

Wild Arctic Char (*Salvelinus alpinus*) Upregulate Gill Na^+ , K^+ -ATPase during Freshwater Migration

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ABSTRACT

The successful acclimation of euryhaline fishes from seawater to freshwater requires the gills to stop actively secreting ions and start actively absorbing ions. Gill Na^+ , K^+ -ATPase is known to be an integral part of the active ion secretion model of marine fishes, but its importance in the active ion uptake model of freshwater fishes is less clear. This study, conducted in the high Arctic, examines gill Na^+ , K^+ -ATPase regulation in wild anadromous arctic char returning to freshwater from the ocean. Gill Na^+ , K^+ -ATPase activity, protein expression, and mRNA expression of Na^+ , K^+ -ATPase isoforms $\alpha 1a$ and $\alpha 1b$ were monitored in arctic char at three points along their migration route to and from Somerset Island, Nunavut, Canada: out at sea (Whaler's Point), in seawater near the river mouth (Nat's Camp), and after entering the Union River. Arctic char collected from the Union River had more than twofold greater gill Na^+ , K^+ -ATPase activity. This was associated with a significant increase (threefold) in Na^+ , K^+ -ATPase isoform $\alpha 1a$ mRNA expression and a significant increase in plasma sodium and osmolality levels compared with seawater char. Compared with char sampled from Whaler's Point, Na^+ , K^+ -ATPase isoform $\alpha 1b$ mRNA expression was decreased by ~50% in char sampled at Nat's Camp and the Union River. These results suggest that the upregulation of gill Na^+ , K^+ -ATPase activity is involved in freshwater acclimation of arctic char and implicate a role for Na^+ , K^+ -ATPase isoform $\alpha 1a$ in this process. In addition, we

discuss evidence that arctic char go through a preparatory phase, or "reverse smoltification," before entering freshwater.

Introduction

Anadromous salmonid fishes have a remarkable capacity to migrate between freshwater and seawater environments. This ability is dependant on the dynamic regulation of water and ions by the gills, intestine, and kidney. The gills are ultimately responsible for active ion uptake in freshwater and the active excretion of Na^+ and Cl^- in seawater (see Evans et al. 2005 for review). Extensive research has been conducted on the changes to gill physiology during the acclimation of fish from freshwater to seawater, where the gills must quickly change from a system of active ion uptake to active ion excretion. In contrast, few studies have examined the physiological changes that gills undergo during acclimation of salmonid fishes from seawater to freshwater. This is surprising because ion gradients between the fish and its environment are greater in freshwater than they are in seawater, which should make the freshwater migration more challenging.

Gill Na^+ , K^+ -ATPase plays an integral role in regulating plasma Na^+ and Cl^- levels. The functional Na^+ , K^+ -ATPase consists of two protein subunits, a catalytic α subunit and a glycoprotein β subunit, which assemble in a 1 : 1 ratio and create an $\alpha\beta$ heterodimer. The α subunit contains the binding sites for ATP, Na^+ , K^+ , and ouabain as well as a phosphorylation site (Lingrel and Kuntzweiler 1994) and is responsible for the reciprocal translocation of three Na^+ and two K^+ across the plasma membrane. We recently showed that seawater acclimation of rainbow trout involves the differential regulation of two isoforms of the α subunit of gill Na^+ , K^+ -ATPase (Richards et al. 2003; Bystriansky et al. 2006). The $\alpha 1b$ isoform is up-regulated on exposure to seawater, while the $\alpha 1a$ isoform is rapidly downregulated. This is also seen in Atlantic salmon (*Salmo salar*) and arctic char (*Salvelinus alpinus*) during seawater acclimation (Bystriansky et al. 2006), confirming that the expression pattern of these gill Na^+ , K^+ -ATPase isoforms during salinity change is consistent across the three genera of anadromous salmonids. During seawater exposure of salmonids (Richards et al. 2003; Bystriansky et al. 2006), the rise in Na^+ , K^+ -ATPase activity is preceded by increased expression of isoform $\alpha 1b$, suggesting that this isoform is important for successful acclimation to seawater. This is supported by the ob-

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servation that landlocked arctic char that fail to acclimate to seawater do not upregulate isoform $\alpha 1b$ (Bystriansky 2005). However, the rapid decrease in $\alpha 1a$ expression on seawater exposure (Richards et al. 2003; Bystriansky et al. 2006) suggests the $\alpha 1a$ isoform is not required in the marine environment and therefore may be involved in the gill-active ion uptake model of freshwater fishes.

Gill Na⁺,K⁺-ATPase is widely accepted to be the driving force for active excretion of excess plasma Na⁺ and Cl⁻ in marine fishes. However, its role in freshwater fish ionoregulation is less clear (Evans et al. 2005). Current ion uptake models in salmonids focus on the action of the apical H⁺ V-ATPase, which generates an electrochemical gradient that drives Na⁺ uptake via an apical Na⁺ channel (Avella and Bornancin 1989). Na⁺,K⁺-ATPase shuttles the accrued Na⁺ across the basolateral membrane into the blood. In doing so, Na⁺,K⁺-ATPase acts to maintain a low intracellular Na⁺ concentration, which should improve the gradient for apical Na⁺ uptake. However, as Kirschner (2004) points out, the energy required to drive Na⁺ uptake in freshwater can largely be generated by Na⁺,K⁺-ATPase alone. This suggests the regulation of gill Na⁺,K⁺-ATPase could be equally as important as the H⁺ V-ATPase during freshwater acclimation of fishes. In fact, many studies of euryhaline marine or brackish-water fishes (nonsalmonids) show that freshwater acclimation involves the upregulation of Na⁺,K⁺-ATPase activity (Lasserre 1971; Gallis et al. 1979; Stuenkel and Hillyard 1980; Doneen 1981; Stagg and Shuttleworth 1982; Ciccotti et al. 1994; Woo and Chung 1995; Kelly et al. 1999; Lin et al. 2003; Scott et al. 2004a, 2004b) or mRNA expression (Lin et al. 2003; Scott et al. 2004a, 2004b). Many of these studies display a U-shaped pattern of regulation (Lasserre 1971), where transfer of fish to both higher and lower salinities involves increased gill Na⁺,K⁺-ATPase activity. Unfortunately, few studies have examined gill Na⁺,K⁺-ATPase regulation during the acclimation of salmonid fishes to freshwater. Eliassen et al. (1998) showed a decline in gill Na⁺,K⁺-ATPase activity of arctic char following transfer from seawater to freshwater, which was associated with a decrease in plasma Na⁺ and Cl⁻ levels. Similar decreases in plasma ion levels were also reported by Avella et al. (1990) during freshwater acclimation of coho salmon (*Oncorhynchus kisutch*). In addition, Shrimpton et al. (2005) provides evidence that sockeye salmon (*Oncorhynchus nerka*) reduce gill Na⁺,K⁺-ATPase activity, plasma Cl⁻, and osmolality levels while still in seawater during their oceanic migration toward their native river. This suggests salmonid fishes may exhibit a preparatory phase before freshwater entry. Interestingly, for one of the four salmon stocks examined in that study, gill Na⁺,K⁺-ATPase activity did not decline immediately following entry into freshwater, and this was associated with a significant increase in plasma osmolality compared with that of salmon collected in seawater (Shrimpton et al. 2005). The mRNA level for the $\alpha 1a$ isoform of gill Na⁺,K⁺-ATPase was also observed to increase following freshwater ex-

posure of sockeye salmon (Shrimpton et al. 2005), opposite to what is seen during seawater acclimation of rainbow trout, arctic char, and Atlantic salmon (Richards et al. 2003; Bystriansky et al. 2006). This suggests gill Na⁺,K⁺-ATPase may play an important role during freshwater acclimation of salmonid fishes.

This study monitored gill Na⁺,K⁺-ATPase activity, protein, and isoform-specific mRNA expression in wild arctic char (*Salvelinus alpinus*) that were naturally migrating from the Arctic Ocean into freshwater. Anadromous arctic char make annual seaward migrations each spring before returning every fall to freshwater, where they overwinter. Arctic char are considered relatively poor osmoregulators compared to other anadromous salmonids because they have a larger minimum size before they are able to tolerate seawater, take longer to acclimate to seawater, and spend only a short period in seawater each year (Hoar 1976, 1988). This more limited osmoregulatory capacity may also be evident during their acclimation to freshwater.

Material and Methods

Experimental Procedure

This study was carried out in the area of Creswell Bay on Somerset Island, Nunavut, Canada, approximately 225 km south of the town of Resolute Bay. The study area consisted of three collection sites along a natural migration path of a large population of anadromous arctic char (Fig. 1). The only freshwater habitat available to this population is Stanwell-Fletcher Lake, which connects to Creswell Bay (Arctic Ocean) via the Union River. Anadromous arctic char return from the sea to this freshwater refuge each fall. Collection site 1 is situated on Creswell Bay, approximately 500 m north of the mouth of the Union River, and will be referred to as Nat's Camp (as it is located near the hunting camp of a local Inuit named Nathaniel Kalluk). The seawater conditions of this site fluctuated slightly, ranging from 25‰–32‰ salinity, and surface water temperature ranged from -1.5° to 1°C. Prevailing wind and ocean currents deflect the Union River freshwater outflow to the south, which keeps this area saline. For 5 d before and also at the time of sampling, the salinity was always >30‰ salinity and the temperature was <0°C. As char found in the region of Nat's Camp could easily move into the estuary, a second sampling site was chosen approximately 9 km from the mouth of the Union River. This site will be referred to as Whaler's Point (due to its use as a whale research site). This site was found to have full-strength seawater (32‰ salinity) and a temperature of 0°C. The char collected from this site were suspected to always be exposed to full-strength seawater. The third site was the Union River, approximately 20–100 m upstream from the mouth of the river. The river water conditions were 6°C at the time of sampling, and the salinity was 0‰. The rapid flow and downhill grade of the river near its mouth prevents the entry of seawater to the sampling site.

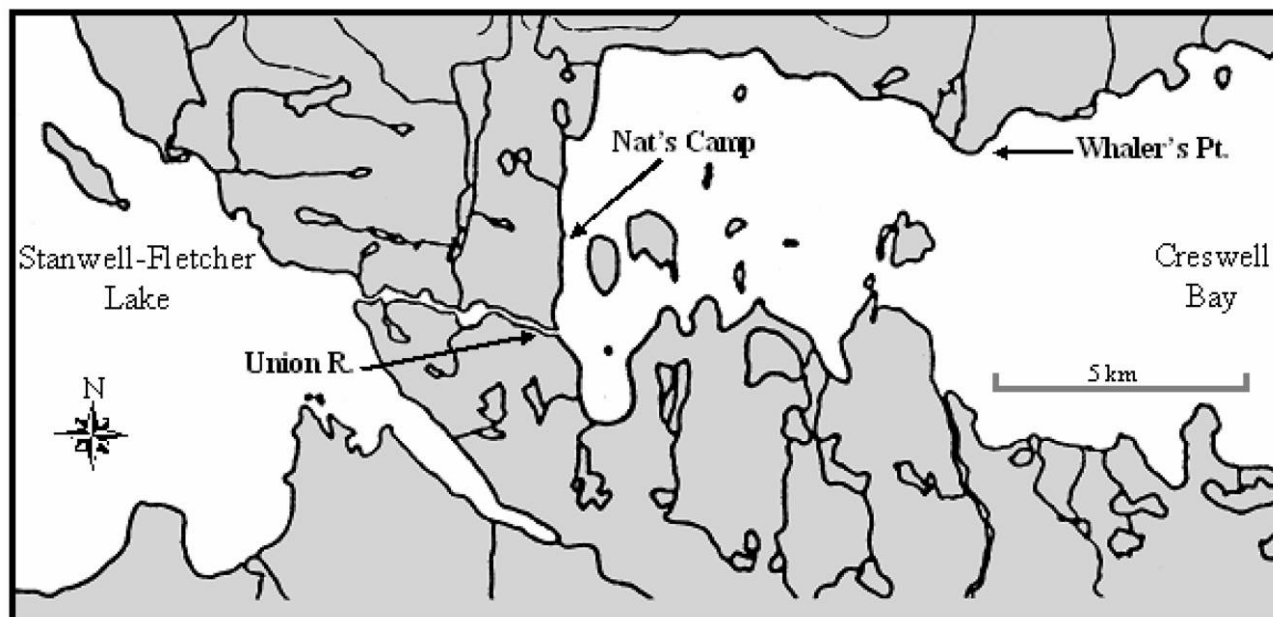


Figure 1. Map of Creswell Bay/Stanwell-Fletcher Lake region of Somerset Island, Nunavut, Canada, with the location of the three collection sites for wild arctic char used in this study. Locations were Whaler's Point (seawater, 32‰ salinity), Nat's Camp (seawater, 32‰ salinity), and the Union River (freshwater).

Char were first seen migrating up the Union River on August 16, 2001. On that day, arctic char were caught with a large hand net as they moved along the riverbank. A blood sample was taken from each fish by caudal puncture with a heparinized (500 U mL^{-1}) syringe. Char were then killed by a blow to the head, and the second gill arch on the right side was quickly excised and immediately frozen in a liquid nitrogen-chilled cryoshipper. Blood samples were immediately centrifuged for 5 min ($3,000 \text{ g}$), and plasma was removed and frozen in the cryoshipper. Samples remained in the cryoshipper and were transported back to the University of Guelph and stored at -80°C until analyzed. The same procedure was carried out the following day at Nat's Camp and Whaler's Point, except at these sites, the char were caught by rod and reel. The average fight time for these char was approximately 2 min. Any char that took longer than 4 min to catch or any that appeared exhausted were released. Mean char size was similar between sites ($3.84 \pm 0.56 \text{ kg}$).

Determination of Plasma Osmolality and Ion Levels

Plasma osmolality was determined using a vapor pressure osmometer (model 5500, Wescor, Logan, UT). Cl^- levels were measured using a chloride titrator (model CMT10, Radiometer, Copenhagen). Na^+ levels were measured using a flame photometer (model FLM2, Radiometer, Copenhagen).

Determination of Na^+, K^+ -ATPase Activity

Gill filaments were homogenized on ice in SEI ($\text{pH} = 7.5$, 150 mmol L^{-1} sucrose, 10 mmol L^{-1} EDTA, 50 mmol L^{-1} imidazole) buffer using a ground-glass homogenizer. Homogenates were centrifuged for 30 s (4°C) at $5,000 \text{ g}$ to remove filaments and other insoluble material. The supernatant was used directly in the assay of enzyme activity. Na^+, K^+ -ATPase activity was determined spectrophotometrically using a NADH-linked assay modified from the methods of 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 Na^+, K^+ -ATPase-specific inhibitor ouabain (final concentration 1 mmol 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 Na^+, K^+ -ATPase activity. Optimal assay conditions to give maximal enzyme activity were determined to be 100 mmol L^{-1} NaCl, 20 mmol L^{-1} KCl, 5 mmol L^{-1} MgCl_2 , 50 mmol L^{-1} imidazole, 3 mmol L^{-1} ATP, 2 mmol L^{-1} PEP, 0.2 mmol L^{-1} NADH, 4 U LDH, and 5 U PK, $\text{pH} 7.5$. Na^+, K^+ -ATPase activity is expressed as micromoles of ADP $\text{h}^{-1} \text{ mg protein}^{-1}$. Maximal Na^+, K^+ -ATPase activity was determined using a Cary 300 Bio spectrophotometer (Varian, Palo Alto, CA) equipped with a Pelletier-controlled, thermostated cell

changer. Gill Na⁺,K⁺-ATPase activity was determined at 1.5° and 6°C.

Determination of Na⁺,K⁺-ATPase α -Subunit mRNA Expression

Total RNA was extracted from gill filaments (which were removed from arches) using TriPure Isolation Reagent (Boehringer, Mannheim) following the guanidine thiocyanate method (Chomczynski and Sacchi 1987). Isolated total RNA was quantified spectrophotometrically and run (2 μ g) on an agarose gel (1%) to check for RNA integrity. First-strand cDNA was synthesized from 2 μ g of total RNA using oligo(dT₁₅) primer and RevertAid H Minus M-MuLV reverse transcriptase (RT), following the manufacturer's instructions (MBI Fermentas). Quantitative RT-PCR (qRT-PCR) was performed on an ABI Prism 7000 sequence analysis system (Applied Biosystems, Foster City, CA). PCR reactions contained 1 μ L of cDNA, 150 pmol of each primer, and universal SYBR Green Master Mix (Applied Biosystems). Forward and reverse primers used were designed to be Na⁺,K⁺-ATPase α -subunit specific for the α 1a and α 1b isoforms and for the control gene elongation factor 1 α (EF1 α ; Richards et al. 2003). Primer sequences were as follows: Na⁺,K⁺-ATPase α 1a forward 5' GGC CGG CGA GTC CAA T 3', Na⁺,K⁺-ATPase α 1a reverse 5' GAG CAG CTG TCC AGG ATC CT 3' (product size 66); Na⁺,K⁺-ATPase α 1b forward 5' CTG CTA CAT CTC AAC CAA CAA CAT T 3', Na⁺,K⁺-ATPase α 1b reverse 5' CAC CAT CAC AGT GTT CAT TGG AT 3' (product size 81); and EF1 α forward 5' GAG ACC CAT TGA AAA GTT CGA GAA G 3', EF1 α reverse 5' GCA CCC AGG CAT ACT TGA AAG 3' (product size 71). The qRT-PCR reaction conditions were as follows: 2 min at 50°C and 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 products from three individuals (one from each collection site) for each gene were cloned into a T vector (pGEM T-easy, Promega; Fisher Scientific, Nepean, ON) and transformed into heat-shock-competent *Escherichia coli* (strain JM109; Promega; Fisher Scientific), and colonies were 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) and sequenced using an ABI Prism 377 DNA sequencer (Applied Biosystems) at the University of Guelph Molecular Supercenter (Guelph, ON). Negative control reactions 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 : 6,246 starting copies for Na⁺,K⁺-ATPase isoform α 1a, 1 : 4,896 starting copies for Na⁺,K⁺-ATPase isoform α 1b,

and 1 : 6,224 starting copies for EF1 α . Relative quantities of each target gene were determined using the comparative C_T method (Applied Biosystems 2001). The relative quantity of Na⁺,K⁺-ATPase α 1a and α 1b mRNA in gill samples was normalized to an endogenous reference (EF1 α) and expressed relative to the mean value for char collected from Whaler's Point according to the formula

$$\text{amount} = 2^{-\Delta\Delta C_T},$$

where $\Delta\Delta$ refers to the difference in expression between the target gene and EF1 α and the relative expression relative to the mean Whaler's Point char, 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 (using a standard curve ranging from 1 μ L to 1 μ L of a \times 625 dilution; Applied Biosystems 2001). All samples were run in duplicate.

Determination of Na⁺,K⁺-ATPase Protein Expression

Gill Na⁺,K⁺-ATPase protein expression was determined using the method of Else and Wu (1999), by monitoring binding of ³H-ouabain (0.588 TBq mmol⁻¹, obtained from PerkinElmer, Boston) to gill tissue homogenate. Briefly, gill homogenates were prepared as described for Na⁺,K⁺-ATPase activity determination. Homogenates were diluted to a concentration of 1 mg protein mL⁻¹, and 15 μ L were added to 250 μ L of incubation medium (10 mmol L⁻¹ NaH₂PO₄, 5 mmol L⁻¹ MgCl₂, 5 \times 10⁻⁵ mol L⁻¹ unlabeled ouabain plus 1.5 \times 10⁻⁷ mol L⁻¹ ³H-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 μ L of incubation medium (10 mmol L⁻¹ NaH₂PO₄, 5 mmol L⁻¹ MgCl₂, 10⁻² mol L⁻¹ unlabeled ouabain plus 1.5 \times 10⁻⁷ mol L⁻¹ ³H-ouabain, pH 7.4) were run for each sample to determine nonspecific binding (NSB). Sample and NSB tubes were run in duplicate and incubated for 2.5 h at 25°C. Tubes were then centrifuged (4,000 g for 5 min), and Na⁺,K⁺-ATPase remained on filters. Filters were washed five times with 50 μ L of wash solution (10 mmol L⁻¹ NaH₂PO₄, 5 mmol L⁻¹ MgCl₂, pH 7.4), allowed to dry, removed from their tubes and placed in scintillation vials containing 15 mL of Scintisafe Econo F scintillation fluid (Fisher Scientific), and left in the dark overnight. Vials were read using a Beckman LS 6500 multipurpose scintillation counter (Beckman, Fullerton, CA) with DPM (disintegrations per minute) correction. Ouabain was assumed to bind to Na⁺,K⁺-ATPase in a 1 : 1 ratio, and Na⁺,K⁺-ATPase density was 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 the amount of homogenate

Table 1: Plasma Na^+ , Cl^- (mEq L^{-1}) and total osmolality (mOsm kg^{-1} ; mean \pm SEM) from arctic char (*Salvelinus alpinus*) collected from Whaler's Point (seawater, 32‰ salinity), Nat's Camp (seawater, 32‰ salinity), and the Union River (freshwater)

Plasma parameter	Whaler's Point	Nat's Camp	Union River
Na^+	161.4 \pm 3.5	161.9 \pm 3.3	172.8 \pm 2.9*
Cl^-	140.1 \pm 2.2	139.0 \pm 2.3	141.1 \pm 3.5
Osmolality	331.9 \pm 3.9	333.6 \pm 2.0	346.3 \pm 5.3*

Note. Asterisk denotes significant difference from char collected from Whaler's Point and Nat's Camp sites. $n = 8$ for each group.

and calculated picomoles of Na^+ , K^+ -ATPase. The incubation conditions (time and temperature) used were tested to ensure maximal ^3H -ouabain binding, and the number of washes (and volume) performed was found to not change results (between four and six washes [at 50 μL] were used and returned consistent values for NSB tubes). Na^+ , K^+ -ATPase density is expressed as picomoles of Na^+ , K^+ -ATPase mg protein^{-1} . Molecular activity (ATP min^{-1}) was calculated using Na^+ , K^+ -ATPase activities determined at 6°C. Protein content of tissue homogenates for Na^+ , K^+ -ATPase activity and density were determined using the Bio-Rad standard protein assay (Bio-Rad, Hercules, CA) and standardized with bovine serum albumin (BSA).

Chemicals

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

Statistical Analysis

All data are presented as means \pm SEM. Sample size for Whaler's Point, Nat's Camp, and Union River char was eight for each parameter determined except for Na^+ , K^+ -ATPase density and molecular activity, which were determined with $n = 5$ for each group (chosen at random). Comparisons of Na^+ , K^+ -ATPase activity, isoform mRNA expression, and protein expression for the three sites were performed using a one-way ANOVA. When required, a Tukey HSD multiple comparison test was used to determine significance. Comparisons of relative amounts of Na^+ , K^+ -ATPase $\alpha 1a$ and $\alpha 1b$ isoform mRNA within each collection site were made using an appropriate Student's t -test. For all comparisons, $P < 0.05$ was considered significant.

Results

Arctic char were collected from three sites along their natural migration route in Creswell Bay and the Union River (Fig. 1). Char collected from the Union River were found to have sig-

nificantly higher plasma Na^+ and osmolality than char collected in seawater from Whaler's Point or Nat's Camp, while plasma Cl^- levels were not significantly different between sites (Table 1). Gill Na^+ , K^+ -ATPase activity determined at both 1.5° and 6°C was significantly higher in the Union River char (~2.5–threefold) when compared with char collected from Whaler's Point and Nat's Camp (Fig. 2). Gill Na^+ , K^+ -ATPase activity did not differ between Whaler's Point and Nat's Camp arctic char. Gill Na^+ , K^+ -ATPase protein density was not significantly different between the three sites, but Na^+ , K^+ -ATPase molecular activity was significantly higher (~threefold) in Union River char compared with fish collected from Whaler's Point or Nat's Camp (Fig. 3).

The relative mRNA expression of gill Na^+ , K^+ -ATPase isoform $\alpha 1a$ was more than threefold higher in arctic char migrating up the Union River than in fish collected in seawater at the other two sites (Fig. 4A). Conversely, compared to arctic char collected at Whaler's Point, expression of mRNA for isoform $\alpha 1b$ was significantly lower in char collected at Nat's Camp (62% lower) and in the Union River (70% lower; Fig. 4B). For all three sites, the relative expression of isoform $\alpha 1b$ was significantly higher than isoform $\alpha 1a$; however, the ratio of isoform $\alpha 1b$ to $\alpha 1a$ was lowest in fish collected from the Union River and significantly lower than char from Whaler's Point but not statistically different from fish collected at Nat's Camp (Table 2). The ratios found for Whaler's Point and Nat's Camp char were also not significantly different from each other.

Discussion

Arctic char migrating up the Union River were found to have more than threefold higher Na^+ , K^+ -ATPase activity than their counterparts residing in seawater. To our knowledge, this is the first report of gill Na^+ , K^+ -ATPase upregulation in a salmonid fish acclimating to freshwater. Gill Na^+ , K^+ -ATPase activity of salmonid fishes is widely accepted to be higher in seawater acclimated individuals (Hoar 1988); however, another recent study on freshwater migration of sockeye salmon (Shrimpton et al. 2005) shows that for one population sampled soon after freshwater entry, gill Na^+ , K^+ -ATPase was not lower than that seen in seawater. In our study, enzyme measurements were

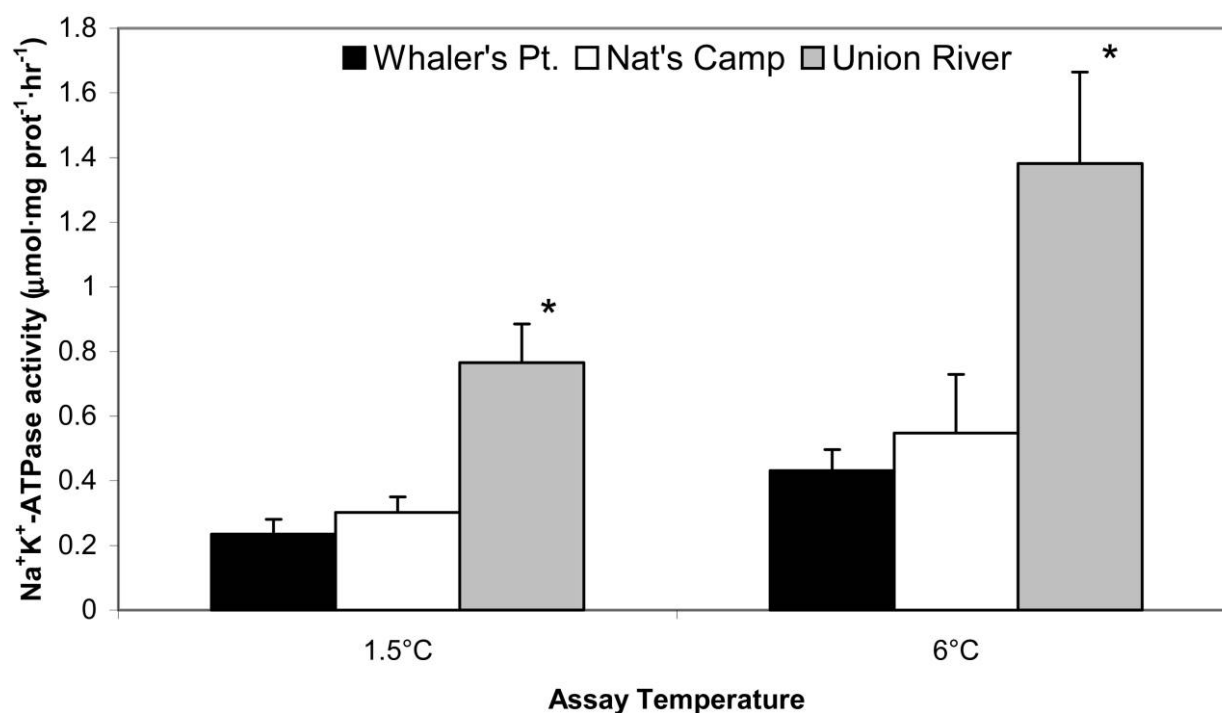


Figure 2. Gill Na^+, K^+ -ATPase activity (mean \pm SEM) of arctic char (*Salvelinus alpinus*) collected from Whaler's Point (seawater, 32‰ salinity), Nat's Camp (seawater, 32‰ salinity), and the Union River (freshwater). Enzyme activity was determined at 1.5° and 6°C. Asterisks denote that activity of Union River char was significantly higher ($P < 0.05$) than the activity of fish collected from Whaler's Point and Nat's Camp at both assay temperatures. $n = 8$ for each group.

determined at 1.5° and 6°C to ensure that the difference seen in Na^+, K^+ -ATPase activity was not a factor of environmental temperature acclimation. Many previous studies show that seawater acclimation of fishes induces an increase in gill Na^+, K^+ -ATPase activity (Kirschner 1980; McCormick 1996). In seawater, gill Na^+, K^+ -ATPase maintains Na^+ and K^+ gradients across the basolateral membrane of chloride cells, which drive the excretion of blood Na^+ and Cl^- via the gill. Due to the role of gill Na^+, K^+ -ATPase in active salt excretion in marine fishes, it seems counterintuitive that fish acclimating to freshwater would increase gill Na^+, K^+ -ATPase activity. However, many studies on euryhaline, brackish-water, and marine species also show increased gill Na^+, K^+ -ATPase activity during freshwater acclimation. These include sea bass *Dicentrarchus labrax* (Lasserre 1971; Jensen et al. 1998), killifish *Fundulus heteroclitus* (Scott et al. 2004a, 2004b), pupfish *Cyprinodon salinus* (Stuenkel and Hillyard 1980), mullet *Mugil cephalis* (Ciccotti et al. 1994) and *Chelon labrosus* (Gallis et al. 1979), flounder *Platichthys flesus* (Stagg and Shuttleworth 1982) and *Gillichthys mirabilis* (Doneen 1981), milkfish *Chanos chanos* (Lin et al. 2003), and sea bream *Mylio macrocephalus* (Kelly et al. 1999).

These findings draw attention to the role of gill Na^+, K^+ -ATPase in freshwater fish ionoregulation. Na^+, K^+ -ATPase has been localized to mitochondria-rich cells (MRCs) in freshwater

rainbow trout gill (Witters et al. 1996; Wilson et al. 2000), where it is responsible for the uphill transport of Na^+ across the basolateral membrane into the blood. The electrochemical gradient necessary to drive Na^+ uptake is probably generated by the apical H^+ V-ATPase and the basolateral Na^+, K^+ -ATPase. However, as Kirschner (2004) points out, the energy required to drive Na^+ uptake in freshwater can be solely generated by Na^+, K^+ -ATPase if external $[\text{Na}^+]$ is in the 1 mmol L^{-1} range. It is only in freshwater of very low $[\text{Na}^+]$ ($\mu\text{mol L}^{-1}$) that Na^+, K^+ -ATPase may be incapable of generating the gradient required for Na^+ uptake. The Na^+ content of freshwater from Stanwell-Fletcher lake (from which the Union River originates) has been reported as 4 mg L^{-1} (approximately 174 $\mu\text{mol L}^{-1}$; de March et al. 1977). Under these conditions, the combined action of Na^+, K^+ -ATPase and H^+ V-ATPase are required to make active Na^+ uptake possible (Kirschner 2004). The relative contribution of each of these pumps to the electrochemical gradient required for Na^+ uptake is not clear but probably varies with tissue, cell type, and expression patterns for the two transporters. In frog skin granular cells, gradients for Na^+ uptake are generated by Na^+, K^+ -ATPase, while in MRCs, the H^+ V-ATPase works in concert with Na^+, K^+ -ATPase (Ehrenfeld and Klein 1997). In freshwater rainbow trout gills, H^+ V-ATPase has been localized to MRCs and pavement cells (Lin et al. 1994).

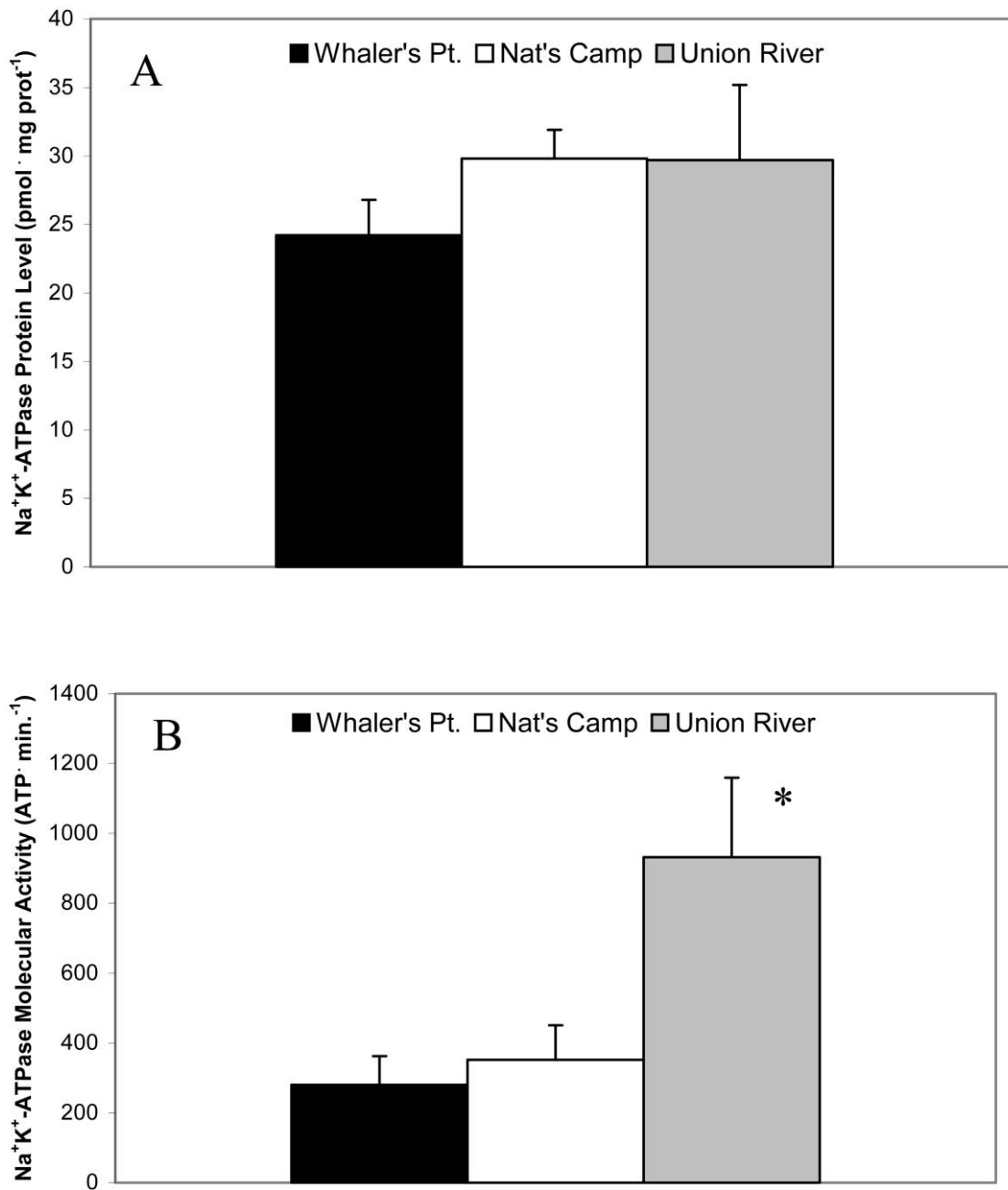


Figure 3. Gill Na⁺,K⁺-ATPase protein expression (A) and molecular activity (B) (mean ± SEM) from arctic char (*Salvelinus alpinus*) collected from Whaler's Point (seawater, 32‰ salinity), Nat's Camp (seawater, 32‰ salinity), and the Union River (freshwater). Molecular activity is calculated based on gill Na⁺,K⁺-ATPase activity assayed at 6°C. Asterisk denotes significant difference from Whaler's Point and Nat's Camp. *n* = 5 for each group.

Regardless of the system described, gill Na⁺,K⁺-ATPase is probably responsible for generating a significant proportion of the electrochemical gradient required for Na⁺ uptake, and therefore, its upregulation probably enhances Na⁺ uptake during freshwater acclimation. An enhanced pattern of Na⁺,K⁺-ATPase upregulation during freshwater acclimation has been described

by Scott et al. (2004b) for a northern population of killifish that ionoregulate better than individuals from a southern population who do not upregulate gill Na⁺,K⁺-ATPase to the same extent. The observed increase in gill Na⁺,K⁺-ATPase activity of arctic char collected from the Union River may also enhance Na⁺ uptake in the freshwater environment.

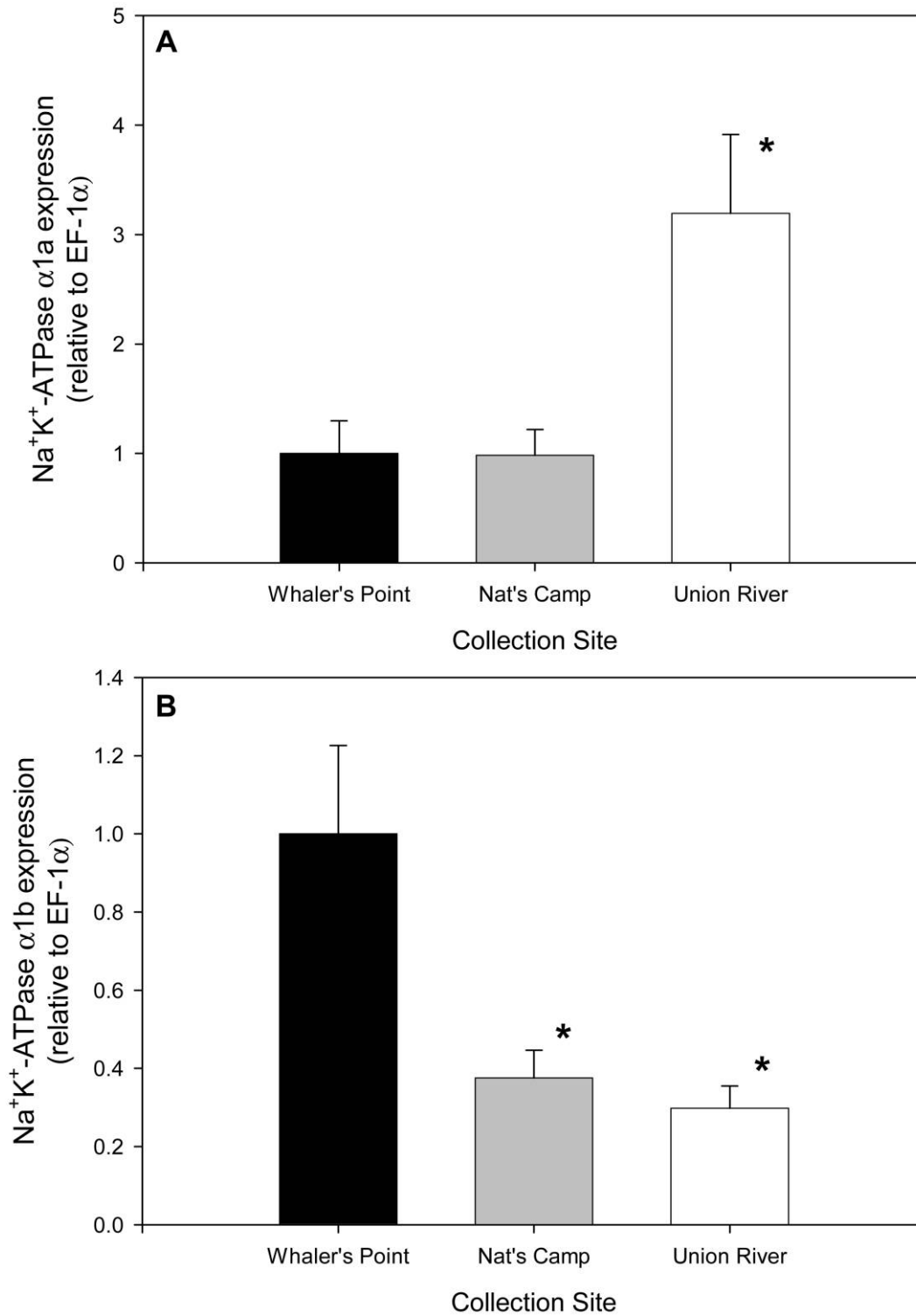


Figure 4. Gill Na⁺K⁺-ATPase α 1a (A) and α 1b (B) isoform mRNA expression in arctic char collected from Whaler's Point (seawater, 32‰ salinity), Nat's Camp (seawater, 32‰ salinity), and the Union River (freshwater). The mRNA expression is relative to the control gene elongation factor 1 α (EF1 α) and normalized to the Whaler's Point group (normalized to a value of 1). A, asterisk denotes significant difference ($P < 0.05$) from Whaler's Point and Nat's Camp; B, asterisks denote significant difference ($P < 0.05$) from Whaler's Point. $n = 8$ for each group.

Table 2: Amounts (relative to the control gene EF1 α), sum, and ratio (mean \pm SEM) of gill Na⁺,K⁺-ATPase isoform α 1a and α 1b mRNA from arctic char collected from Whaler's Point (seawater, 32‰ salinity), Nat's Camp (seawater, 32‰ salinity), and the Union River (freshwater)

Parameter	Whaler's Point	Nat's Camp	Union River
α 1a expression	.3 \pm .1	.3 \pm .1	1.0 \pm .2
α 1b expression	8.8 \pm 2.3 ^a	3.3 \pm .9 ^a	2.6 \pm .5 ^a
Sum (α 1b + α 1a)	9.0 \pm 1.9	3.6 \pm .7 ^b	3.6 \pm .63 ^b
Ratio α 1b : α 1a	57.1 \pm 25.0	19.4 \pm 10.9	3.5 \pm .9 ^b

^a The α 1b expression is significantly different ($P < 0.05$) from the α 1a expression within fish collected from the same site.

^b Denotes significantly different ($P < 0.05$) from Whaler's Point char. For comparisons for each isoform between sites, see Figure 3. $n = 8$ for both groups.

The expression of multiple Na⁺,K⁺-ATPase isoforms may be related to different cellular roles of Na⁺,K⁺-ATPase, which may change under different environmental conditions. The α 1b isoform is clearly upregulated during seawater acclimation of salmonids (Richards et al. 2003; Bystriansky et al. 2006) and downregulated during freshwater acclimation of arctic char in this study. The opposite is true for the α 1a isoform, which is upregulated on entry to freshwater (this study) and quickly reduced during seawater acclimation (Richards et al. 2003; Bystriansky et al. 2006). Whether these two isoforms are functionally different is not yet known, but differences in projected amino acid sequences in several of the membrane-spanning regions (Richards et al. 2003) suggest the two isoforms may interact differently with their basolateral membrane (BLM) environment. We recently showed that gill Na⁺,K⁺-ATPase activity from seawater-acclimated arctic char is negatively correlated to BLM cholesterol content and positively correlated to BLM percent phosphatidylethanolamine and percent 18:2n6, while in freshwater-acclimated char, Na⁺,K⁺-ATPase activity appears to be independent of membrane composition (Bystriansky 2005). In addition, Lingwood et al. (2004, 2005) provides evidence that gill Na⁺,K⁺-ATPase in seawater- but not freshwater-acclimated rainbow trout is associated with BLM lipid raft microdomains and may be regulated by the glycosphingolipid sulfatide, which has been proposed to be a cofactor for Na⁺,K⁺-ATPase (Karlsson 1977). Lingwood et al. (2006) also proposes an interesting mechanism for the non-raft-associated gill Na⁺,K⁺-ATPase in freshwater-acclimated trout in which Na⁺ and K⁺ transport are decoupled, which may improve the efficiency of Na⁺ transport across the BLM. This increased efficiency of basolateral Na⁺ transport by freshwater gill Na⁺,K⁺-ATPase may generate a greater electrochemical gradient for Na⁺ uptake. Such posttranslational membrane-based regulation of Na⁺,K⁺-ATPase may be possible due to the observed sequence differences found in several transmembrane regions of the α 1a and α 1b isoforms (Richards et al. 2003).

In addition, several other rapid modulators of Na⁺,K⁺-ATPase activity are known that may also explain the rapid increase in Na⁺,K⁺-ATPase activity seen in char gill after freshwater exposure. These include several protein kinases that direct phosphorylation/dephosphorylation of Na⁺,K⁺-ATPase and the trafficking of Na⁺,K⁺-ATPase proteins between the plasma membrane and intracellular endosomal pools (reviewed in Blanco and Mercer 1998). Protein kinase A (Tipsmark and Madsen 2001) and C (Crombie et al. 1996) have been shown to alter Na⁺,K⁺-ATPase activity in brown trout *Salmo trutta* and Atlantic cod *Gadus morhua* gills, respectively. Further examination of these potential modulators of gill Na⁺,K⁺-ATPase activity is needed to better understand Na⁺,K⁺-ATPase regulation during salinity acclimation of euryhaline fishes. It is also important to consider that the observed "isoform switching" seen in this study (and others) is not entirely involved in whole-animal Na⁺, K⁺, and Cl⁻ regulation. Na⁺,K⁺-ATPase is ultimately responsible for many cellular processes that depend on Na⁺ and K⁺ gradients (e.g., acid-base balance, cellular uptake of energy-rich metabolites). It is possible that changes in isoform expression are also a response to changes in other cellular requirements seen during salinity acclimation.

The number of Na⁺,K⁺-ATPase molecules in the gill was similar between fish sampled from the three collection sites and therefore does not explain the increased Na⁺,K⁺-ATPase activity seen in the Union River char. Protein densities in arctic char were similar to densities found in other studies on a wide range of animals (Else et al. 1996; Else and Wu 1999). Molecular activities of Na⁺,K⁺-ATPase were very low, but if extrapolated to cover a range of temperatures assuming a Q₁₀ of 3 (determined from Arrhenius plots generated using Union River and Whaler's Point char gills over a 20°C temperature range; data not shown), arctic char Na⁺,K⁺-ATPase molecular activity was similar to or slightly lower than what is seen in other ectotherms, including fish (Else et al. 1996; Else and Wu 1999). The molecular activity of gill Na⁺,K⁺-ATPase from Union River

char was significantly higher than that of char collected in seawater. This increased molecular activity may be indicative of the expression of a different Na⁺,K⁺-ATPase isoform (e.g., $\alpha 1a$) and/or may have been caused by certain modulators of Na⁺,K⁺-ATPase activity (e.g., protein kinases) and/or may be due to changes in the composition and properties of the gill BLM. Migration from the ocean to freshwater that is 6°C warmer would probably cause a change in gill BLM composition and fluidity. The effects of seawater acclimation on arctic char (Bystriansky 2005) and rainbow trout (Lingwood et al. 2004, 2005) BLMs discussed earlier may also induce changes in the molecular activity of gill Na⁺,K⁺-ATPase. Several other studies have also found that membrane composition and its properties may confer a change in the molecular activity of Na⁺,K⁺-ATPase (Else and Wu 1999; Wu et al. 2001, 2004).

Most anadromous salmonids, including arctic char (Aas-Hansen et al. 2005), are known to undergo a process of smoltification before migration from freshwater to seawater (see Hoar 1988 for review). The process involves many physiological changes that prepare them for seawater acclimation, including upregulation of gill Na⁺,K⁺-ATPase. The results of this study suggest that arctic char may undergo a "reverse smoltification" before migrating up river. The expression of the $\alpha 1b$ isoform was significantly reduced in arctic char collected while still in seawater at Nat's Camp (compared with the Whaler's Point fish), to levels similar to those seen in fish collected from the Union River. This suggests that there is anticipation for a reduced requirement for the $\alpha 1b$ protein in the near future. Arctic char collected at Nat's Camp were only ~500 meters from the river mouth and could easily move in and out of the river outflow. The movement in and out of the estuary may be an important behavioral adaptation that allows time for necessary physiological modifications to occur that are needed for successful acclimation to freshwater. If isoform $\alpha 1b$ is specialized for ion excretion, a reduction in its expression might cause these fish to exhibit signs of reduced osmoregulatory capacity while in seawater. However, plasma ion and osmolality levels in these fish were maintained at levels very similar to Whaler's Point char. Interestingly, the Union River char were found to have higher plasma Na⁺ and total osmolality than the seawater char collected from the other two sites. Typically, fish moving to an environment with reduced salinity experience a rapid drop in plasma ions and total osmolality (e.g., Jensen et al. 1998). One explanation for this observation is that char preparing to go upriver reduce their active secretion of ions by reducing expression of the $\alpha 1b$ isoform. This leads to the slow accumulation of salts and a rise in plasma osmolality. Once plasma osmolality increases to a threshold level, char move into the river, carrying with them a reserve of Na⁺ that may compensate for the expected decrease in plasma osmolality that they will encounter in freshwater. Associated with the movement into freshwater is the rapid increase in $\alpha 1a$ expression, the isoform that is quickly shut down during seawater accli-

mation (Richards et al. 2003; Bystriansky et al. 2006). This isoform may be specialized to function in the gill active ion uptake model in freshwater. The increased expression of $\alpha 1a$ may also occur while the fish are still in the estuary. Increased expression of this protein and the net reversal of gill ion flux from an outward to an inward direction would also explain the elevated plasma osmolality and Na⁺ levels. However, this does not explain why Cl⁻ levels were not similarly elevated in the river fish, since the increase in Na⁺ accounts for nearly all of the observed increase in plasma osmolality. The increase in plasma Na⁺ levels should be balanced by an equivalent increase in a counteracting anion; however, Cl⁻ levels did not change, suggesting another anion may have increased. An increase in plasma HCO³⁻ (or a decrease in plasma H⁺) would suggest an acid-base imbalance in these fish. The mechanisms for Na⁺ and Cl⁻ uptake are known to be independent in freshwater fish (Maetz and Garcia Romeu 1964), and Galvez et al. (2002) have proposed independent models for active Na⁺ and Cl⁻ uptake by freshwater rainbow trout by α and β MRCs. It is also possible that modifications in gill ion permeability or changes in drinking rates may have led to the observed increase in plasma Na⁺ levels. Interestingly, Morisawa et al. (1979) found that naturally migrating chum salmon collected within 12 h of entering freshwater had plasma Na⁺ levels similar to fish collected from the ocean, while plasma Cl⁻ concentration was significantly decreased.

The idea that salmonids undergo a reverse smoltification before migrating into freshwater is also supported by the work of Uchida et al. (1997) and Hirano et al. (1990). In these studies, chum salmon returning from sea lose their osmoregulatory capacity if they are maintained in seawater and die due to increased plasma osmolality, while salmon transferred to freshwater are able to regulate their plasma osmolality and survive. Chum salmon maintained in seawater also experience a decrease in gill Na⁺,K⁺-ATPase activity and lamellar chloride cell number (Uchida et al. 1997), opposite to what is seen in the same species as fry during smoltification (Uchida et al. 1996). This decrease in gill Na⁺,K⁺-ATPase may be due to a down-regulation in Na⁺,K⁺-ATPase $\alpha 1b$ isoform expression without a concomitant rise in $\alpha 1a$. Interestingly, chum salmon developed distinct vertical bars on their sides while still in seawater before moving upriver, which sounds reminiscent of the parr marks seen in juvenile salmon before smoltification. Also of note, Nilssen et al. (1997) found that arctic char that returned from the ocean and were collected in freshwater displayed a decreasing capacity for seawater acclimation (based on plasma Na⁺ and osmolality levels after a 24-h seawater challenge test) the longer they had been in freshwater. Despite this, Nilssen et al. (1997) concluded that arctic char retained their seawater tolerance even after 3 wk in freshwater and that their return to freshwater is not related to any decreased seawater tolerance. If reverse smoltification occurs, once triggered, it may be irreversible, with entry to freshwater necessary for survival. This

would explain the observed “winter mortality” of arctic char maintained in seawater beyond the time of year they would normally return to freshwater (Gjedrem 1975). For arctic char maintained in seawater in winter, gill Na^+, K^+ -ATPase activity is depressed and osmoregulatory capacity is reduced as indicated by high plasma osmolality (Staurnes 1993). Seasonal changes in gill Na^+, K^+ -ATPase activity and isoform-specific expression may make overwintering in freshwater necessary for survival of arctic char and other *Salvelinus* spp.

The results of this study, as well as those of Shrimpton et al. (2005), suggest that gill Na^+, K^+ -ATPase may play a more important role in freshwater acclimation of salmonid fishes than was previously appreciated. Reciprocal expression of $\alpha 1a$ and $\alpha 1b$ gill Na^+, K^+ -ATPase α -subunit isoforms appear to be an integral part of salinity acclimation. Further study is needed to determine isoform-specific protein expression and to understand what independent roles the different isoforms may play under different environmental conditions.

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