# Failure to up-regulate gill $Na^+$,$K^+$-ATPase α-subunit isoform α1b may limit seawater tolerance of land-locked Arctic char (*Salvelinus alpinus*)☆

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## Abstract

Many populations of Arctic char (*Salvelinus alpinus*) are land-locked, physically separated from the ocean by natural barriers and unable to migrate to sea like anadromous populations. Previous studies which experimentally transferred land-locked Arctic char to seawater report high mortality rates due to osmoregulatory failure and an inability to up-regulate gill $Na^+$,$K^+$-ATPase activity. This study examined the mRNA expression of two recently discovered α-subunit isoforms of gill $Na^+K^+$-ATPase (α1a and α1b) during seawater exposure of land-locked Arctic char. mRNA levels of these gill $Na^+$,$K^+$-ATPase α-subunit isoforms were compared to $Na^+$,$K^+$-ATPase activity and protein levels and related to osmoregulatory performance. Land-locked Arctic char were unable to regulate plasma osmolality following seawater exposure. Seawater exposure did not induce an increase in gill $Na^+$,$K^+$-ATPase activity or protein levels. $Na^+$,$K^+$-ATPase isoform α1a mRNA quickly decreased upon exposure to seawater, while isoform α1b levels were unchanged. These results suggest the inability of land-locked Arctic char to acclimate to seawater is due a failure to up-regulate gill $Na^+$,$K^+$-ATPase activity which may be due to their inability to increase $Na^+$,$K^+$-ATPase α1b mRNA expression.
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## 1. Introduction

Many species of salmonid fishes are euryhaline, able to migrate between freshwater and seawater environments. Of the three major salmonid genera, it is generally accepted that *Oncorhynchus* and *Salmo* species have a greater capacity for seawater acclimation than members of the genus *Salvelinus* (Hoar, 1976, 1988). Of the anadromous salmonids, Arctic char (*Salvelinus alpinus*) are considered to have the lowest tolerance for seawater (Rounsefell, 1958 discussed in Hoar, 1976), based on the fact that they do not migrate to sea until they are at least 10–17 cm, which can take 2–9 years in many Arctic lakes (Johnson, 1980). When Arctic char migrate to sea in the late spring or early summer they remain in seawater for only a few weeks or months before returning to freshwater in the fall to overwinter (Johnson, 1980). Arctic char are considered optionally anadromous (Hoar, 1976), as many individuals do not go to sea every year, choosing to remain in freshwater through the summer. The reason why some individuals do not migrate to sea to take advantage of the rich food resources of the ocean is not clear, but may relate to their seawater hardiness. In addition, many Arctic char populations are land-locked, separated from the sea by natural barriers. This is mainly due to the iso-static rebound of land since the last glaciation (Wilson et al., 1996), which has eliminated natural connections of many Arctic lakes to the ocean. This phenomenon has isolated populations from the sea, supplying us with an interesting natural experiment to examine if seawater tolerance has been lost in such populations.

Most studies suggest the seawater tolerance of land-locked populations of Arctic char is diminished compared to anadromous populations (Roberts, 1971; Staurnes et al., 1992;

Eliassen et al., 1998). However, Schmitz (1995) concluded that land-locked Arctic char have retained their ability to tolerate seawater, and Roberts (1971) suggests that land-locked populations can tolerate salinities of up to 25‰. The limited seawater tolerance of land-locked Arctic char may be related to an insufficient up-regulation of gill $Na^+,K^+$-ATPase activity. Gill $Na^+,K^+$-ATPase activity increases during the acclimation of most euryhaline fish species to seawater (McCormick, 1996), including anadromous Arctic char (Finstad et al., 1989; Staurnes et al., 1992; Bystriansky et al., 2006; Bystriansky and Ballantyne, 2007), and is thought to be ultimately responsible for regulating plasma $Na^+$ and $Cl^-$ levels. However, exposure of land-locked (Staurnes et al., 1992; Eliassen et al., 1998) or resident (Arnesen et al., 1995) Arctic char to seawater does not induce the expected increase in gill $Na^+,K^+$-ATPase activity. This suggests the limited (or nil) seawater tolerance of land-locked Arctic char is due to their inability to increase gill $Na^+,K^+$-ATPase activity following seawater exposure.

We have recently shown that the acclimation of an anadromous strain of Arctic char, to seawater involves the differential mRNA expression of two gill $Na^+,K^+$-ATPase α-subunit isoforms (Bystriansky et al., 2006). Isoform α1a levels quickly decrease following seawater exposure while isoform α1b levels increase and precede the observed increase in gill $Na^+,K^+$-ATPase typically seen when anadromous salmonids acclimate to seawater. This same pattern of gill $Na^+,K^+$-ATPase α-subunit isoform regulation appears to be conserved between anadromous salmonids as it is also seen in rainbow trout (Richards et al., 2003) and Atlantic salmon (Bystriansky et al., 2006) during seawater acclimation. This relationship suggests the α1b isoform may be involved in the gill active ion excretion mechanism of marine fishes and may therefore be responsible for increased seawater tolerance. If this is true we might expect that land-locked Arctic char, which have a limited ability to acclimate to seawater, do not increase the expression of the α1b isoform upon exposure to seawater. This study determined if mRNA expression of these gill $Na^+,K^+$-ATPase isoforms are similarly regulated in land-locked Arctic char during seawater exposure. Gill mRNA expression is compared to $Na^+,K^+$-ATPase protein levels and activity in an attempt to explain the limited seawater tolerance of land-locked Arctic char populations.

## 2. Materials and methods

### 2.1. Experimental procedure

Twenty-eight Arctic char ($31.2 \pm 1.5$ cm) were caught by rod and reel in land-locked Small Lake, approximately 8 km from the town of Resolute Bay, Nunavut, Canada on July 22–23, 2002. Average fight time was less than 2 min. Char that took more than 3 min to catch or those that looked exhausted were not used in the experiment and released. Lake surface water temperature was ~2 °C. Char were gently placed in large coolers filled with Small lake water and transferred by truck to the Department of Fisheries and Oceans Resolute Bay laboratory. Char were placed in an outdoor 2-meter diameter fiberglass tank filled with water obtained from nearby Char Lake. The freshwater holding tank

was a static system since the nearest freshwater (Char lake) was approximately 2 km away, but a pump circulated the water from the holding tank to a second smaller header tank to maintain oxygen levels. One quarter of the water was changed daily and sea ice was supplemented to the tank to maintain the temperature at ~0 °C on days when air temperatures were above freezing. The salinity of the holding tank was checked regularly to ensure the addition of the sea ice did not introduce salt contamination, but was always found to remain at 0‰. Char were maintained under these conditions for 5 days to allow for acclimation to the tank and to allow recovery from the stress associated with their collection/transfer. There were no mortalities and all fish appeared to be in good condition at the start of the experiment. Throughout the collection and experiment photoperiod was 24 h daylight at this latitude.

Pumps were placed in Resolute Bay by S.C.U.B.A. below ice scour levels and deep enough to ensure that water collected was full strength seawater (32‰). The pumping rate of seawater varied due to the height of the tide, with higher pumping rates occurring at high tide. Ice was removed from the holding tank and seawater was then pumped into the header tank, which then entered the holding tank (via the circulating pump). Water was allowed to over flow a standpipe in the holding tank and the freshwater in the system was gradually replaced by seawater. Full water turnover took 2.5 h, and for the remainder of the experiment, the holding tank was continually supplied with fresh seawater. The temperature in the holding tank fluctuated between $-0.5$ °C and $0.8$ °C and was dependant on seawater pumping rate and air temperature. Six char were collected from the holding tank immediately before seawater exposure began and acted as a control group. Six char were also sampled after each of 1, 2 and 7 days of seawater exposure. There were no significant differences in fish size between experimental groups. The experimental design is the best possible given time constraints and limited equipment (pumps, tanks etc.). At each sampling time, six char were collected from the holding tank and a blood sample was taken by caudal puncture with a heparinized (500 U/mL) syringe. Char were then killed by a blow to the head and gill samples were taken and immediately frozen in a liquid nitrogen chilled cryoshipper. Blood samples were immediately centrifuged for 5 min ($3000 \times g$, 4 °C) and plasma was removed and frozen in the cryoshipper. Samples remained in the cryoshipper until they were transported to the University of Guelph (Guelph, ON, Canada) and stored at $-80$ °C until analyzed.

### 2.2. Determination of plasma osmolality levels

Plasma osmolality was determined using a vapour pressure osmometer (Model 5500, Wescor, Utah, USA).

### 2.3. Determination of $Na^+,K^+$-ATPase activity

Gill filaments were homogenized on ice in SEI buffer (pH = 7.5, 150 mmol $L^{-1}$ sucrose, 10 mmol $L^{-1}$ EDTA, 50 mmol $L^{-1}$ imidazole) using a ground glass homogenizer. Homogenates were centrifuged for 30 s (4 °C) at $5000 \times g$ 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 method 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 $\varepsilon_{340}=6.22$) between the two conditions was used to calculate $Na^+,K^+$-ATPase activity. Optimal assay conditions to give maximal enzyme activity were determined as; 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 $L^{-1}$ phosphoenolpyruvate, 0.2 mmol $L^{-1}$ NADH, 4U LDH and 5U PK, pH 7.5. $Na^+,K^+$-ATPase activity is expressed as $\mu$mol ADP·$h^{-1}$·mg protein$^{-1}$. Maximal $Na^+,K^+$-ATPase activity was determined using a Cary 300bio spectrophotometer (Varian Inc., Palo Alto, CA, USA), equipped with pelletier controlled thermostated cell changer maintained at 1 °C.

## 2.4. Determination of $Na^+,K^+$-ATPase protein levels

Gill $Na^+,K^+$-ATPase protein levels were determined by the method of Else and Wu (1999) by monitoring binding of $^3H$-ouabain (0.588 TBq mmol$^{-1}$, obtained from Perkin Elmer, Boston, MA) 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 $\mu$L added to 250 $\mu$L of incubation medium containing (10 mmol $L^{-1}$ $NaH_2PO_4$, 5 mmol $L^{-1}$ $MgCl_2$, $5\times10^{-5}$ mol $L^{-1}$ unlabelled ouabain plus $1.5\times10^{-7}$ 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 $\mu$L of incubation medium containing (10 mmol $L^{-1}$ $NaH_2PO_4$, 5 mmol $L^{-1}$ $MgCl_2$, $10^{-2}$ mol $L^{-1}$ unlabelled ouabain plus
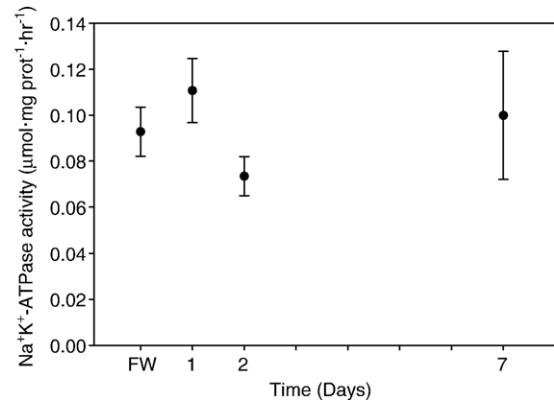
Fig. 2. Gill $Na^+,K^+$-ATPase activity (mean$\pm$S.E.M.) in Arctic char (*Salvelinus alpinus*) held in freshwater (FW) and following transfer to seawater (32‰) for 7 days. No significant differences found. $n=6$ for each group.

$1.5\times10^{-7}$ 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 (4000$\times$g for 5 min) and $Na^+,K^+$-ATPase remained on filters. Filters were washed five times with 50 $\mu$L of wash solution (10 mmol $L^{-1}$ $NaH_2PO_4$, 5 mmol $L^{-1}$ $MgCl_2$, 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) and left in the dark overnight. Vials were read using a Beckman LS 6500 multi-purpose scintillation counter (Beckman Instruments, Fullerton, CA, USA) with DPM correction. Ouabain was assumed to bind to $Na^+,K^+$-ATPase in a 1:1 ratio and $Na^+,K^+$-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 pmol $Na^+,K^+$-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 when between four and six washes (at 50 $\mu$L) were used and returned consistent values for NSB tubes. $Na^+,K^+$-ATPase number is expressed as pmol $Na^+,K^+$-ATPase·mg protein$^{-1}$. Molecular activities (ATP min$^{-1}$) were calculated using $Na^+,K^+$-ATPase activities determined at 1 °C. Protein content of tissue homogenates for $Na^+,K^+$-ATPase activity and number were determined using the Bio-Rad standard protein assay (Bio-Rad Laboratories, Hercules, CA, USA), standardized with bovine serum albumin (BSA).

## 2.5. Determination of $Na^+,K^+$-ATPase $\alpha$-subunit mRNA levels

Total RNA was extracted from gill samples using TriPure Isolation Reagent (Boeringer Mannheim) following the guanidine thiocyanate method (Chomczynski and Sacchi, 1987). Isolated total RNA was quantified spectrophotometrically and run (2 $\mu$g) on an agarose gel (1%) to check for RNA integrity. First strand cDNA was synthesized from 2 $\mu$g of total RNA using oligo(dT$_{15}$) primer and RevertAid™ H Minus M-MuLV reverse transcriptase
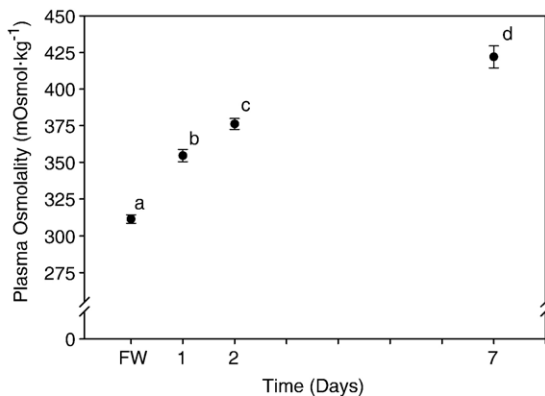
Fig. 1. Plasma osmolality (mean$\pm$S.E.M.) of Arctic char (*Salvelinus alpinus*) held in freshwater (FW) and following transfer to seawater (32‰) for 7 days. Data points with different letters are significantly different. $n=6$ for each group.

Table 1
Gill Na$^+$,K$^+$-ATPase protein levels and molecular activity (mean±S.E.M.) from land-locked Arctic char (*Salvelinus alpinus*) in freshwater (control) or exposed to seawater (SW, 32‰) for up to 7 days

| Parameter | Freshwater | SW — 1 day | SW — 2 days | SW — 7 days |
|---|---|---|---|---|
| Na$^+$,K$^+$-ATPase protein levels (pmol·mg protein$^{-1}$) | 8.0±3.7 | 9.5±1.9 | 11.0±4.5 | 13.7±3.4 |
| Na$^+$,K$^+$-ATPase molecular activity (ATP·min$^{-1}$) | 173.4±35.4 | 232.5±49.8 | 179.5±51.3 | 151.1±36.4 |

Na$^+$,K$^+$-ATPase molecular activity based on activity measurements determined at 1 °C.
$n=5$ for each group, no significant differences found.

following the manufacturer's instructions (MBI Fermentas). 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 μL of cDNA, 150 pmoles 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 α1a and α1b isoforms and for the control gene elongation factor 1α (EF1α) (Bystriansky et al., 2006). Primer sequences were as follows: Na$^+$,K$^+$-ATPase α1a forward 5′ GGC CGG CGA GTC CAA T 3′, Na$^+$,K$^+$-ATPase α1a reverse 5′ GAG CAG CTG TCC AGG ATC CT 3′ (product size 66bp); Na$^+$,K$^+$-ATPase α1b forward 5′ CTG CTA CAT CTC AAC CAA CAA CAT T 3′, Na$^+$, K$^+$-ATPase α1b reverse 5′ CAC CAT CAC AGT GTT CAT TGG AT 3′ (product size 81bp); EF1α 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 71bp). 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 2 individuals (1 freshwater, 1 seven day seawater) for 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) 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) at the University of Guelph, Molecular Supercenter (Guelph, ON, Canada). 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 1:4286 starting copies for Na$^+$,K$^+$-ATPase isoform α1a, 1:6356 starting copies for Na$^+$,K$^+$-ATPase isoform α1b and 1:6426 starting copies for EF1α. Relative quantities of each target gene were determined using the comparative $C_T$ method (Applied Biosystems Inc., 2001). The relative quantity of Na$^+$,K$^+$-ATPase α1a and α1b mRNA in gill samples was normalized to an endogenous reference (EF1α) and expressed relative to the mean value for char acclimated to freshwater (controls) according to the formula:

$$Amount = 2^{-\Delta\Delta C_T}$$

where $^{\Delta\Delta}$ refers to the difference in levels between the target gene and EF1α and the relative levels relative to the mean value for the freshwater (control) 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
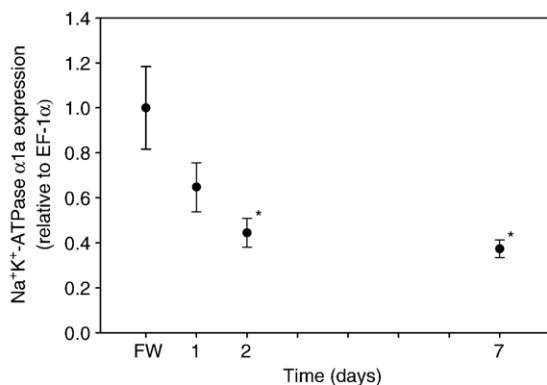


Fig. 3. Gill Na$^+$,K$^+$-ATPase α1a mRNA levels (mean±S.E.M.) in Arctic char (*Salvelinus alpinus*) held in freshwater (FW) or following transfer to seawater (32‰) for 7 days. mRNA levels are relative to the control gene EF1α (elongation factor 1α) with the FW (control) group standardized to equal an level of one. * denotes group is significantly different from FW (control) group. $n=6$ for each group.
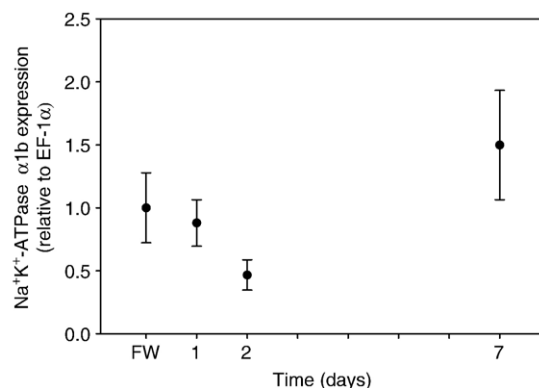


Fig. 4. Gill Na$^+$,K$^+$-ATPase α1b mRNA levels (mean±S.E.M.) in Arctic char (*Salvelinus alpinus*) held in freshwater (FW) or following transfer to seawater (32‰) for 7 days. mRNA levels are relative to the control gene EF1α (elongation factor 1α) with the FW (control) group standardized to equal a level of one. No significant differences found. $n=6$ for each group.

Table 2
Amounts (relative to control gene EF1α), sum and ratio of gill Na$^+$,K$^+$-ATPase isoform α1a and α1b mRNA from Arctic char (*Salvelinus alpinus*) collected in freshwater (control) and after 1, 2 and 7 days of seawater (SW, 32‰) exposure

| Parameter | Freshwater | SW — 1 day | SW — 2 days | SW — 7 days |
|---|---|---|---|---|
| α1a | 0.41±0.07 | 0.28±0.05 | 0.21±0.03 | 0.16±0.02* |
| α1b | 0.63±0.18 | 0.53±0.12 | 0.29±0.08 | 0.94±0.29 |
| Sum (α1b+α1a) | 1.03±0.22 | 0.81±0.15 | 0.49±0.09 | 1.11±0.30 |
| Ratio α1b : α1a | 1.59±0.41 | 1.90±0.36 | 1.43±0.29 | 5.39±1.19[a] |

* denotes α1a level is significantly lower ($p < 0.05$) than α1b level for fish exposed to seawater for 7 days. [a] denotes α1b:α1a ratio is significantly different ($p < 0.05$) from all other sampling times. Comparisons made over time series for each isoform found in Figs. 3 and 4.
$n = 6$ for each group.

amounts (using a standard curve ranging from 1 µl to 1 µl of a 625×dilution) (Applied Biosystems Inc., 2001). All samples were run in duplicate.

## 2.6. Chemicals

Chemicals not identified 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.

## 2.7. Statistical analysis

All data are presented as means±S.E.M. Sample size for all four groups was 6 for each parameter determined except for Na$^+$,K$^+$-ATPase number and molecular activity which was determined with $n = 5$ for each group (chosen at random). Comparisons of plasma osmolality, gill Na$^+$,K$^+$-ATPase activity, isoform mRNA levels and Na$^+$,K$^+$-ATPase protein levels for the four groups were performed using a one-way analysis of variance (ANOVA). When required, a Tukey multiple comparison test was used to determine significance. Comparisons of relative amounts of Na$^+$,K$^+$-ATPase α1a and α1b isoform mRNA within each group were determined using an appropriate Students *t*-test. For all comparisons $p < 0.05$ was considered significant.

## 3. Results

No char died during their collection, transport or acclimation to the experimental tank. Following seawater exposure, four mortalities occurred, one died on day two, two on day three and one on day five of seawater acclimation presumably due to osmoregulatory failure. Arctic char plasma osmolality increased significantly over the time course of salinity exposure (Fig. 1), from 311.6±2.9 mOsmol·kg$^{-1}$ for char in freshwater to 422.0± 7.5 mOsmol·kg$^{-1}$ following seven days in seawater. Gill Na$^+$, K$^+$-ATPase activity of Arctic char transferred to seawater was not significantly different from control fish at any time following seawater exposure (Fig. 2). The same was true for gill Na$^+$,K$^+$-ATPase protein levels as control char had similar

levels to those exposed to seawater (Table 1). Molecular activity of gill Na$^+$,K$^+$-ATPase from Arctic char exposed to seawater was also not significantly different from freshwater acclimated char (Table 1).

Expression of mRNA for both the α1a and α1b isoform of Na$^+$,K$^+$-ATPase was detected in gill from both freshwater acclimated and seawater exposed Arctic char. Expression of the α1a isoform was found to be highest in freshwater acclimated char. Seawater exposure induced a rapid reduction in isoform α1a mRNA, with levels being significantly depressed after 2 and 7 days in seawater (Fig. 3). mRNA levels of the α1b isoform were not different between freshwater and seawater exposed Arctic char (Fig. 4). The relative amounts of the α1a and α1b isoforms were not different in char acclimated to freshwater, nor in fish exposed to seawater for 1 or 2 days (Table 2). By 7 days of seawater exposure, the relative amount of α1a was found to be significantly lower than α1b. This led to a significantly higher ratio of α1b to α1a in Arctic char exposed to seawater for 7 days compared to the other three groups (Table 2).

## 4. Discussion

The successful acclimation of salmonid fishes to seawater typically involves a crisis and stabilization phase of ionoregulatory adjustment (Gordon, 1959). During the crisis phase, plasma Na$^+$ and Cl$^-$ levels quickly rise due to salt absorption from ingested seawater. To regulate this salt load, the physiology of the gill changes from a system that actively accumulates salts to one that actively secretes Na$^+$ and Cl$^-$ (Eddy, 1982). As the osmoregulatory capacity of the gill is increased, plasma osmolality peaks, then declines and stabilizes at a new steady state. The stabilization of plasma ion levels at an acceptable level indicates the successful acclimation to seawater. The exposure of land-locked Arctic char to seawater in this study induced a rapid and significant rise in plasma osmolality to over 420 mOsmol·kg$^{-1}$ within 7 days. A rise in plasma osmolality to this degree suggests osmoregulatory problems as the upper lethal plasma osmolality for rainbow trout is thought to be approximately 425 mOsmol·kg$^{-1}$ (Conte and Wagner, 1965). Typically, the crisis period for anadromous Arctic char lasts up to 96 h with peak plasma osmolality reaching levels of 340–380 mOsmol·kg$^{-1}$ (Arnesen et al., 1992; 1995 Halvorsen et al., 1993; Dumas et al., 1995; Nilssen et al., 1997; Bystriansky

et al., 2006) before slowly declining over many days. It is clear the Arctic char used in this study were not successfully acclimating to seawater. The inability of land-locked Arctic char to acclimate to full strength seawater has also been shown in several other studies (Roberts, 1971; Staurnes et al., 1992; Eliassen et al., 1998) and suggests that these populations may have lost their capacity to acclimate to seawater. This leads us to question what limits the seawater tolerance of land-locked Arctic char.

The inability of land-locked Arctic char to up-regulate gill Na$^+$,K$^+$-ATPase activity following seawater exposure is the most likely cause of osmoregulatory failure. Gill Na$^+$,K$^+$-ATPase activity is increased in nearly all euryhaline fish species during acclimation from freshwater to seawater (McCormick, 1996), including anadromous Arctic char (Finstad et al., 1989; Staurnes et al., 1992; Arnesen et al., 1995; Eliassen et al., 1998; Bystriansky et al., 2006, Bystriansky and Ballantyne, in press). This inability to up-regulate gill Na$^+$,K$^+$-ATPase activity following seawater exposure has also been observed in several other studies of resident (Arnesen et al., 1995) and land-locked (Staurnes et al., 1992; Eliassen et al., 1998) Arctic char. Na$^+$,K$^+$-ATPase activity can be up-regulated in several ways including increased protein number or molecular activity. In the present study, neither gill Na$^+$,K$^+$-ATPase protein levels nor molecular activity increase following seawater exposure. However, during the acclimation of anadromous Arctic char to seawater, gill Na$^+$,K$^+$-ATPase protein levels are increased facilitating an increase in enzyme activity (Bystriansky et al., 2006). The failure of land-locked Arctic char to increase Na$^+$,K$^+$-ATPase activity may be due to several factors including an inability to increase mRNA transcription and/or protein translation. Increased gill Na$^+$,K$^+$-ATPase activity during seawater acclimation of salmonid fish is typically preceded by an increase in mRNA expression for the protein (Seidelin et al., 2000; Tipsmark et al., 2002). We have recently shown that rainbow trout gills express mRNA for four distinct Na$^+$,K$^+$-ATPase α-subunit isoforms (Richards et al., 2003). The α1a and α1b isoforms are expressed at significantly higher levels than the α1c and α3 isoforms and are differentially regulated during salinity acclimation of rainbow trout (Richards et al., 2003). Acclimation of anadromous rainbow trout, Atlantic salmon and Arctic char from freshwater to seawater involves a rapid decrease in α1a mRNA levels and an increase in α1b levels (Richards et al., 2003; Bystriansky et al., 2006). This increase in α1b mRNA precedes a similar increase in gill Na$^+$,K$^+$-ATPase activity and protein expression (Bystriansky et al., 2006), suggesting α1b is the isoform responsible for the active secretion of Na$^+$ and Cl$^-$ by marine fish gills. The opposite pattern of gill mRNA expression is seen in anadromous Arctic char during their migration from the ocean into freshwater, as mRNA levels of the α1a isoform increase upon freshwater exposure while levels of the α1b isoform decrease just prior to movement up river (Bystriansky et al., in press). Similar to anadromous Arctic char, the land-locked char in the present study were found to reduce mRNA levels of the α1a isoform following seawater exposure. This suggests the α1a isoform is less important in the marine environment. What we do not see in this study is the expected rise in α1b mRNA levels following seawater acclimation. This may explain why Na$^+$,K$^+$-ATPase protein levels and overall activity

are not up-regulated during seawater exposure of land-locked Arctic char. If the up-regulation of isoform α1b is required for successful seawater acclimation, the failure to increase mRNA expression of α1b may explain the reduced osmoregulatory capacity of land-locked Arctic char in this study following seawater exposure.

Interestingly, mRNA for the α1b isoform is still expressed in land-locked Arctic char in both freshwater and seawater environments. This suggests the α1b isoform may have other cellular roles not associated with salt excretion. Further examination of this isoform in land-locked Arctic char or in stenohaline freshwater salmonid species may grant further insight into the role of this Na$^+$,K$^+$-ATPase isoform. The ratio of α1b to α1a expression was slightly above unity for both freshwater acclimated and seawater exposed Arctic char. The increase seen in this ratio following 7 days in seawater is explained by the declining levels of α1a and not due to any significant increase in α1b mRNA. In contrast, the ratio of α1b to α1a in gills of wild anadromous Arctic char living in seawater is ~57 (Bystriansky et al., 2007), suggesting that the relative expression of mRNA for the α1b isoform is quite low in land-locked Arctic char, even after 1 week in seawater.

The limited capacity for seawater acclimation of these land-locked Arctic char may have also been related to the timing of this study. The osmoregulatory capacity of Arctic char acclimating to seawater has been shown to be greatest during the spring and summer and much lower in the winter months (Finstad et al., 1989; Arnesen et al., 1992; Staurnes et al., 1992; Staurnes, 1993). Other studies indicate anadromous populations of Arctic char (Eliassen et al., 1998; Aas-Hansen et al., 2005) make preparatory increases in gill Na$^+$,K$^+$-ATPase activity while still in freshwater during the spring and summer months akin to the parr-smolt transformation seen in other anadromous salmonids (Hoar, 1988). Eliassen et al. (1998) also offers evidence that this preparatory increase in gill Na$^+$,K$^+$-ATPase activity may occur in the land-locked Skjomen population of Arctic char during the months of May and June. As this study was conducted in late July, the char may not have been optimally prepared to acclimate to seawater. However, Schmitz (1995) does suggest the seawater adaptability of land-locked char is similar to anadromous strains, remaining high until the end of July or into early August.

The inability to up-regulate gill Na$^+$,K$^+$-ATPase activity is the likely cause of osmoregulatory failure in land-locked Arctic char exposed to seawater in this study. The failure to increase gill Na$^+$,K$^+$-ATPase activity may be caused by the inability to increase mRNA expression of the α1b isoform. It should be noted, that one individual in the 7-day seawater exposed group did have a relatively high gill Na$^+$,K$^+$-ATPase activity (0.24 μmol·mg protein$^{-1}$·h$^{-1}$), Na$^+$,K$^+$-ATPase protein level (23.8 pmol·mg protein$^{-1}$) α1b mRNA expression (1.45 relative to EF1a) and α1b to α1a ratio (9.13) when compared to all other individuals in this study. That individual also had the lowest plasma osmolality (395 mOsmol·kg$^{-1}$) in the 7-day seawater exposed group. This suggests that seawater tolerance of land-locked Arctic char may not be completely lost from this population. Further investigation into the isoform specific roles

of gill Na⁺,K⁺-ATPase in both freshwater and seawater ionoregulation is required to understand the capacity of land-locked populations of Arctic char to acclimate to seawater.

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