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Gill Na^+ - K^+ -ATPase activity correlates with basolateral membrane lipid composition in seawater- but not freshwater-acclimated Arctic char (*Salvelinus alpinus*)

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Bystriansky JS, Ballantyne JS. Gill Na^+ - K^+ -ATPase activity correlates with basolateral membrane lipid composition in seawater- but not freshwater-acclimated Arctic char (*Salvelinus alpinus*). *Am J Physiol Regul Integr Comp Physiol* 292: R1043–R1051, 2007. First published October 12, 2006; doi:10.1152/ajpregu.00189.2005.—The successful migration of euryhaline teleost fish from freshwater to seawater requires the upregulation of gill Na^+ - K^+ -ATPase, an ion transport enzyme located in the basolateral membrane (BLM) of gill chloride cells. Following 39 days of seawater exposure, Arctic char had similar plasma sodium and chloride levels as individuals maintained in freshwater, indicating they had successfully acclimated to seawater. This acclimation was associated with an eightfold increase in gill Na^+ - K^+ -ATPase activity but only a threefold increase in gill Na^+ - K^+ -ATPase protein number, suggesting that other mechanisms may also modulate gill Na^+ - K^+ -ATPase activity. We therefore investigated the influence of membrane composition on Na^+ - K^+ -ATPase activity by examining the phospholipid, fatty acid, and cholesterol composition of the gill BLM from freshwater- and seawater-acclimated Arctic char. Mean gill BLM cholesterol content was significantly lower (~22%) in seawater-acclimated char. Gill Na^+ - K^+ -ATPase activity in individual seawater Arctic char was negatively correlated with BLM cholesterol content and positively correlated with %phosphatidylethanolamine and overall %18:2n6 (linoleic acid) content of the BLM, suggesting gill Na^+ - K^+ -ATPase activity of seawater-acclimated char may be modulated by the lipid composition of the BLM and may be especially sensitive to those parameters known to influence membrane fluidity. Na^+ - K^+ -ATPase activity of individual freshwater Arctic char was not correlated to any membrane lipid parameter measured, suggesting that different lipid-protein interactions may exist for char living in each environment.

salinity; phospholipid; cholesterol; fatty acid

GILLS PLAY A PIVOTAL ROLE in the osmoregulation of both freshwater and marine teleost fishes. The gill epithelium is the site of active ion uptake in freshwater fishes and active sodium and chloride excretion in marine fishes (14). In both environments, the transport of sodium and chloride is driven by sodium and potassium gradients generated by Na^+ - K^+ -ATPase, an enzyme located in the basolateral membrane (BLM) of gill chloride cells (25). In freshwater, this energy for transport is supplemented by an apical H^+ -ATPase. When euryhaline teleosts move from freshwater to seawater, the gill is modified to stop accumulating ions and to start excreting excess sodium and chloride. Associated with the acclimation of most euryhaline fish species to seawater is an increase in gill Na^+ - K^+ -ATPase activity (30). This upregulation of gill Na^+ -

K^+ -ATPase is likely critical because of its role in the regulation of plasma sodium and chloride levels.

Many studies have shown increased mRNA (38, 41) and protein (11, 23, 28) expression of Na^+ - K^+ -ATPase in gills of teleosts following seawater exposure. Increased Na^+ - K^+ -ATPase protein expression is thought to be a major determinant of increased activity in fish gills during seawater acclimation. However, rapid increases in killifish (47) and brown trout (46) gill Na^+ - K^+ -ATPase activity following seawater exposure has been shown to precede any increase in enzyme number. In addition, we show in the present study that, following 30 days of seawater acclimation, Arctic char (*Salvelinus alpinus*) increase gill Na^+ - K^+ -ATPase activity approximately eightfold, while this increase in activity is only accompanied by a threefold increase in gill Na^+ - K^+ -ATPase protein levels. This suggests that gill Na^+ - K^+ -ATPase activity is regulated by multiple mechanisms and is not determined solely by enzyme number. Towle et al. (47) proposed that Na^+ - K^+ -ATPase activity is also modulated posttranslationally, potentially through modifications to the membrane environment that surrounds the enzyme.

The activity of Na^+ - K^+ -ATPase has been shown to be sensitive to the composition of its surrounding membrane environment. The complete removal of lipid inactivates Na^+ - K^+ -ATPase; however, activity is fully restored by reintroducing it to a proper membrane environment (32, 42). Na^+ - K^+ -ATPase activity has been correlated to several physical membrane properties including membrane thickness (24), phospholipid composition (48), fatty acyl chain length (29), and membrane fluidity (26). Na^+ - K^+ -ATPase activity has also been reported to be negatively correlated to membrane cholesterol content in many tissues [see Yeagle (51) for discussion] including rainbow trout erythrocytes (36), kidney, and intestine (8). In general, alterations to the lipid environment that increase membrane fluidity tend to also increase Na^+ - K^+ -ATPase activity (44).

The influence of changing salinity on the membrane composition of fish gills has not been adequately studied. Crockett (7) found no significant change in American eel (*Aguilla rostrata*) gill BLM lipid composition following seawater acclimation and concluded that lipid restructuring of the gill is not responsible for increased Na^+ - K^+ -ATPase activity in this catadromous species. However, studies on guppy (10) and the anadromous rainbow trout (33) report changes in whole gill lipid composition following seawater exposure. If changing

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environmental salinity alters the gill lipid composition of some fish species, it may also lead to a modulation of gill Na⁺-K⁺-ATPase activity. Therefore, the mechanism for regulating gill Na⁺-K⁺-ATPase activity may differ between fish species. Strong osmoregulators may be capable of upregulating Na⁺-K⁺-ATPase activity solely through increased protein number, while poorer osmoregulators may rely on a slower or more limited increase in enzyme number accompanied by manipulations to the membrane environment that allow for maximal enzyme activity. Thus it may be informative to examine osmoregulatory strategies in a species with a more limited osmoregulatory capacity. Of the anadromous salmonids, Arctic char are considered relatively poor osmoregulators, requiring a longer period to adjust to changing salinity (20, 21). Since the regulation of gill Na⁺-K⁺-ATPase activity is so crucial for successful acclimation of fishes to changing salinity, we examined the potential relationship between gill Na⁺-K⁺-ATPase activity and observed changes in gill BLM phospholipid/fatty acid and cholesterol composition of freshwater- and seawater-acclimated Arctic char.

MATERIALS AND METHODS

Experimental animals. All procedures were conducted under Animal Utilization Protocols approved by the Animal Care Committee of the University of Guelph and were compliant with Guidelines of the Canadian Council for Animal Care.

Arctic char (463 ± 17 g) were reared at the University of Guelph Hagen Aqualab in freshwater (raw well water, 1 mM Na⁺, 1.4 mM Cl⁻, 1.5 mM Mg²⁺, 2.6 mM Ca²⁺, pH 8.0) at 10°C for 2 yr under a simulated natural photoperiod that mimicked light conditions at a 45° latitude. This study consists of two separate experiments in which Arctic char were transferred from freshwater to seawater. The two experiments were identical except for the duration of seawater exposure. In the first experiment, Arctic char were exposed to seawater for 30 days. From these fish, we determined gill Na⁺-K⁺-ATPase activity, Na⁺-K⁺-ATPase protein levels, and isoform-specific Na⁺-K⁺-ATPase α-subunit mRNA levels. The results of that experiment formed the rationale for the remainder of the study in which we examined the potential relationship between gill Na⁺-K⁺-ATPase activity and gill BLM lipid composition in Arctic char exposed to seawater for 39 days. During the month of June, eight individuals were transferred directly to each of two full-strength seawater (32‰, 10°C) tanks with identical lighting conditions. These Arctic char were exposed to seawater for either 30 (*group 1*) or 39 days (*group 2*). Eight additional char were removed from their holding tank and transferred to each of two identical freshwater tanks. These groups were exposed to freshwater for 30 or 39 days and acted as control fish. For both experimental groups, char were not fed for 48 h before or after transfer. Feeding (trout chow pellets; Martin Feeds, Elmira, ON, Canada) continued (for both freshwater and seawater char) at the rate of satiation of the seawater-exposed Arctic char, since feeding rates decreased on transfer. After 2 wk in seawater, feeding rates had returned to ~60% of pretransfer levels, and by the end of the experiment, all fish were feeding well. After 30 (*group 1*) or 39 days (*group 2*), blood samples were collected from each individual by caudal puncture using a heparinized (500 units/ml) syringe. Arctic char were then killed by a sharp blow to the head. For Arctic char in the 30-day group, the gills were quickly excised and frozen in liquid nitrogen and stored at -80°C for analysis of Na⁺-K⁺-ATPase activity, Na⁺-K⁺-ATPase protein levels, and isoform-specific Na⁺-K⁺-ATPase α-subunit mRNA levels. For char in the 39-day group, the gills were quickly excised, and a small portion was frozen in liquid nitrogen and stored at -80°C for analysis of Na⁺-K⁺-ATPase activity. The remaining gill was used immediately in the isolation of gill BLM. Three Arctic char in the 30-day seawater-exposed group died during the experiment, reducing the sample size to five. For the

39-day group there were no mortalities, and all fish appeared to be in good health at the end of the experiment.

Measurement of Na⁺-K⁺-ATPase α-subunit mRNA levels. Total RNA was extracted from gill samples using TriPure isolation reagent (Boehringer Mannheim), following the guanidine thiocyanate method (5). Isolated total RNA was quantified spectrophotometrically and run (2 μg) on an agarose gel (1%) to check for RNA integrity. First-strand cDNA was synthesized from 2 μg of total RNA using oligo(dT)₁₅ primer and RevertAid H Minus Moloney murine leukemia virus 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 μl 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 α1a- and α1b-isoforms and for the control gene elongation factor-1α (EF1α) (37). 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 66 bp); 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 81 bp); EF1α forward 5' GAG ACC CAT TGA AAA GTT CGA GAA G 3', EF1α reverse 5' GCA CCC AGG CAT ACT TGA AAG 3' (product size 71 bp). 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 that only one band (of the appropriate size) was present. In addition, amplified product from two individuals for each isoform was cloned into a T-vector (pGEM T-easy, Promega; Fisher Scientific, Nepean, ON, Canada) 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 the 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 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 1:4,996 starting copies for Na⁺-K⁺-ATPase isoform-α1a, 1:5,240 starting copies for Na⁺-K⁺-ATPase isoform-α1b, and 1:4,786 starting copies for EF1α. Relative quantities of each target gene were determined using the comparative cycle threshold (C_T) method (1). The relative quantity of Na⁺-K⁺-ATPase α1a and α1b mRNA in gill samples was determined using the following formulas: Efficiency^{-C_T}, where efficiency is the slope of the standard curve, and C_T refers to the fractional cycle number at which the amplified target reaches a fixed threshold. These quantities were also expressed relative to EF1α. 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 that utilized cDNA concentration ranging from 1 μl of undiluted cDNA to 1 μl of a 625× dilution of cDNA) (1). All samples were run in duplicate.

Measurement of Na⁺-K⁺-ATPase protein levels. Gill Na⁺-K⁺-ATPase protein levels were measured by the method of Else and Wu (13) by monitoring binding of [³H]ouabain (0.588 TBq/mmol; obtained from Perkin Elmer, Boston, MA) to gill tissue homogenate. Briefly, gill homogenates were prepared as described for Na⁺-K⁺-ATPase activity measurement. Homogenates were diluted to a concentration of 1 mg protein/ml, and 15 μl were added to 250 μl of incubation medium (containing 10 mmol/l NaH₂PO₄, 5 mmol/l

MgCl₂, 5 × 10⁻⁵ mol/l unlabeled ouabain plus 1.5 × 10⁻⁷ mol/l [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 μl of incubation medium (containing 10 mmol/l NaH₂PO₄, 5 mmol/l MgCl₂, 10⁻² mol/l unlabeled ouabain plus 1.5 × 10⁻⁷ mol/l [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 μl of wash solution (10 mmol/l NaH₂PO₄, 5 mmol/l MgCl₂, pH 7.4), allowed to dry, and 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 counted using a Beckman LS 6500 multipurpose scintillation counter (Beckman Instruments, Fullerton, CA) with 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 amount of homogenate 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 not to change results when between four and six washes (at 50 μl) were used and returned consistent values for NSB tubes. Na⁺-K⁺-ATPase levels are expressed as picomoles Na⁺-K⁺-ATPase per milligram protein.

Isolation of gill BLM. Gill BLM were isolated using the method of Perry and Flik (34). All steps were performed at 0–4°C. Briefly, soft tissue was scraped from gill arches with a glass slide and added to 15 ml of hypotonic homogenization buffer (pH 8.0, 25 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA, 1 mM HEPES, 1 mM Tris·HCl, and 100 U/ml aprotinin). Samples were homogenized with 30 strokes of a loose-fitting (*type A*) followed by 30 strokes of a tight-fitting (*type B*) pestle in a 50-ml Dounce homogenizer (Kontes Glass, Vineland, NJ). The volume was adjusted to 50 ml with the same buffer and further homogenized with 10 additional strokes with the tight pestle. An aliquot of this homogenate was frozen in liquid nitrogen and stored at –80°C for the analysis of marker enzyme activities. The homogenate was centrifuged at 550 g for 15 min to remove nuclei and cellular debris. The supernatant was decanted into a clean centrifuge tube and centrifuged at 50,000 g for 1 h. The resulting supernatant was discarded, leaving a pellet containing a dark solid portion that tightly adhered to the tube below a white fluffy layer that was easily loosened and removed with a mild swirling motion in 15 ml of sucrose buffer (pH 7.4, 250 mM sucrose, 5 mM MgCl₂, 5 mM HEPES, and 5 mM Tris·HCl). The white layer in sucrose buffer was then homogenized in the Dounce homogenizer with 100 strokes of the tight-fitting (*type B*) pestle. The sample was then centrifuged at 1,000 g for 10 min followed immediately by 10,000 g for 10 min, producing a pellet containing most of the remaining contaminating membranes. The supernatant was decanted into a clean centrifuge tube and centrifuged at 30,000 g for 45 min, producing a final pellet enriched with BLM. This pellet was resuspended in 0.5 ml of suspension buffer (pH 7.4, 5 mM MgCl₂, 150 mM NaCl, 20 mM HEPES, and 20 mM Tris·HCl) by drawing it through a 22-gauge needle 10 times. The final membrane suspension was frozen in liquid nitrogen and stored at –80°C until it was analyzed for marker enzyme activities, protein, phospholipid, fatty acid, and cholesterol composition.

Determination of gill Na⁺-K⁺-ATPase and BLM marker enzyme activities. Gill Na⁺-K⁺-ATPase activity was determined spectrophotometrically using a NADH-linked assay modified from the method of Gibbs and Somero (15). 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). Gills were scraped with a glass slide to remove cells from gill filaments and homogenized on ice in SEI buffer

(pH 7.5, 150 mM sucrose, 10 mM EDTA, 50 mM imidazole) using a ground glass homogenizer. Homogenates were centrifuged for 30 s (4°C) at 5,000 g to remove insoluble material, and the supernatant was used directly in the assay of Na⁺-K⁺-ATPase activity. Gill homogenates were assayed for ATPase activity in the presence and absence of the Na⁺-K⁺-ATPase-specific inhibitor ouabain (final concentration 1 mM). Samples were run in duplicate with and without ouabain, and the difference in the rate of NADH oxidation (λ = 340 nm) between the two conditions was used to calculate (millimolar extinction coefficient ε₃₄₀ = 6.22) Na⁺-K⁺-ATPase activity. Optimal assay conditions to give maximal enzyme activity were determined as follows: 100 mM NaCl, 20 mM KCl, 5 mM MgCl₂, 50 mM imidazole, 3 mM ATP, 2 mM phosphoenolpyruvate (PEP), 0.2 mM NADH, 4 U LDH, and 5 U PK, pH 7.5. These conditions yielded a maximal rate of enzyme activity, suggesting that substrate binding sites on both the cytoplasmic and extracellular surface were accessible. Treatment of whole gill homogenates with a range of sodium deoxycholate concentrations did not yield higher enzyme activity.

Marker enzyme activities were determined in the initial homogenate and final BLM suspension to assess the relative purity of the preparation. Na⁺-K⁺-ATPase activity was used as a marker for BLM and measured as stated above. Cytochrome *c* oxidase (CCO), a marker enzyme for the inner mitochondrial membrane, was determined using the method of Blier and Guderley (2). NADPH cytochrome *c* reductase (NADPH CC reductase) was assayed as an endoplasmic reticulum marker under the following conditions, modified from Ray (35): pH 7.4, 50 mM imidazole, 500 μM oxidized cytochrome *c*, 1 mM KCN, and 80 μM NADPH (omitted for control). Because the procedure for BLM isolation produces a proportion of sealed membrane vesicles, maximal marker enzyme activities were determined on vesicles treated with 0.1% sodium deoxycholate, which was found to yield maximal enzyme activity. Because the use of detergents can alter lipid composition and lipid-protein interactions, correlations between Na⁺-K⁺-ATPase activity and lipid composition were made using enzyme activities determined with whole gill homogenates, which did not require treatment with detergents.

All enzymes were analyzed using a Hewlett Packard HP8452 diode array spectrophotometer (Hewlett Packard, Mississauga, ON, Canada) equipped with a temperature-controlled cell changer maintained at 10°C with a Haake G circulating water bath (Haake Buchler Instruments, Saddlebrook, NJ). Na⁺-K⁺-ATPase, CCO, and NADPH CC reductase activities are expressed as micromoles per hour per mg protein.

Determination of gill BLM phospholipid/fatty acid composition. Gill BLM total lipid was extracted by the method of Bligh and Dyer (3). Extracted lipid samples were used in the separation of phospholipids by thin-layer chromatography (TLC). TLC separation of BLM phospholipids followed the method of Holub and Skeaff (22). Each TLC plate contained three sample lanes and a blank lane. Five distinct bands were visualized and identified using the commercial phospholipid standards (starting from the origin) sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylethanolamine (PE). The area corresponding to each phospholipid band in both sample and blank lanes on each TLC plate was scraped into a clean Kimax tube containing 2 ml of 6% H₂SO₄ in methanol and 10 μl of the internal standard heptadecanoic acid (17:0, 1 μg/μl). The phospholipid fatty acids were methylated by placing the tubes in an oven at 80°C for 2 h. Tubes were then allowed to cool to room temperature and supplemented with 2 ml of petroleum ether and vortexed for 2 min. Water (1 ml) was then added, and the tubes were vortexed for an additional 30 s. Tubes were then centrifuged at 2,000 g for 6 min, and the upper petroleum ether phase containing the fatty acid methyl esters (FAMES) was removed and separated by gas chromatography (GC). FAME samples were dried down under nitrogen gas and resuspended in 25 μl of carbon disulfide (CS₂). One microliter of each FAME sample was injected into a Hewlett Packard (HP5890 series II) GC fitted with a flame ionization

detector (FID) using an automatic injector (HP7673A). FAMES were separated on a 30-m DB225 megabore-fused silica column (Chromatographic specialties Brockville, ON, Canada) by the method of Stuart et al. (43). FAMES were identified by comparison with retention times of a prepared standard, made up of methylated menhaden oil and several commercially available FAME standards, containing all of the FAMES of interest. Chain lengths shorter than 14:0 were not resolved under these conditions and thus are not reported. Absolute amounts of FAMES were calculated from peak areas using the known amount of internal standard (17:0) added during the methylation process. Phospholipid content of BLM was calculated based on total FAME concentration (per mole) for each of the five phospholipids detected.

Determination of BLM cholesterol and protein content. Cholesterol levels of isolated gill BLM were determined using a Sigma Diagnostic (St. Louis, MO) cholesterol kit. Levels of membrane cholesterol, phospholipid, and fatty acids were expressed per milligram of protein. Gill homogenate and gill BLM protein content was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA), standardized with bovine serum albumin (BSA).

Determination of plasma osmolality and ion levels. Plasma osmolality was determined using a vapor pressure osmometer (model no. 5500; Wescor). Chloride levels were measured using a chloride titrator (model CMT10; Radiometer, Copenhagen, Denmark). Sodium and potassium levels were measured using a flame photometer (model FLM2, Radiometer).

Chemicals not identified previously were purchased from Sigma Chemical (Sigma-Aldrich, Oakville, ON, Canada) with the exception of BSA (purchased from BioShop, Burlington, ON, Canada), methanol and chloroform (purchased from Fisher Scientific, Whitby, ON,

Canada), and fatty acid standards (purchased from Nu Check Prep, Elysian, MN). All chemicals used were of the highest available purity.

Statistical analysis. All data are presented as means \pm SE. Comparisons of each parameter between freshwater (control)- and seawater-acclimated char were performed using a two-tailed *t*-test. Regression analysis was performed using the least squares method. Assumptions for normality, independence, and homoscedasticity were verified by generating appropriate residual plots. Data transformations (log, square root, and inverse square root) were used when appropriate to meet the above assumptions. For all comparisons, $P < 0.05$ was considered significant.

RESULTS

Following 30 days of seawater exposure, Arctic char had significantly higher gill $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity and protein levels compared with freshwater (control) fish (Fig. 1). However, the observed eightfold increase in gill $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity of seawater fish was only accompanied by an approximate threefold increase in gill $\text{Na}^+\text{-K}^+\text{-ATPase}$ protein levels. Examination of gill $\text{Na}^+\text{-K}^+\text{-ATPase}$ α -subunit isoform mRNA expression showed that Arctic char exposed to seawater for 30 days had reduced levels of the $\alpha 1a$ -isoform and significantly increased levels of the $\alpha 1b$ -isoform. This switch in isoform expression, and the fact that the increase in gill $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity following seawater exposure cannot be explained by the observed increase in $\text{Na}^+\text{-K}^+\text{-ATPase}$ protein levels alone, suggested that other mechanisms for modifying enzyme activity may be important. We decided to

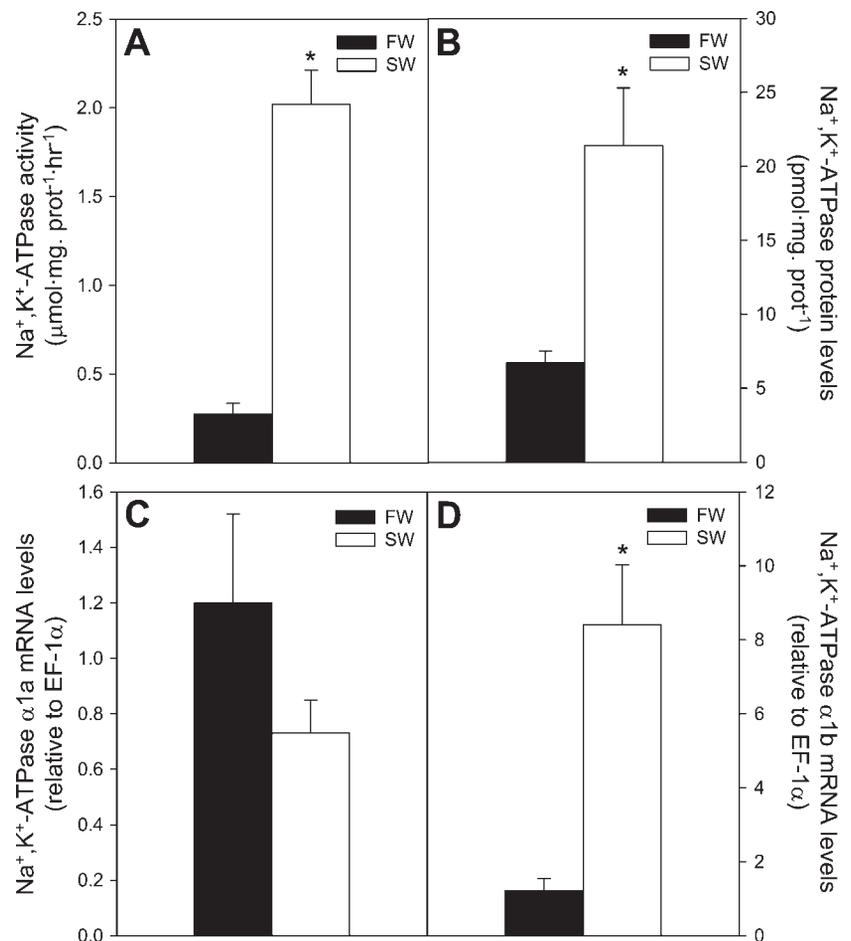


Fig. 1. A: gill $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity. B: gill $\text{Na}^+\text{-K}^+\text{-ATPase}$ protein (prot) levels. C: gill $\text{Na}^+\text{-K}^+\text{-ATPase}$ isoform- $\alpha 1a$. D: $\alpha 1b$ mRNA levels of Arctic char (*Salvelinus alpinus*) exposed to freshwater (FW; control) or seawater (SW; 32‰) for 30 days. EF1 α , elongation factor-1 α . All data are presented as means \pm SE; $n = 8$ for freshwater groups and $n = 5$ for seawater groups. *Significantly different from freshwater char ($P < 0.05$).

examine the role of BLM lipid composition as a potential modulator of gill Na⁺-K⁺-ATPase activity.

The determination of marker enzyme activities verified that BLM preparations were highly enriched with Na⁺-K⁺-ATPase and had only minor contamination with CCO and NADPH CC reductase for both freshwater- and seawater-acclimated Arctic char (Table 1). Plasma sodium, chloride, and potassium levels as well as total osmolality were not significantly different between freshwater and 39-day seawater-exposed Arctic char (Table 2). Following 39 days in seawater, gill Na⁺-K⁺-ATPase activity was significantly elevated (~6-fold) compared with freshwater (control) fish (Table 2).

Analysis of gill BLM phospholipid and fatty acid content showed a remarkable similarity between the two experimental groups. PC was found to be most abundant (~60%), followed by PE (~17%), PS (9–10%), SM (~8%), and PI (4–7%), with the total phospholipid fatty acid content amounting to 334–345 nmol/mg protein (Table 3). PI content of BLM of seawater-acclimated fish was significantly lower (~45%) than that from freshwater control char. The PC/PE ratio was approximately four (Table 3).

The corresponding fatty acid composition for each of the five phospholipids was also determined for freshwater- and seawater-acclimated Arctic char. For brevity, full fatty acid data sets are not included, but the most abundant fatty acids are reported below with numbers in parenthesis referring to freshwater and seawater values, respectively. PC contained a high proportion of saturated fatty acids in both groups (~43%), while monoene and polyene content was 31 and 25%, respectively. The most common fatty acids were 16:0 (38.7 ± 0.7 and 38.3 ± 0.9%), 18:1 (23.2 ± 0.4 and 23.7 ± 0.8%), and 22:6n3 (10.9 ± 0.9 and 12.3 ± 1.1%).

The fatty acid composition of PE in both groups was much more unsaturated than that of PC, containing ~46% polyenes, ~25% monoenes, and 27–29% saturates. Dominant fatty acids included 22:6n3 (27.5 ± 2.1 and 24.3 ± 3.6%), 18:1 (17.2 ± 1.1 and 16.7 ± 1.1%), 16:0 (15.1 ± 1.0 and 14.2 ± 1.2%), and 18:0 (9.7 ± 0.9 and 9.6 ± 1.5%). Several other polyunsaturated fatty acids (PUFAs; 18:2n6, 20:4n6 and 20:5n3) were also detected in both groups at significant levels (~4%). PS contained ~44, 18, and 38% saturates, monoenes, and polyenes, respectively, and had the highest ratio of n3/n6 PUFAs (~7) of all the phospholipids measured because of high levels

Table 1. Maximal activities of marker enzymes in gill homogenates and enrichment factors in BLM of Arctic char exposed to freshwater or seawater for 39 days

Marker Enzyme	Freshwater		Seawater	
	Homogenate activity	BLM enrichment, fold	Homogenate activity	BLM enrichment, fold
Na ⁺ -K ⁺ -ATPase	0.4±0.1	9.2	2.9±0.5*	8.3
CCO	0.9±0.1	0.8	0.9±0.1	1.1
NADPH CC reductase	0.3±0.0	0.9	0.2±0.0	1.1

Maximal activities (μmol · h⁻¹ · Mg protein⁻¹; mean ± SE) of marker enzymes in gill homogenates and enrichment factors in gill basolateral membranes (BLM) of Arctic char (*Salvelinus alpinus*) exposed to freshwater (control) or seawater (32‰) for 39 days. CCO, cytochrome c oxidase; NADPH CC reductase, NADPH cytochrome c reductase. For all enzymes, n = 8. *Significantly different from freshwater (control) char.

Table 2. Plasma ion and osmolality levels and gill Na⁺-K⁺-ATPase activity of Arctic char exposed to freshwater or seawater for 39 days

	Freshwater Char	Seawater Char
Sodium	153.9±1.25	154.0±2.0
Chloride	129.6±1.1	126.8±1.9
Potassium	2.5±0.2	2.3±0.1
Osmolality	301.1±1.1	303.4±1.3
Gill Na ⁺ -K ⁺ -ATPase	0.5±0.1	3.1±0.5*

Plasma ion (mM) and osmolality (mmol/kg) levels and gill Na⁺-K⁺-ATPase activity (μmol · mg protein⁻¹ · h⁻¹; mean ± SE) of Arctic char (*S. alpinus*) exposed to freshwater (control) or seawater (32‰) for 39 days. For all parameters measured, n = 8. *Significantly different from freshwater (control) char.

of 22:6n3 (26.2 ± 2.9 and 31.0 ± 2.2%) in both groups. Total PI fatty acid content was significantly lower in seawater-acclimated char gill BLM, due to ~72 and ~62% decreases in the absolute amount (nmol/mg protein) of 16:0 and 18:1, respectively. These changes were responsible for 56 and 55% decreases in the absolute total saturated and monounsaturated fatty acid levels, respectively. When calculated as a percentage of the total PI fatty acid content, changes in 18:1, total saturates, and total monoenes were not significantly different. PI %16:0 did decrease significantly in seawater-acclimated char (from 14.7 ± 2.3 to 9.7 ± 1.5%) compared with control fish. Absolute levels of PI 20:4n6 did not change with salinity acclimation; however, their percent contribution to the total fatty acid content increased significantly from 17.5 ± 2.9 to 27.2 ± 2.3%, due to the overall decrease in absolute total PI fatty acid levels. PI was found to have the lowest overall n3/n6 fatty acid ratio, with freshwater char BLM having a significantly lower ratio than seawater fish. The low n3/n6 ratio was due to the high levels of 20:4n6 (17.5 ± 2.9 and 27.2 ± 2.3%), the second most abundant fatty acid in PI next to 18:0 (31.6 ± 4.0 and 36.6 ± 1.5%). SM contained high levels of saturated fatty acids (49–54%) and only 9–15% PUFAs. The dominant fatty acids were 16:0 (28.9 ± 2.7 and 25.9 ± 2.5%), 24:1 (17.9 ± 3.4 and 18.0 ± 2.5%), 18:0 (11.2 ± 3.9 and 8.2 ± 1.2%), 14:0 (11.9 ± 2.2 and 10.7 ± 1.5%), and 18:1 (10.1 ± 2.5 and 9.5 ±

Table 3. Total phospholipid composition of gill basolateral membranes from Arctic char exposed to freshwater or seawater for 39 days

Phospholipid	Absolute Amount, nmol/mg protein		% , by mol	
	Freshwater	Seawater	Freshwater	Seawater
SM	27.1±4.9	25.6±5.1	7.7±0.9	8.7±2.0
PC	199.1±12.9	202.3±29.1	58.4±2.0	61.5±1.3
PS	36.5±6.2	32.2±7.7	10.2±1.3	9.1±0.7
PI	24.3±6.7	13.0±2.1*	7.1±1.8	4.0±0.4*
PE	57.6±8.9	60.6±15.5	16.5±2.3	16.8±2.1
Total	344.6±27.7	333.7±52.4	100.0±0.0	100.0±0.0
PC-to-PE ratio	4.1±0.7	4.2±0.7		

Total phospholipid composition (mean ± SE) of gill BLM from Arctic char (*S. alpinus*) exposed to freshwater (control) or seawater (32‰) for 39 days; n = 8 for seawater fish, and n = 7 for freshwater fish. SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine. *Significantly different from freshwater (control) char.

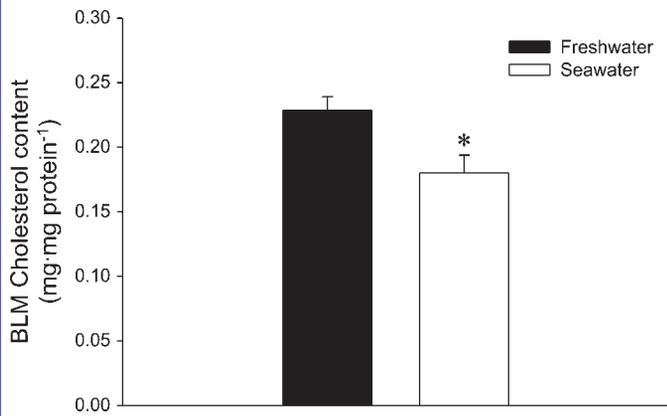


Fig. 2. Gill basolateral membrane (BLM) cholesterol content (mean ± SE) from freshwater (control)- and seawater (32‰)-acclimated Arctic char (*S. alpinus*); *n* = 8 for both groups. *Significantly different from freshwater (control) char (*P* < 0.05).

1.3%). When combined, the overall phospholipid fatty acid composition of the gill BLM for both freshwater and seawater char contained 41–43% saturates, ~29% monoenes, and ~29% polyenes, giving a monoene-to-polyene ratio of ~1:1. The n3/n6 ratio was 2.2–2.4, and the average chain length was 18.3.

The cholesterol content of gill BLM was significantly lower in seawater-acclimated Arctic char (Fig. 2). Regression of gill Na⁺-K⁺-ATPase activity against gill BLM cholesterol content for individual fish was not significant for freshwater char, but there was a significant negative correlation (*P* = 0.03) for seawater-acclimated char (*r*² = 0.56) (Fig. 3). Regression analysis between individual gill Na⁺-K⁺-ATPase activity and several gill BLM phospholipid and fatty acid parameters also revealed significant correlations for seawater-acclimated char. The PC/PE ratio was negatively correlated to gill Na⁺-K⁺-ATPase activity in the seawater-acclimated char (*P* = 0.02, *r*² = 0.61) (data not shown). This was due to a positive correlation between Na⁺-K⁺-ATPase activity and percent PE content of the BLM membrane (*P* = 0.02, *r*² = 0.60) (Fig. 4).

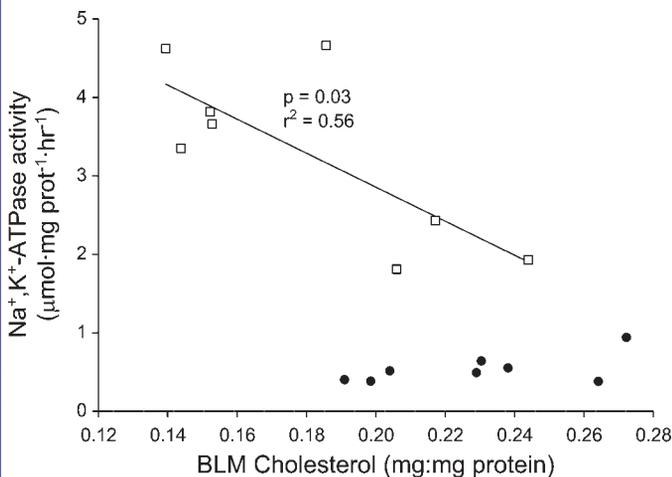


Fig. 3. Gill Na⁺-K⁺-ATPase activity plotted against gill BLM cholesterol composition for freshwater (control; ●)- and seawater (32‰; □)-acclimated Arctic char (*S. alpinus*); *n* = 8 for both groups. Regression for seawater-acclimated char was significant (*P* < 0.05), whereas no significant regression was found for freshwater (control) char.

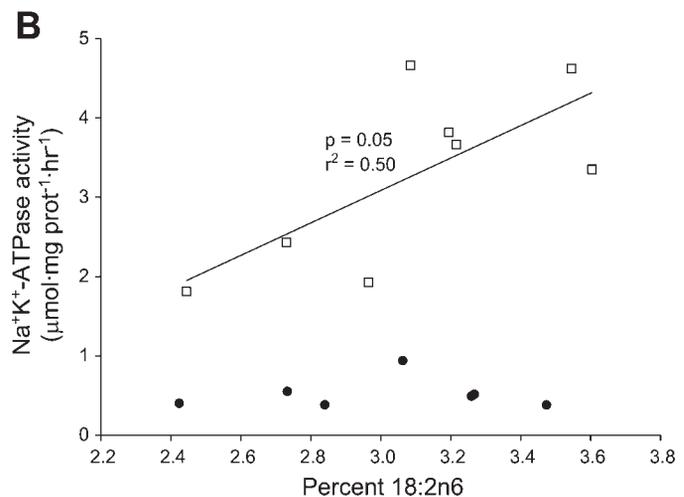
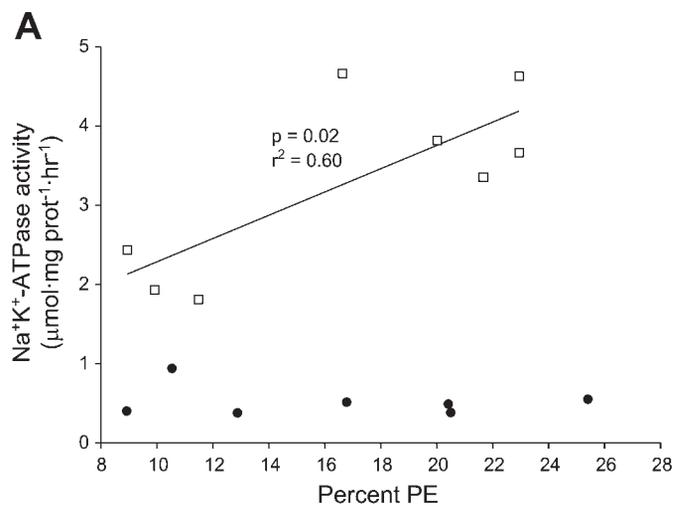


Fig. 4. Gill Na⁺-K⁺-ATPase activity plotted against BLM. A: %phosphatidylethanolamine (%PE). B: overall %18:2n6 (linoleic acid) for freshwater (control; ●)- and seawater (32‰; □)-acclimated Arctic char (*S. alpinus*); *n* = 7 for freshwater char, and *n* = 8 for seawater char. Regressions for seawater-acclimated char were significant (*P* < 0.05) for %PE and %18:2n6, whereas no significant regressions were found for freshwater (control) char.

PC content did not correlate to Na⁺-K⁺-ATPase activity when tested alone (*P* = 0.29). A significant positive correlation was also found between gill Na⁺-K⁺-ATPase activity and total BLM 18:2n6 composition (*P* = 0.05, *r*² = 0.50) (Fig. 4). No significant relationship was found between gill Na⁺-K⁺-ATPase activity and any BLM lipid parameter for freshwater-acclimated char.

DISCUSSION

In this study, Arctic char acclimated to seawater had significantly elevated gill Na⁺-K⁺-ATPase activity compared with freshwater char. This observed increase in gill Na⁺-K⁺-ATPase activity is very typical of euryhaline fishes following seawater exposure (31) and is thought to be an important adaptation enabling them to successfully acclimate to the marine environment. The increase in gill Na⁺-K⁺-ATPase activity is thought to be largely due to an increase in protein number, as several studies report increased Na⁺-K⁺-ATPase protein levels following seawater exposure of various fish species (11, 23, 28). However, our observation of an eightfold

increase in Arctic char gill Na⁺-K⁺-ATPase activity is accompanied by a more limited threefold increase in gill Na⁺-K⁺-ATPase protein number. This disconnect between Na⁺-K⁺-ATPase activity and protein levels has also been seen in other studies where rapid increases in gill Na⁺-K⁺-ATPase activity precede an increase in enzyme number (46, 47). Although increased gill Na⁺-K⁺-ATPase protein levels are likely an important determinant of enzyme activity, it is clear that other mechanisms are involved in modulating the activity of the enzyme. Because changes in membrane composition can have profound effects on the activity of embedded enzymes, this study examined the potential influence of gill BLM lipid composition on Na⁺-K⁺-ATPase activity.

Few studies have examined the influence of changing salinity on fish gill phospholipid/fatty acid composition. Levels of PC, PE, PS, and SM were not different between seawater- and freshwater-acclimated char, and the percent composition of each phospholipid was very similar to what is seen in American eel gill BLM (7), with PC being the most abundant phospholipid followed by PE, PS, SM, and PI. Crockett (7) found no change in any of these phospholipids in eel gill BLM following seawater exposure, whereas Daikoku et al. (10) found increased whole gill PC and decreased PE and SM content in seawater-exposed guppies. The main change in membrane phospholipids in the present study occurred in PI. Not only did the overall content of this phospholipid decline in seawater-acclimated fish, but the nature of its component fatty acids also changed. PI is a precursor of several important second messengers including phosphatidylinositol 4,5-bisphosphate, which has been shown to regulate many ion transporters and channels (19). The increased proportion of 20:4n6 (arachidonic acid; AA) in PI of seawater-acclimated char may also indicate a change in signal transduction pathways, as it is a precursor for eicosanoid synthesis. This area of fish gill physiology has not been adequately studied, and further work is needed to establish whether PI and AA play a role in the regulation of gill Na⁺-K⁺-ATPase. Crockett (7) found no major change in overall fatty acid content of American eel gill BLM following seawater acclimation, with the dominant fatty acids being similar to what is reported in the present study. The percent PUFA content of guppy whole gill was found to increase substantially following seawater exposure, from 18 to 29% (10). PUFA levels did not change in Arctic char gill BLM following seawater exposure but did account for ~30% of the total fatty acid content. Other studies on whole gill membrane composition of rainbow trout (33) and European eel (45) also showed minor changes in fatty acid composition following exposure to increased salinity. However, because these studies do not differentiate between plasma membrane and intracellular membranes, it is impossible to assess how the BLM specifically was influenced by seawater acclimation. Overall, the structural changes in Arctic char gill BLM phospholipids and their component fatty acids during salinity acclimation could be judged to be minor and confined to PI, the main phospholipid involved in cell signaling.

Gill BLM cholesterol content was significantly lower in seawater-acclimated Arctic char. Cholesterol has an ordering effect on membrane lipids and acts to reduce membrane fluidity and permeability (50). Changes in membrane cholesterol can have a profound effect on membrane protein function (51). Na⁺-K⁺-ATPase is known to be sensitive to changes in mem-

brane cholesterol. Na⁺-K⁺-ATPase activity has been negatively correlated to membrane cholesterol content in many tissues including human erythrocytes (6, 49), bovine kidney (52), dog brain (12), rabbit smooth muscle (4), and human endothelial cells (27). This relationship has also been shown in rainbow trout erythrocytes (36), kidney, and intestine (8). A significant, negative correlation between gill Na⁺-K⁺-ATPase activity and BLM cholesterol content of individual fish was observed for seawater-acclimated Arctic char. This relationship suggests that cholesterol may play a role in modulating Na⁺-K⁺-ATPase activity and may explain some of the variation in Na⁺-K⁺-ATPase activity among individuals. Part of the observed increase in Arctic char gill Na⁺-K⁺-ATPase activity following seawater exposure may be stimulated by the reduction in gill BLM cholesterol content. This is the first demonstration of a correlation between Na⁺-K⁺-ATPase and membrane lipid composition in fish during salinity acclimation. The potential inhibitory effect of cholesterol on Na⁺-K⁺-ATPase activity may be mediated through its effects on membrane structure and fluidity. A negative correlation between membrane cholesterol and Na⁺-K⁺-ATPase activity has also been correlated to membrane fluidity (12), and Sinensky et al. (40) present a near perfect relationship between increased membrane order and decreased Na⁺-K⁺-ATPase activity. The mechanism by which cholesterol influences protein function is not clear. Yeagle (51) suggests that cholesterol's ordering effect on membranes effectively reduces the "free volume" available to membrane proteins. This reduction in free volume limits protein conformational change and reduces protein activity.

Other correlations between phospholipid/fatty acid composition of gill BLM and Na⁺-K⁺-ATPase activity of individual seawater-acclimated fish also suggest that membrane fluidity is involved in the modulation of gill Na⁺-K⁺-ATPase activity. Gill Na⁺-K⁺-ATPase activity from seawater-acclimated Arctic char was positively correlated to BLM percent PE and overall percent 18:2n6 and negatively correlated to the PC/PE ratio. These membrane parameters influence membrane fluidity. The PC/PE ratio is known to decrease in animals acclimating to decreased temperature, as PE keeps the membrane fluid at lower temperatures (18). Membrane fluidity also increases with increased unsaturation of the membrane (17). This also suggests that the positive correlation of Na⁺-K⁺-ATPase activity to percent 18:2n6 content may be related to the overall fluidity of the membrane. The consensus of these correlations is the consistent relationship between parameters responsible for increasing membrane fluidity and increased Na⁺-K⁺-ATPase activity.

Of considerable interest is the fact that freshwater Arctic char gill Na⁺-K⁺-ATPase activity appears to be independent of the lipid parameters determined in this study. One possible explanation for this is the recent discovery of multiple Na⁺-K⁺-ATPase isoforms in teleost fish (9, 16, 39). On acclimation to seawater, rainbow trout differentially express mRNA for two Na⁺-K⁺-ATPase α -subunit isoforms, α 1a, which is down-regulated on entry to seawater, and α 1b, whose expression increases with seawater exposure (37). In this study, we confirm that this same isoform relationship exists for Arctic char. The amino acid sequences for the α 1a and α 1b rainbow trout isoforms differ quite significantly in their fifth and sixth transmembrane domains (37). The differences in amino acid se-

quence between the $\alpha 1a$ - and $\alpha 1b$ -isoforms in these transmembrane regions may confer different protein-lipid interactions and explain why Na⁺-K⁺-ATPase activities of freshwater- and seawater-acclimated Arctic char differ in their apparent sensitivity to the lipid composition of the BLM. The lack of correlation between gill Na⁺-K⁺-ATPase activity and BLM cholesterol content of freshwater Arctic char may also be explained if the isoform present in freshwater char gill is actually far more sensitive to cholesterol than the isoform present in seawater fish. It is possible that, at the higher cholesterol levels seen in freshwater char gill BLM, the lipid-sensitive activity of Na⁺-K⁺-ATPase is maximally inhibited. The number of cholesterol molecules available to associate with Na⁺-K⁺-ATPase is likely higher in the freshwater condition, as freshwater-acclimated Arctic char gills contain approximately threefold fewer Na⁺-K⁺-ATPase molecules and ~20% more cholesterol than seawater-acclimated char. Clearly, the potential mechanistic relationship between Na⁺-K⁺-ATPase and cholesterol in the gills of freshwater- and seawater-acclimated fish requires further examination.

There is a relatively large variation in gill Na⁺-K⁺-ATPase activity for seawater-acclimated Arctic char compared with the freshwater control char. Interestingly, the relatively low gill Na⁺-K⁺-ATPase activity of some seawater-acclimated individuals did not appear to limit their osmoregulatory capacity, as their plasma ion and osmolality levels were similar to those of individuals with the highest gill Na⁺-K⁺-ATPase activity. In fact, there was very little variation in plasma ion and osmolality levels between seawater-exposed individuals. There is also a relatively large variation in gill BLM cholesterol content between seawater-acclimated individuals compared with the freshwater control fish. In general, fish with high Na⁺-K⁺-ATPase activity had low membrane cholesterol levels and vice versa. Higher cholesterol content and lower membrane fluidity reduce membrane permeability to water and ions across barrier epithelia (53). The apical surface of the gill is suspected to be the primary barrier between the outside environment and the internal composition of the fish. However, the overall permeability of the gill epithelium is likely influenced by the permeability of the BLM, as it also acts to regulate intracellular composition. The higher cholesterol levels seen in the BLM of some individuals may reduce membrane permeability to partially compensate for the lower Na⁺-K⁺-ATPase activity seen in those individuals. A strategy that allowed higher BLM cholesterol content may be favored by some individuals, as supplementing the BLM with cholesterol may be less energetically costly than maintaining large numbers of Na⁺-K⁺-ATPase proteins and sustaining higher sodium pump activity.

In conclusion, our data support the contention that gill Na⁺-K⁺-ATPase activity may be modulated by the lipid composition of the BLM. The mechanism by which the BLM exerts control over gill Na⁺-K⁺-ATPase activity is not known but may be manifested through modifications to membrane fluidity. The observed negative correlation between BLM cholesterol composition and gill Na⁺-K⁺-ATPase activity of seawater-acclimated Arctic char and the apparent lack of correlation seen in freshwater-acclimated char suggest that there is a change in membrane-protein interactions during salinity acclimation. The successful acclimation of Arctic char and other anadromous fishes to increased salinity may require modifica-

tions to gill BLM composition, facilitating the required up-regulation in Na⁺-K⁺-ATPase activity. These results also support recent findings that multiple Na⁺-K⁺-ATPase isoforms are differentially expressed in freshwater- and seawater-acclimated salmonid fishes and suggest that these different isoforms differ in their membrane lipid sensitivities.

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