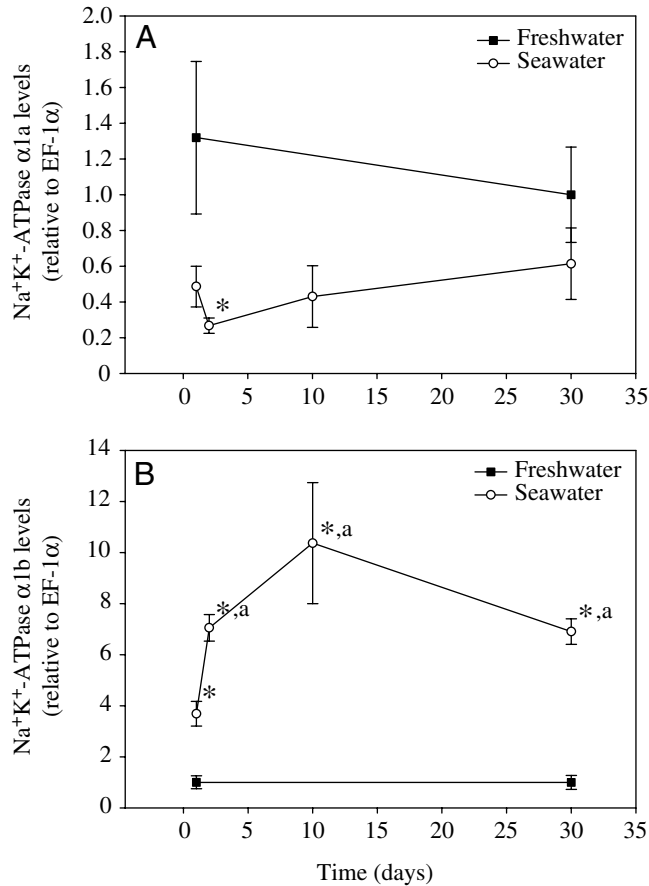


Fig. 3. Gill  $\text{Na}^+/\text{K}^+$ -ATPase (A)  $\alpha 1a$  and (B)  $\alpha 1b$  isoform mRNA levels in Arctic char acclimated to freshwater (control) or to seawater (32‰) for 30 days. mRNA level is relative to that of the control gene, elongation factor 1 $\alpha$  (EF1 $\alpha$ ), and is normalized to the Arctic char freshwater 30 day (control) group (normalized to a value of one). \*Significantly different ( $P < 0.05$ ) from both freshwater control points; <sup>a</sup>significantly different ( $P < 0.05$ ) from the 1 day seawater group (B only). Sample size ranged from 5 to 8, and is listed in Results.

acclimated group. Similarly,  $\alpha 1a$  levels were significantly reduced in Atlantic salmon exposed to seawater for 4 days and remained low in the 10 and 30 day acclimated groups (Fig. 4A). In gills of seawater-acclimated rainbow trout (Fig. 5A) levels of isoform  $\alpha 1a$  were significantly lower than the levels in freshwater trout at all time points.

Isoform  $\alpha 1b$  had the opposite expression pattern to  $\alpha 1a$ , being lowest in freshwater fish and increasing significantly upon exposure to seawater. All three species had similar expression patterns for isoform  $\alpha 1b$ . For Arctic char there was a significant increase in  $\alpha 1b$  levels after just 1 day in seawater, with levels increasing further on days 2, 10 and 30 (Fig. 3B). Similarly, for Atlantic salmon,  $\alpha 1b$  levels were significantly higher after 4, 10 and 30 days of seawater exposure compared with both freshwater control groups (Fig. 4B). In rainbow trout,  $\alpha 1b$  levels were significantly higher in all seawater-acclimated groups compared with freshwater controls (Fig. 5B).

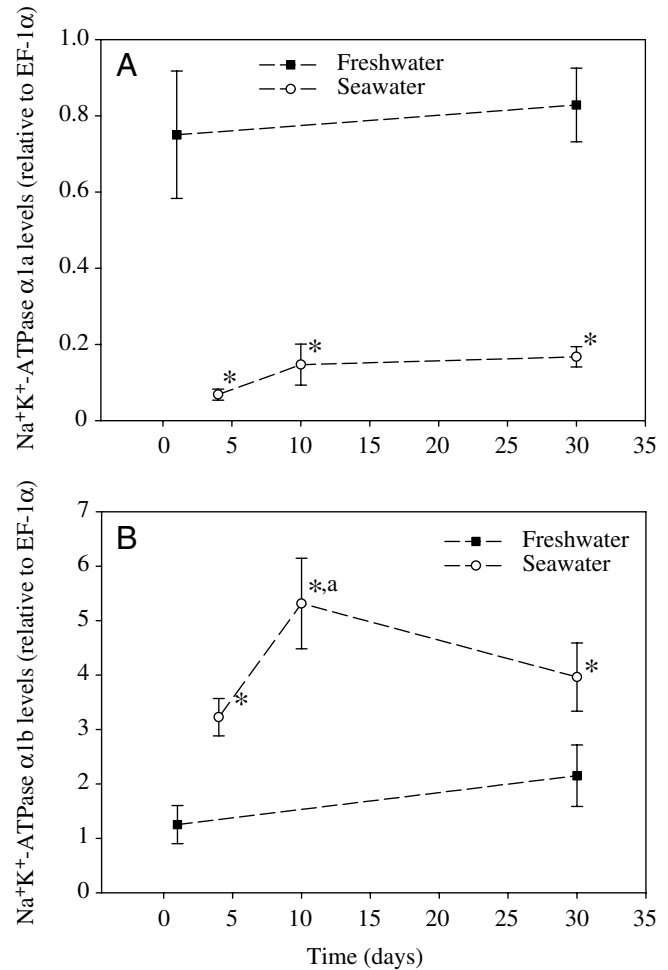


Fig. 4. Gill  $\text{Na}^+/\text{K}^+$ -ATPase (A)  $\alpha 1a$  and (B)  $\alpha 1b$  isoform mRNA levels in Atlantic salmon acclimated to freshwater (control) or to seawater (32‰) for 30 days. mRNA levels are relative to that of the control gene elongation factor 1 $\alpha$  (EF1 $\alpha$ ) and normalized to the Arctic char freshwater 30 day (control) group (normalized to a value of one). \*Significantly different ( $P < 0.05$ ) from both freshwater control points; <sup>a</sup>significantly different ( $P < 0.05$ ) from the 4 day seawater group. Sample size equals 8 for all groups.

The ratio of isoform  $\alpha 1b$  to  $\alpha 1a$  generally increased with time in seawater for all three species (Table 2). Similarly, total (sum of  $\alpha 1a + \alpha 1b$ )  $\text{Na}^+/\text{K}^+$ -ATPase mRNA levels also increased with time in seawater for all three species (Table 2). Freshwater Atlantic salmon were found to have a significantly higher  $\alpha 1b:\alpha 1a$  ratio than freshwater Arctic char in the 30 day group, and even in the 1 day groups this value was nearly significant ( $P = 0.053$ ).

#### Gill $\text{Na}^+/\text{K}^+$ -ATPase protein levels and molecular activity

Freshwater Atlantic salmon (at both day 1 and 30) had significantly higher (approx. threefold) gill  $\text{Na}^+/\text{K}^+$ -ATPase protein content when compared with freshwater Arctic char and rainbow trout (Table 3). Atlantic salmon and Arctic char gill  $\text{Na}^+/\text{K}^+$ -ATPase protein content was found to be

significantly higher after 10 and 30 days of seawater acclimation compared with control fish. A significant rise (compared with controls) in gill  $\text{Na}^+/\text{K}^+$ -ATPase protein content for rainbow trout was only seen in the 30 day seawater-acclimated group. Seawater-acclimated Atlantic salmon gill  $\text{Na}^+/\text{K}^+$ -ATPase protein content was also significantly higher than both Arctic char and rainbow trout after 10 and 30 days in seawater (Table 3).  $\text{Na}^+/\text{K}^+$ -ATPase molecular activity was similar between species and did not change with seawater acclimation, except in the case of the 30 day seawater-acclimated Arctic char which differed from the 1 and 2 day seawater-acclimated groups (Table 3).

### Discussion

Most studies that involve exposing anadromous salmonid fishes to seawater report an increase in gill  $\text{Na}^+/\text{K}^+$ -ATPase activity [Folmar and Dickhoff provide an extensive list (Folmar and Dickhoff, 1980; McCormick and Saunders, 1987)], suggesting that it is an integral part of their successful acclimation. Not surprisingly, this was also true for all three species examined in this study. In order to better understand what limits the seawater tolerance of different salmonid species (or individuals), it is important to understand the regulation of gill  $\text{Na}^+/\text{K}^+$ -ATPase. Our previous discovery that rainbow trout differentially express multiple gill  $\text{Na}^+/\text{K}^+$ -ATPase isoforms following seawater exposure (Richards et al., 2003) unveils a new level of complexity in the physiological response of fishes to changing salinity.

Following exposure to seawater, the level of  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha 1a$  mRNA is rapidly reduced in all three species examined. This decreased amount of isoform  $\alpha 1a$  is in contrast to many studies that report an increase in gill  $\text{Na}^+/\text{K}^+$ -ATPase mRNA expression during salinity acclimation of salmonid fishes (Madsen et al., 1995; D'Cotta et al., 2000; Seidelin et al., 2000; Singer et al., 2002; Tipsmark et al., 2002). However, those studies did not distinguish between individual isoforms of  $\text{Na}^+/\text{K}^+$ -ATPase, as they used probes designed for northern blot analysis that were created based on highly conserved regions of  $\text{Na}^+/\text{K}^+$ -ATPase. Therefore their observations probably include the expression of all  $\text{Na}^+/\text{K}^+$ -ATPase isoforms present in the gill. The present study reports increased levels of gill  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha 1b$  mRNA following seawater exposure of all three species of salmonid examined. The increase in  $\alpha 1b$  levels outweighs the observed decrease in  $\alpha 1a$  levels following seawater exposure and is therefore responsible for the observed increase in overall ( $\alpha 1a + \alpha 1b$ )  $\text{Na}^+/\text{K}^+$ -ATPase levels. Therefore, the observed increase in  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha$ -subunit mRNA expression seen in other studies (Madsen et al., 1995; D'Cotta et al., 2000; Seidelin et al., 2000; Singer et al., 2002; Tipsmark et al., 2002) is probably due to a specific increase in  $\alpha 1b$  isoform expression. This suggests  $\alpha 1b$  might be the specific  $\text{Na}^+/\text{K}^+$ -ATPase isoform associated with the typical upregulation of gill  $\text{Na}^+/\text{K}^+$ -ATPase seen in salmonids during seawater acclimation.

During seawater acclimation, levels of  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha 1b$

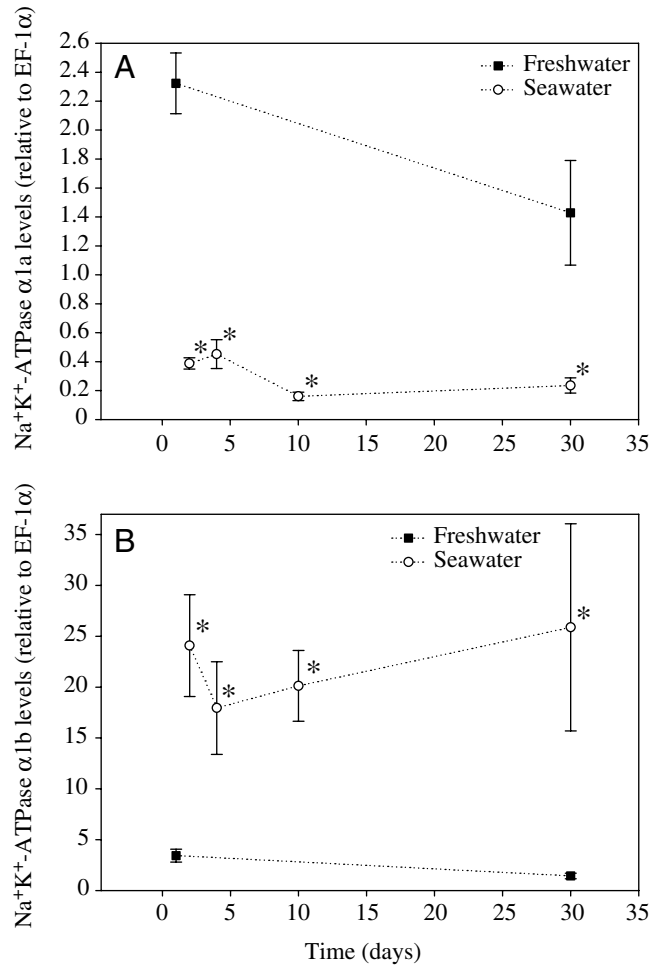


Fig. 5. Gill  $\text{Na}^+/\text{K}^+$ -ATPase (A)  $\alpha 1a$  and (B)  $\alpha 1b$  isoform mRNA levels in rainbow trout acclimated to freshwater (control) or to seawater (32‰) for 30 days. mRNA level is relative to that of the control gene, elongation factor 1 $\alpha$  (EF1 $\alpha$ ) and normalized to the Arctic char freshwater 30 day (control) group (normalized to a value of one). \*Significantly different ( $P < 0.05$ ) from both freshwater control points. Sample size ranged from 6 to 8, and is listed in Results.

mRNA was increased in each of the three species by the first sampling period (day 1 for Arctic char, 2 for rainbow trout, 4 for Atlantic salmon). This is similar to other studies that report increased  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha$ -subunit mRNA levels within 1 day of seawater exposure of salmonids, including Atlantic salmon (D'Cotta et al., 2000; Singer et al., 2002) and brown trout (Madsen et al., 1995; Seidelin et al., 2000; Tipsmark et al., 2002). In the present study, the pattern of increased  $\alpha 1b$  precedes a similar increase in gill  $\text{Na}^+/\text{K}^+$ -ATPase activity and protein content. This increase in enzyme activity is seen by day 10 of seawater exposure in all three species. The time lag between increased mRNA levels and activity is probably due to the time it takes for *de novo* synthesis of  $\text{Na}^+/\text{K}^+$ -ATPase protein. This has been estimated to take from 4 to 6 days in salmonid fishes (Conte and Lin, 1967). Similarly, other studies report 1- to 11-day time lags between increased  $\text{Na}^+/\text{K}^+$ -

Table 2. Individual amounts, sum and ratio of gill Na<sup>+</sup>/K<sup>+</sup>-ATPase isoforms  $\alpha 1a$  and  $\alpha 1b$  mRNA from Atlantic salmon, Arctic char and rainbow trout acclimated to freshwater or seawater for up to 30 days

Species	Isoform	Days in freshwater (control)		Days in seawater				
		1	30	1	2	4	10	30
Arctic char	$\alpha 1a$	1.58±0.51	1.20±0.32	0.58±0.14	0.32±0.05	ND	0.51±0.20	0.73±0.12
	$\alpha 1b$	1.22±0.31	1.21±0.33	4.49±0.59*	8.59±0.63*	ND	12.63±2.88*	8.41±1.62*
	$\alpha 1a+\alpha 1b$	2.81±0.82	2.42±0.63	5.08±0.51	8.91±0.64 <sup>a,b,c</sup>	ND	13.15±2.68 <sup>a,b,c</sup>	9.15±2.45 <sup>a,b</sup>
	$\alpha 1b:\alpha 1a$	0.82±0.06	1.04±0.15	10.12±1.80	30.91±6.81 <sup>a,b,c</sup>	ND	36.03±14.62 <sup>a,b,c</sup>	11.41±2.65
Atlantic salmon	$\alpha 1a$	0.90±0.20	0.99±0.11	ND	ND	0.08±0.02	0.17±0.06	0.20±0.03
	$\alpha 1b$	1.52±0.42	2.62±0.68*	ND	ND	3.93±0.42*	6.48±1.01*	4.83±0.76*
	$\alpha 1a+\alpha 1b$	2.43±0.57	3.61±0.79	ND	ND	4.01±0.44	6.65±1.07 <sup>a</sup>	5.03±0.76
	$\alpha 1b:\alpha 1a$	1.89±0.59	2.49±0.53 <sup>δ</sup>	ND	ND	52.99±7.08 <sup>a,b</sup>	51.20±10.90 <sup>a,b</sup>	28.41±7.91
Rainbow trout	$\alpha 1a$	2.79±0.25	1.72±0.43	ND	0.47±0.05	0.54±0.12	0.19±0.04	0.28±0.06
	$\alpha 1b$	4.18±0.77	1.75±0.33	ND	29.34±6.11*	21.86±5.54*	24.52±4.24*	31.53±12.42*
	$\alpha 1a+\alpha 1b$	6.97±0.84	3.46±0.54	ND	29.80±6.12	22.40±5.53	24.71±4.24	31.81±12.44 <sup>b</sup>
	$\alpha 1b:\alpha 1a$	1.55±0.29	1.47±0.60	ND	64.46±11.08	49.40±13.05	140.71±33.65 <sup>a,b</sup>	120.86±36.97 <sup>a,b</sup>

ND, not determined (species not sampled on that day).

Individual isoform mRNA levels are relative to that of the control gene EF1 $\alpha$ .

Values are mean  $\pm$  s.e.m.; sample size ranged from 5 to 8, and is listed in Results.

\* $\alpha 1b$  level is significantly different ( $P<0.05$ ) from corresponding  $\alpha 1a$  level.

<sup>a,b,c</sup>Significantly different ( $P<0.05$ ) from corresponding freshwater 1 day, freshwater 30 day and seawater 1 day groups, respectively. <sup>δ</sup>Level is significantly higher ( $P<0.05$ ) than the corresponding 30 day freshwater Arctic char group.

Comparisons made over the time series for each isoform for each species as shown in Figs 3–5.

ATPase mRNA levels and increased Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in salmonids acclimating to seawater (Madsen et al., 1995; D'Cotta et al., 2000).

The differential regulation of these two gill Na<sup>+</sup>/K<sup>+</sup>-ATPase isoforms during salinity acclimation suggests they may differ physiologically, and play specific roles in each environment. Examination of projected amino acid sequences [from rainbow trout  $\alpha 1a$  and  $\alpha 1b$  cDNA sequences (Richards et al., 2003)] for the two isoforms suggest that there are major differences that may confer different physiological properties on the two isoforms. Salmonid  $\alpha 1a$  and  $\alpha 1b$  are identical to the mammalian  $\alpha 1$  isoform in several highly conserved regions, including seven ( $\alpha 1a$ ) or eight ( $\alpha 1b$ ) of the eight known amino acid residues that control ATP binding and the aspartate 369 phosphorylation site (Lingrel and Kuntzweiler, 1994). However, salmonid  $\alpha 1a$  and  $\alpha 1b$  differ considerably in several transmembrane regions, which are known to control cation binding (Mobasher et al., 2000). In these transmembrane regions the  $\alpha 1a$  isoform is less similar than the  $\alpha 1b$  when compared to the mammalian  $\alpha 1$ . These sequence differences between the Salmonid  $\alpha 1a$  and  $\alpha 1b$  isoforms may explain the gill Na<sup>+</sup>/K<sup>+</sup>-ATPase kinetic differences between freshwater- and seawater-acclimated rainbow trout reported by Pagliarani et al. (Pagliarani et al., 1991) and may also explain why gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity is correlated to membrane composition in seawater- but not freshwater-acclimated Arctic char (Bystriansky, 2005). The function of the  $\alpha 1a$  isoform is not known, but it appears to be of lesser importance in the marine environment. It is tempting to speculate that Na<sup>+</sup>/K<sup>+</sup>-

ATPase  $\alpha 1a$  is the isozyme involved in the gill ion uptake of freshwater salmonids. Pagliarani et al. (Pagliarani et al., 1991) showed that in freshwater-acclimated rainbow trout gill, the Na<sup>+</sup>/K<sup>+</sup>-ATPase  $K_m$  values for Na<sup>+</sup> and K<sup>+</sup> were less than half of that determined for seawater-acclimated trout. This significantly lower  $K_m$  for Na<sup>+</sup> may allow Na<sup>+</sup>/K<sup>+</sup>-ATPase to maintain intracellular Na<sup>+</sup> concentrations at lower levels than those seen in gills of seawater-acclimated trout. A lower intracellular Na<sup>+</sup> concentration would significantly improve the gradient for apical Na<sup>+</sup> uptake and would suggest that Na<sup>+</sup>/K<sup>+</sup>-ATPase might play a more important role in the gill Na<sup>+</sup> uptake model than previously appreciated. The importance of gill Na<sup>+</sup>/K<sup>+</sup>-ATPase in regulating Na<sup>+</sup> uptake in freshwater fish is also supported by Hirata et al., who provide evidence that Osorezan dace can regulate internal Na<sup>+</sup> levels following exposure to pH 3.5 by significantly increasing gill Na<sup>+</sup>/K<sup>+</sup>-ATPase expression, while an increase in the expression of the apical vacuolar proton ATPase (V-H<sup>+</sup>-ATPase) is much more limited (Hirata et al., 2003). In addition, Piermarini and Evans present an interesting model for Na<sup>+</sup> uptake by elasmobranch gill that relies on Na<sup>+</sup>/K<sup>+</sup>-ATPase and not the V-H<sup>+</sup>-ATPase to drive Na<sup>+</sup> uptake (Piermarini and Evans, 2001). Clearly, further research is required to understand the potentially important role of Na<sup>+</sup>/K<sup>+</sup>-ATPase in ion uptake of fishes.

When compared to Atlantic salmon, the Arctic char and rainbow trout had obvious difficulties acclimating to seawater. This was evident from their highly elevated plasma Na<sup>+</sup>, Cl<sup>-</sup> and osmolality following seawater exposure and mortalities of several char and trout. The observed changes in gill Na<sup>+</sup>/K<sup>+</sup>-



Table 3. Gill Na<sup>+</sup>/K<sup>+</sup>-ATPase protein levels and molecular activity from Arctic char, rainbow trout and Atlantic salmon acclimated to freshwater or seawater for up to 30 days

Species	Isoform	Days in freshwater (control)		Days in seawater (32‰)				
		1	30	1	2	4	10	30
Arctic char	Protein expression	8.1±2.9	6.7±0.8	9.3±4.6	14.8±2.1	ND	28.8±6.7 <sup>*,a,b</sup>	21.4±3.9 <sup>*,a</sup>
	Molecular activity	703.4±259.4	828.7±322.4	507.3±114.7	311.6±86.0	ND	1288.3±212.0	1669.8±278.4 <sup>b,c</sup>
Atlantic salmon	Protein expression	19.4±1.6 <sup>**</sup>	21.0±5.7 <sup>**</sup>	ND	ND	22.5±4.1	74.5±6.6 <sup>*,a,l,**</sup>	46.5±3.0 <sup>*,a,**</sup>
	Molecular activity	608.3±127.5	807.9±274.5	ND	ND	639.5±162.6	328.9±30.7	667.2±93.4
Rainbow trout	Protein expression	6.6±1.2	5.9±1.7	ND	7.3±1.8	10.1±0.9	14.3±1.0	32.7±0.9 <sup>*,a,c,d,**</sup>
	Molecular activity	721.0±181.2	1150.9±434.9	ND	950.7±215.8	807.4±107.2	1230.1±113.8	671.9±81.4

Molecular activity: ATP min<sup>-1</sup>; protein expression: pmol mg<sup>-1</sup> protein.

Values are means ± s.e.m.; sample size for all groups is 5.

ND, not determined (species not sampled on that day).

<sup>\*,a,b,c,d</sup>Significantly different ( $P < 0.05$ ) from corresponding freshwater 1 day, freshwater 30 day, seawater 1 day, seawater 2 day and seawater 4 day groups, respectively.

<sup>\*\*</sup>Level is significantly higher ( $P < 0.05$ ) than the corresponding Arctic char and rainbow trout group.

ATPase mRNA levels do not explain why Atlantic salmon performed far better in seawater than both Arctic char and rainbow trout. For all three species, the patterns of  $\alpha 1a$  and  $\alpha 1b$  isoform levels were quite similar. The absolute increases in isoform  $\alpha 1b$  were actually largest in the Arctic char and rainbow trout, the two species that performed far worse than the Atlantic salmon. Following seawater exposure, the three species also had similar gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activities at each of the times sampled, with a significant increase in activity seen after 10 and 30 days acclimation. The only observed difference that could explain this discrepancy in osmoregulatory capacity is the gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity of these species while in freshwater. Prior to seawater exposure, freshwater Atlantic salmon had threefold higher gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity than both rainbow trout and Arctic char. This heightened gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity was correlated to a threefold higher gill Na<sup>+</sup>/K<sup>+</sup>-ATPase protein content when compared with both Arctic char and rainbow trout. This suggests the Atlantic salmon were better prepared for a seawater challenge.

The present study was conducted in the early summer coinciding with the time when these species would be well prepared to acclimate to seawater. Prior to moving into seawater, most anadromous salmonids undergo a process of smoltification, which prepares them for a life in seawater (Hoar, 1988). This preparatory period often includes an increase in gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity (McCormick and Saunders, 1987; Hoar, 1988). Atlantic salmon (Saunders and Henderson, 1978; McCormick and Saunders, 1987), rainbow trout (Zaugg and

Wagner, 1973) and Arctic char (Aas-Hansen et al., 2005) are all known to display a smolting response at this time of year, which includes an increase in gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity prior to seawater exposure. The fish used in the present study were exposed to a lighting regime that mimicked natural photoperiod changes occurring outside. These conditions may have allowed these fish to undergo a smolting response, which prepared them for 'a seawater migration'. The changing photoperiod may have triggered typical endocrine changes (e.g. rise in cortisol) known to be important during smoltification of most salmonid species, which are thought to lead to an increase in gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity and expression (Hoar, 1988). Both Atlantic salmon and rainbow trout exhibit a strong smolting response (Hoar, 1976), the Atlantic salmon had much higher gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity and protein levels. This heightened Na<sup>+</sup>/K<sup>+</sup>-ATPase activity may have enabled them to osmoregulate far more efficiently than the Arctic char or rainbow trout upon transfer to seawater. The significantly higher Na<sup>+</sup>/K<sup>+</sup>-ATPase protein content of Atlantic salmon was also evident after 10 and 30 days in seawater, although this did not translate into higher Na<sup>+</sup>/K<sup>+</sup>-ATPase activity compared with char and trout at those times.

Species differences in osmoregulatory capacity were anticipated, as Atlantic salmon are known to be relatively good osmoregulators. However, rainbow trout were expected to perform far better, as other studies have reported they only require 4–5 days to regulate their plasma ion content (Houston, 1959). Unfortunately, at this point it is not known and not possible to ascertain whether the higher gill Na<sup>+</sup>/K<sup>+</sup>-ATPase

activity or protein expression seen in Atlantic salmon was related to a specific Na<sup>+</sup>/K<sup>+</sup>-ATPase isoform. If isoform  $\alpha 1b$  is important during seawater acclimation, the typical increase in gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity of salmonids during smoltification may be due to increased  $\alpha 1b$  protein expression. Freshwater Atlantic salmon did have significantly higher ratios of  $\alpha 1b:\alpha 1a$  mRNA than Arctic char when the 30 day freshwater groups were compared, however at the start of the experiment (freshwater day 1) this difference was not statistically significant ( $P=0.053$ ). Although speculative, the presence of a higher proportion of Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha 1b$  protein prior to seawater migration may enable some salmonid species to acclimate to seawater more quickly than others.

In this study we have only examined the potential role of gill Na<sup>+</sup>/K<sup>+</sup>-ATPase regulation to explain the observed species differences in osmoregulatory performance during seawater acclimation. As the limiting mechanisms to seawater acclimation are still unknown, many other factors may be at play. Gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity may not be the limiting factor in euryhalinity. Acclimation to changing salinity is clearly a complicated physiological response, which requires many coordinated physiological changes in multiple tissues. The direct comparison of different fish species that vary in their salinity tolerance is a good approach to understanding what limits salinity tolerance. Other factors that may be responsible for the observed species differences include the expression and regulation of the gill apical chloride channel (CFTR) and the basolateral sodium potassium chloride cotransporter (NKCC). These proteins must act in conjunction with Na<sup>+</sup>/K<sup>+</sup>-ATPase for efficient ion secretion. Each of these needs to be examined in depth before we can ascertain the true limiting mechanism(s) for seawater acclimation.

The results of the present study clearly indicate that two isoforms of gill Na<sup>+</sup>/K<sup>+</sup>-ATPase are reciprocally expressed during seawater acclimation of salmonid fishes. Levels of the  $\alpha 1a$  isoform are quickly down regulated whereas  $\alpha 1b$  levels increase in response to seawater exposure. This pattern of Na<sup>+</sup>/K<sup>+</sup>-ATPase regulation is seen in representative species from three genera of anadromous salmonids, suggesting it is a widespread phenomenon. This information significantly improves our understanding of gill Na<sup>+</sup>/K<sup>+</sup>-ATPase regulation during salinity acclimation of salmonid fishes and leads to many new questions. In this study, the observed pattern of Na<sup>+</sup>/K<sup>+</sup>-ATPase mRNA isoform switching does not explain the greater osmoregulatory capacity of Atlantic salmon. However, patterns of isoform-specific Na<sup>+</sup>/K<sup>+</sup>-ATPase protein expression may not be proportional to the observed mRNA expression of each isoform. Some salmonid species may have an enhanced seawater tolerance because they increase gill Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha 1b$  protein expression prior to seawater exposure. Future studies need to examine isoform-specific protein expression of gill Na<sup>+</sup>/K<sup>+</sup>-ATPase over the parr-smolt transformation and during seawater acclimation of salmonid fishes, to further elucidate the potential roles of isoforms  $\alpha 1a$  and  $\alpha 1b$ . It is clear that the regulation of gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity is quite complicated, probably controlled by many

different mechanisms including mRNA transcription, protein translation and other post-translational factors.

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