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RESEARCH ARTICLE

Freshwater adaptation in prickly sculpin (Pisces: Cottidae): intraspecific comparisons reveal evidence for water pH and Na^+ concentration driving diversity in gill H^+-ATPase and ion regulation

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ABSTRACT

Phenotypic divergence is a hallmark of adaptive radiation. One example involves differentiation in physiological traits involved in ion regulation among species with contrasting lifestyles and living in distinct environments. Differentiation in ion regulation and its ecological implications among populations within species are, however, less well understood. To address this knowledge gap, we collected prickly sculpin (*Cottus asper*) from distinct habitat types including coastal rivers connected to estuaries, coastal lakes and interior lakes, all from British Columbia, Canada. We tested for differences in plasma Na^+ and Cl^-, gill Na^+/K^+-ATPase and H^+-ATPase activity and protein abundance as well as changes in body mass and arterial blood pH in fish sampled from the field and acclimated to two different freshwater conditions in the laboratory: artificial lake water (ALW) and ion-poor water (IPW). We also tested for links between environmental water chemistry and the physiological characteristics associated with ion regulation. Transfer to IPW resulted in upregulation of gill Na^+/K^+-ATPase and H^+-ATPase activity as well as increases in gill H^+-ATPase protein expression level in each habitat compared with that in the common ALW treatment. Despite the presence of population-within-habitat-type differences, significant habitat-type effects were revealed in most of the ion regulation characteristics examined under different acclimation conditions. Significantly lower plasma Cl^- was detected in fish from coastal rivers than in fish from the other two habitat types during the IPW treatment, which was also significantly lower compared with that in ALW. Similarly, gill Na^+/K^+-ATPase activity was lower in the coastal river populations in IPW than in fish from coastal and interior lakes, which was not in accordance with the protein expression in the gill. For gill H^+-ATPase, fish from interior lake populations had the highest level of activity across all habitat types under all conditions, which was related to the protein levels in the gill. The activity of gill H^+-ATPase was positively correlated with the combined effect of water Na^+ and pH under the ALW treatment. Our results suggest that variation in habitat may be an important factor driving differences in gill Na^+/K^+-ATPase and H^+-ATPase activity across populations of *C. asper*. Further, the combined effect of water Na^+ and pH may have played a key role in physiological adaptation in *C. asper* during post-glacial freshwater colonization and dispersal.

KEY WORDS: Fish, *Cottus asper*, Ion regulation, Ecological implication, Na^+/K^+-ATPase activity, H^+-ATPase activity

INTRODUCTION

Adaptive radiation is associated with the modification of existing traits or the evolution of novel traits, both of which can lead to phenotypic divergence. There are numerous well-known examples of morphological divergence in closely related groups, such as Darwin's finches (Geospizinae; Grant and Grant, 1993; Schluter, 1996), Caribbean anoline lizards (*Anolis*; Losos et al., 1996), threespine stickleback (*Gasterosteus aculeatus*; Rundle et al., 2000) and Hawaiian silverswords (Compositae-Madiinae; Robichaux et al., 1990); however, relatively few studies have examined divergence in physiological traits. Those studies that have examined such divergence have demonstrated associations between physiological traits and whole-animal phenotypes (e.g. intraspecific variation in aerobic swimming performance in threespine stickleback; Dalziel et al., 2012), but the mechanism(s) of natural selection driving divergence in these traits is not well understood.

Fish are the most diverse group of vertebrates and they have successfully exploited and adaptively radiated within diverse aquatic environments including those ranging in salinity from hypersaline to fresh water. During the movement of animals between seawater and freshwater environments, differences in osmolality likely represented a major physiological challenge (Lee and Bell, 1999; Lee et al., 2003; Hwang et al., 2011). To maintain homeostasis in plasma osmolality, fish use distinct ion regulatory processes in marine and freshwater environments. In both environments, gills and their ion-transporting cells (ionocytes) play the major role in ion regulation. There are numerous transport proteins involved in ion regulation, but two important proteins are the ATP-dependent enzymes Na^+/K^+-ATPase and H^+-ATPase. Na^+/K^+-ATPase is embedded within the basolateral membrane of the gill ionocytes, where it generates a transepithelial electrochemical gradient (negative charge inside the cell) by extruding three Na^+ ions from the cell in exchange for the cellular uptake of two K^+ ions. In freshwater fish, H^+-ATPase is located in the apical membrane of some gill ionocytes, where it extrudes H^+ into the aqueous environment to facilitate Na^+ uptake via a putative Na^+ channel (Evans, 2011) and it contributes to whole-animal pH regulation. Studies have found that Na^+/K^+-ATPase enzyme activity is upregulated when transferring marine-adapted animals into fresh

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water or vice versa (Bystriansky et al., 2007; Henriksson et al., 2008). Higher H^+-ATPase activity was found in freshwater-adapted animals compared with marine-adapted animals in freshwater environments (Lee et al., 2011). Previous studies have demonstrated that changes in the activity of Na^+/K^+-ATPase and H^+-ATPase are, at least in part, mediated by changes in protein abundance (Tipsmark et al., 2008; Tresguerres et al., 2005; Wilson et al., 2007a). However, little is known about how the function and regulation of these enzymes evolved in fish during contemporary invasions of fresh water by marine ancestral lineages.

The prickly sculpin (Cottidae: *Cottus asper* Richardson 1836) is a euryhaline freshwater fish found in western North America and is part of a radiation of freshwater sculpins from marine ancestors (Scott and Crossman, 1973; McPhail, 2007; Goto et al., 2015). Early work on this species identified two morphological and life-history forms of *C. asper*: a 'coastal' and an 'inland' form. The coastal form of *C. asper* is typically distributed in coastal rivers and lakes, tends to be weakly 'prickled' (prickles are small hair-like projections in the skin that appear to have a sensory function) and, where possible, moves to estuarine areas for spawning (McAllister and Lindsey, 1959; Krejsa, 1967). In contrast, the inland form tends to have more prickles, is distributed in rivers, tributaries and lakes in more upstream areas and does not undergo seaward movements for spawning (McAllister and Lindsey, 1959; Krejsa, 1967). More recent studies have suggested that the populations residing in environments that differ in osmolality also differ in their ability to retain electrolytes and have divergent genomic signatures across loci related to Na^+ and Cl^- regulation (Na^+/K^+-ATPase and Na^+/Cl^--co-transporter) and gill permeability (claudins; Bohn and Hoar, 1965; Dennenmoser et al., 2014, 2017). These studies, however, collected *C. asper* from the coastal region of British Columbia, Canada (i.e. rivers and lakes associated with the lower Fraser River in southwestern BC), which does not account for the diverse geographical history of freshwater habitats in BC. Indeed, populations of *C. asper* from coastal lakes and rivers, as used in previous studies, are likely to have had recent or current access to brackish or marine environments (e.g. Krejsa, 1965, 1967). In contrast, inland populations from lakes in the interior of BC (i.e. east of the Coast Mountain range) have probably been confined to their habitats since early in postglacial times and are the descendants of postglacial colonists with a preglacial history in fresh waters of unglaciated portions of the Columbia River basin (Krejsa, 1965). Despite the historical diversity among freshwater lake habitats, no studies have examined whether there is differentiation among populations of *C. asper* that inhabit waters from these distinct regions.

The objective of this study was to determine whether the physiological mechanisms of freshwater ion regulation have diverged among populations of *C. asper* that live in diverse habitats ranging from coastal areas to lakes and streams from interior areas hundreds of kilometres from the sea (McPhail, 2007). This study addresses three hypotheses. First, according to differences in the historical and current connectivity to the marine environment, we hypothesized that populations from different habitat types have diverged in gill Na^+/K^+-ATPase and H^+-ATPase activity, such that the prickly sculpin from interior lake populations would have the highest gill Na^+/K^+-ATPase and H^+-ATPase activity under freshwater conditions, followed by coastal lake populations, with coastal river populations having the lowest activity of these two enzymes in fresh water. The higher enzyme activity from the interior lake populations would be consistent with an adaptation to fresh water and a greater capacity to maintain plasma ion homeostasis, especially in ion-poor environments, compared with the coastal river populations, which would have the lowest enzyme activity, and the coastal lake populations would be intermediate. Second, we hypothesized that intraspecific variation in gill Na^+/K^+-ATPase and H^+-ATPase activity would be related to the amount of each protein in the gill, suggesting that differences in protein expression underlie putatively adaptive differences. Third, irrespective of freshwater evolutionary history in *C. asper*, we hypothesized that a driving force of diversification of freshwater ion regulation is related to the water chemistry of their local aquatic habitat. To test these hypotheses, we collected *C. asper* from multiple coastal estuaries/rivers, coastal isolated lakes and interior lakes and sampled plasma and gill tissue for characterization of ion regulation immediately upon collection and after lab rearing under common artificial lake water (ALW) or ion-poor water (IPW) conditions. Ion regulation was characterized via measurement of plasma Na^+ and Cl^-, arterial blood pH and body mass changes, and the activity and protein content of Na^+/K^+-ATPase and H^+-ATPase in the gills. Associations between environmental water chemistry and ion regulation characteristics were assessed by correlation analyses.

MATERIALS AND METHODS

Experimental animals and acclimation conditions

We collected *C. asper* from three independent populations within each of coastal river, coastal lake and interior lake habitat types (Fig. S1) in September and October (2017 and 2018). Fraser River Park (FRP), Little Campbell River (LCR) and Cowichan River (CR) are three coastal rivers that drain into estuaries and hereafter are collectively referred to as 'coastal rivers'. Garden Bay Lake (GBL), Frederick Lake (FL) and Pachena Lake (PL) are three coastal, isolated freshwater lakes (i.e. that have no current connections to the sea) and are hereafter referred to as 'coastal lakes'. Nicola Lake (NL), Kamloops Lake (KL) and Okanagan Lake (OK) are three interior freshwater lakes and are hereafter referred to as 'interior lakes'. Although NL and KL are connected to the sea via the Fraser River and OK is connected to the sea via the Columbia River, migration distances to the sea are in excess of 350 km and all three are populations are considered permanent residents of fresh water (see Krejsa, 1965, 1967).

All fish were captured using Gee minnow traps, which were baited with bacon and left overnight 5–15 m from the shore. Captured fish were transferred to Gee minnow keepers and kept in their native water. At the time of fish capture, water conductivity was measured using a conductivity meter with automatic temperature compensation (TDSTestr™, WD-35661-10, Oakton Instruments, Vernon Hills, IL, USA) and two 50 ml water samples were placed into polypropylene tubes (BD Falcon) for later chemical analysis. Surface water temperature was not recorded because prickly sculpins are benthic and the temperature the fish experience is expected to be lower than that at the surface. One tube was used for the analysis of water alkalinity and pH, which was completed within 2 days of collection at The University of British Columbia (UBC) and the other water sample was frozen for analysis of water ion composition (see below). After ∼12 h in the minnow keeper, 8–10 fish from each locality were killed at the field site to sample blood and gill tissue (details below). The remaining fish were placed in plastic bags containing water from their field site. The headspace of each bag was filled with 100% oxygen and then the bags were placed into a cooler lined with ice and transported to UBC. Fish were monitored for signs of stress during transport and the ice and oxygen were replenished if required. At UBC, the fish from different localities were held in separate tanks within the same recirculating aquaculture system filled with

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Table 1. Water chemical characteristics of studied locations and two experimental treatment conditions

Habitat	Location	Na^+ (mmol l^{-1})	Mg^{2+} (mmol l^{-1})	Ca^{2+} (mmol l^{-1})	Alkalinity	pH	Conductivity (µS)
Coastal river	FRP	48.8	1829.2	1.7	17.9	7.6	6500
Coastal river	LCR	27.1	887.5	1.0	19.1	7.4	3600
Coastal river	CR	11.6	291.7	0.7	10.6	7.2	1380
Coastal lake	GBL	0.2	10.0	0.1	6.3	7.0	60
Coastal lake	FL	0.1	0.4	0.1	3.8	6.9	30
Coastal lake	PL	0.1	1.7	0.1	2.3	6.7	20
Interior lake	KL	0.1	21.3	0.3	10.9	7.8	70
Interior lake	NL	0.3	98.8	0.6	27.1	8.1	170
Interior lake	OK	0.4	131.7	0.7	30.8	8.0	230
ALW		0.7	170	0.2	N/A	7.8	150
IPW		0.1	6.7	0.1	N/A	7.3	20

FRP, Fraser River Park; LCR, Little Campbell River; CR, Cowichan River; GBL, Garden Bay Lake; FL, Frederick Lake; PL, Pachena Lake; KL, Kamloops Lake; NL, Nicola Lake; OK, Okanagan Lake; ALW, artificial lake water; IPW, ion-poor water.

dechlorinated Metro Vancouver City tap water supplemented with $NaHCO_3$, $CaSO_4{\cdot}2H_2O$, $MgSO_4$ and KCl to achieve a moderately soft water (Table 1). This water was defined as ALW. All fish were maintained at 11°C, under a 12 h:12 h light:dark photoperiod and they were fed to satiation every second day with blood worms and brine shrimp. Fish were acclimated to this common condition for at least 1 month before experimentation. All experimental procedures involving animals were approved by the UBC Animal Care Committee under animal use protocol number A17-0293.

Water chemistry

Water samples from the fish collection sites and from the acclimation conditions were diluted 10–100 times with deionized water for Na^+ and Ca^{2+} concentration measurements, or 100–200 times for Mg^{2+} concentration measurements. Water Na^+, Mg^{2+} and Ca^{2+} concentrations were determined using flame atomic absorption spectrophotometry (SpectrAA-220FS, Varian, Mulgrave, VIC, Australia) and certified commercial standards. Water alkalinity was calculated as $CaCO_3$ alkalinity by titration of 10 g water with 0.01 mol l^{-1} HCl (BDH®, VWR analytical) to pH 4.5 (Hach®, 2017, DOC316.53.01151) at room temperature. The temperature at which this analysis was done was different from the temperature at the collection site, but even if the temperature differed by 10°C it would only result in a 0.16 or 9.3×10^{-5} mg l^{-1} difference in pH or alkalinity, respectively. Therefore, we do not believe that minor differences in temperature would have a major impact on our conclusions.

Lake/river sampling

To assess the ionoregulatory phenotype of the fish in their native habitats, tissue sampling was conducted at each collection site. Before tissue sampling, fish were transferred from the minnow keeper into a 20 l covered bucket with aerated water from the collection site. Fish were allowed to recover for 30 min before being individually netted and transferred to a separate container with MS-222 (500 mg l^{-1}; buffered to pH 7 with KOH) in lake or river water. Once the fish lost equilibrium, it was removed from MS-222, blotted dry with a paper towel, and weighed to the nearest 0.1 g. The caudal peduncle was severed with a razor blade and blood was sampled using capillary tubes and transferred into a microcentrifuge tube, and the blood cells were separated by centrifugation. Plasma was removed, placed into a new microcentrifuge tube and frozen in liquid nitrogen for later determination of plasma Na^+ and Cl^- concentration. The right gill basket was dissected, placed in a microcentrifuge tube and frozen in liquid N_2 for later determination of Na^+/K^+ -ATPase and H^+-ATPase activity. All samples were transported to UBC in liquid N_2 and then stored at −80°C for later analysis.

ALW and IPW treatments

To investigate aspects of ion regulation under non-stressful and stressful freshwater conditions, we exposed fish to ALW and to IPW by transferring them from their acclimation conditions (ALW) to experimental conditions (ALW and IPW) in separate experiments. The ALW treatment had the same water chemistry as the water used during laboratory acclimation (see above) and the IPW was dechlorinated Metro Vancouver City tap water (from 2017; see Table 1).

In all transfer experiments, 8–10 fish from each locality were randomly transferred to one of 18, 200 l glass aquaria with pea gravel (typical diameter of 0.1–0.3 cm) as a substrate, and filtration and aeration under the desired treatment, ALW or IPW, in an environmental chamber set to 11°C and a 12 h:12 h light:dark photoperiod. Fish were held under these conditions for 2 weeks before sampling. Water samples were collected before introducing fish to the aquaria. During the 2 week experimental period, fish were fed to satiation once every 2 days until 2 days before sampling, when feeding ceased. Partial water changes were performed 1 day after feeding to maintain water quality. After the 2 week experimental period, fish were netted from their tank and transferred to a 20 l covered bucket filled with aerated water of the same composition as the experimental treatment (ALW or IPW). To minimize any impacts of sampling stress, fish were allowed to recover from transfer to the bucket for 30 min before being individually netted and transferred to a separate container with MS-222 (500 mg l^{-1}; buffered to pH 7 with KOH) in water of the same chemical composition as their treatment water. Once anaesthetized, fish were removed from the anaesthetic, weighed, and blood and gill samples were taken for determination of plasma ion concentration and enzyme activity (see below) as described above for lake/river sampling.

Plasma ions

For analysis of plasma Na^+ and Cl^- concentration, plasma samples were thawed at room temperature and diluted 2000 times with ultrapure water (MilliQ® water purification system). Plasma Na^+ concentration was determined by using flame atomic absorption spectrophotometry (SpectrAA-220FS, Varian) and certified NaCl standards. Plasma Cl^- concentration was measured spectrophotometrically according to Zall et al. (1956) using certified NaCl standards.

Gill Na^+/K^+-ATPase and H^+-ATPase activity

Gill Na^+/K^+-ATPase activity was measured on crude gill homogenates according the protocol outlined by McCormick (1993). Briefly, ∼20 mg of gill filaments from the second gill

arch was dissected on ice and immediately homogenized in SEI buffer (250 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} Imidazole, pH 7.3) containing 1% sodium deoxycholic acid. The homogenate was centrifuged at 5000 ***g*** for 30 s at 4°C and the supernatant was collected and stored at −80°C until analysis. Gill Na^+/K^+-ATPase activity was determined by coupling ouabain-sensitive ATP hydrolysis to NADH oxidation via pyruvate kinase and lactate dehydrogenase. Specifically, crude homogenates were thawed on ice and 10 µl was assayed for ATPase activity in the absence or presence of 0.5 mmol l^{-1} ouabain (Sigma-Aldrich). Gill Na^+/K^+-ATPase activity was calculated by subtracting ouabain-sensitive activity from total activity and expressed as µmol ADP mg^{-1} protein h^{-1}. Gill H^+-ATPase activity was measured in a similar manner, but instead of ouabain, 1 mmol l^{-1} *N*-ethylmaleimide (Sigma-Aldrich) was used as a specific inhibitor of H^+-ATPase activity. The cocktail used for measuring H^+-ATPase activity was the same as that used for Na^+/K^+-ATPase, except that it also included 5 mmol l^{-1} sodium azide (Sigma-Aldrich) and 0.5 mmol l^{-1} ouabain (to reduce background ATPase activity). Gill H^+-ATPase activity was calculated by subtracting *N*-ethylmaleimide-sensitive activity from total activity and expressed as µmol ADP mg^{-1} protein h^{-1}. Total protein in the homogenate was measured using Bradford reagent (Sigma-Aldrich) with bovine serum albumin standards (Bradford, 1976). All assays were run in triplicate and the coefficient of variation among replicates was <10%.

SDS-PAGE and western blot analysis

A portion of the gill homogenates that were used for the determination of gill Na^+/K^+-ATPase and H^+-ATPase activity was then used for western blot analysis. The samples were added to an equal volume of 2× Laemmli buffer (Laemmli, 1970) and heated at 70°C for 15 min. Those samples were then diluted to approximately 1 µg $µl^{-1}$ with 1× Laemmli buffer and 15 µl of each sample was loaded into separate lanes on a polyacrylamide gel (4% stacking gel and 12% separating gel). A common standard was also created from a pool of diluted samples and included in a separate lane on each gel to allow for gel–gel comparisons and normalization to protein content across gels. A protein ladder (PageRuler™ Prestained protein ladder, Thermo Scientific) was also loaded on each gel to monitor protein migration and size. Proteins were separated by SDS-PAGE using mini-protean vertical electrophoresis apparatus (Bio-Rad). The gels were run at 75 V for 15 min to stack protein from a relatively similar starting point, and then 150 V for 90 min in order to size separate the proteins. Following electrophoresis, proteins were transferred to nitrocellulose membranes using a Bio-Rad semi-dry transfer apparatus. Blots were then blocked with 5% non-fat milk in TTBS (0.05% Tween-20 in Tris-buffered saline, TBS: 20 mmol l^{-1} Tris-HCl, 500 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, pH 7.5) for at least 1 h.

Blots were incubated in the primary antibody (diluted 1:250–1:500; details below) in TTBS overnight at room temperature with agitation. After washing 3 times with TTBS, blots were incubated in goat anti-mouse or goat anti-rabbit IgG HRP-conjugated secondary antibody diluted 1:10,000 in TTBS for 1 h at room temperature. The membranes were washed 3 times in TTBS, then once in TBS, and were incubated in enhanced chemiluminescent solution (Thermo Fisher Scientific) according to the manufacturer's instructions and exposed with a Bio-Rad ChemiDoc™ imager. Tissue homogenates were probed with each antibody on separate blots. Digital photos were taken, and band staining intensity was analysed using ImageJ (NIH, Bethesda, MD, USA) in a blind-to-sample-ID manner.

Antibodies

A monoclonal antibody specific for the α-subunit of chicken Na^+/K^+-ATPase was used for detecting gill Na^+/K^+-ATPase (α5 clone; Developmental Studies Hybridoma Bank). This antibody has been used in other fishes for identifying Na^+/K^+-ATPase α-subunit (McCormick et al., 2009; Wilson et al., 2000a,b). Rabbit polyclonal antibody specific to H^+-ATPase B-subunit was used to identify H^+-ATPase (provided by J.M.W.; Wilson et al., 2007b). The proteins β-actin (Abcam) and α-tubulin (Developmental Studies Hybridoma Bank) were used as loading controls for each sample. We used the mean band intensity from the two loading controls to correct the targeted protein band intensity.

Arterial blood pH

To collect arterial blood pH data, we repeated the above experiments the following year using the same ALW and IPW treatments. All conditions and animal collection sites were similar between years, except that we were not able to collect enough fish from Okanagan Lake and therefore they were not included in our analysis of arterial blood pH. To minimize the effect of disturbance of fish during sampling on blood pH, individual fish were placed in 4 l plastic bottles submerged in their original treatment aquaria 1 day before sampling. The 4 l plastic bottles had portions of their sides removed and covered with plastic mesh to allow water flow-through when in the treatment aquaria. A 1 l volume of water was retained below the mesh to hold fish when the bottles were removed from the treatment tanks. To sample fish, a bottle containing an individual fish was gently removed from the aquarium and an overdose of MS-222 (5 g l^{-1} pH 7; final concentration 0.5 g l^{-1}) was added to the sampling bottle. Once a fish lost equilibrium, typically within 1 min, it was removed from the water, blotted dry with a paper towel, and weighed to the nearest 0.1 g. Then, the fish was laid upside down on the dissection table and an arterial blood sample was taken from the dorsal aorta accessed through the mouth using a 23 gauge needle and 1 ml disposable syringe. The blood was then immediately transferred to a 1.5 ml Eppendorf tube (in a water bath set to 11°C) and whole-blood pH was measured using an InLab Micro pH electrode (Mettler Toledo) at 11°C (i.e. the same temperature as the treatment tanks). The InLab Micro pH electrode, 1.5 ml Eppendorf tubes, tube float, water bath, heparin saline (200 IU mg^{-1} in 0.9% NaCl saline; 0.05 ml), 23 gauge needles and 1 ml disposable syringes used in this experiment were equilibrated to the same temperature as the treatment water before sampling started. The needle and disposable syringe were treated with heparinized saline (2 IU ml^{-1} in 0.9% NaCl saline) before use.

Statistical analysis

To determine whether variation in body mass had an influence on physiological traits, we first assessed whether there was an association between body mass and gill Na^+/K^+-ATPase and H^+-ATPase activity under each treatment condition using Pearson correlation analysis. Based upon this analysis, there was no effect of body mass on measured physiological traits (P>0.05; Table S1); therefore, body mass was not included in our subsequent analysis.

To assess differences in water chemistry among coastal rivers, coastal lakes and interior lakes, we performed principal component analysis (PCA) on all water chemistry variables from the nine locations (Table 1). All variables were ln transformed to reduce the extreme values, then centred, and scaled to standardized the variables before PCA analysis in R using the ade4 (Dray et al., 2007) and ggbiplot packages (https://github.com/vqv/ggbiplot/tree/experimental). The mean PC scores along the first two axes for

each habitat were analysed using nested analysis of variance (ANOVA; R function anova).

To evaluate whether our experimental treatments had an effect on body mass, we ensured the data were normally distributed using Shapiro–Wilk normality test and compared the effects of treatment within each population using a Wilcoxon test for non-parametric data and unpaired *t*-tests with Welch's correction in GraphPad Prism version 5.0a (GraphPad Software, San Diego, CA, USA; www.graphpad.com).

To assess the variation in plasma ions, gill enzyme activity and blood pH in fish sampled directly from their native habitats, we used nested ANOVA (R function anova). In this analysis, populations were nested within habitat types (coastal river, coastal lake and interior lake), and individual fish were replicates within a population. Tukey *post hoc* analysis was used to determine where significant differences were present (https://CRAN.R-project.org/package=nlme). To analyse variation within each habitat type, we conducted one-way ANOVA and Tukey tests using GraphPad Prism version 5.0a.

To assess the variation in plasma ions, gill enzyme activity, enzyme protein content and blood pH in fish between the ALW and IPW treatments, among coastal river, coastal lake and interior lake habitats and among populations within each habitat type, we applied a three-factor, nested ANOVA (R function anova). In the model, treatment, habitat type and population were factors, populations were nested in habitats, and individual fish were replicates within a population. When significant main-effects or interactions were identified, *post hoc* analysis was performed using the lsmeans function in the emmeans package (R function emmeans; https://CRAN.R-project.org/package=emmeans).

To assess the relationship between the measured physiological traits and lake/river water chemistry, we conducted Pearson correlation analysis. To assess the potential environmental impact on fish plasma ion composition, we conducted correlation analysis between water chemistry, i.e. water Na^+ and conductivity, and plasma ions, i.e. plasma Na^+ and Cl^-, on the lake/river samples. Moreover, according to the proposed role of gill H^+-ATPase, i.e. to extrude H^+ and take up Na^+, we assessed the combined effect of water Na^+ and pH on gill H^+-ATPase activity from lake/river samples. To assess whether the combined effect of water Na^+ and pH on gill H^+-ATPase has become a selection force, we conducted this analysis on the fish in ALW. To assess whether variation in gill H^+-ATPase is related to pH regulation, we conducted correlation analysis on the arterial blood pH and gill H^+-ATPase activity on the fish from ALW. In addition, to evaluate whether changes in protein content contribute to explaining variation in gill Na^+/K^+-ATPase and H^+-ATPase activity, we conducted the correlation analysis between the protein amount and enzyme activity. All correlation analyses were performed in RStudio V1.1.456 (https://www.rstudio.com/). For all correlation analysis, we did not correct *P*-values for multiple comparisons because each correlation was a test of an independent hypothesis, but the lack of correction inflates type I error and therefore the reader should view some of the results of our correlative analysis as tentative.

RESULTS

Water chemistry

PCA (Fig. S2) of lake/river water chemistry variables (Table 1) revealed three distinct groupings which separated the three habitat types. Principal component (PC) 1 accounted for 79.2% of the variation among habitats and highlighted separation of the coastal lakes from the other two habitat types (Fig. S2). Nested ANOVA results for the PC1 scores revealed that coastal lakes were distinct from coastal rivers (P=0.002) and from interior lakes (P=0.01), whereas coastal rivers and interior lakes were not significantly different from each other (P=0.17). The differentiation along PC1 was largely driven by water Ca^{2+}, Mg^{2+}, alkalinity and conductivity, whereby coastal lakes generally had lower Ca^{2+}, Mg^{2+}, alkalinity and conductivity compared with the other two habitat types. PC2 accounted for 19.3% of the variation where water pH and Na^+ drove most of the differences among all three habitat types (Fig. S2). Nested ANOVA revealed that the PC2 scores were significantly different across all the habitat types (P=0.03 between coastal rivers and coastal lakes; P=0.0000935 between coastal lakes and interior lakes; P=0.000018 between coastal rivers and interior lakes). In general, coastal rivers had higher water Na^+ concentrations and lower pH compared with interior lakes, and coastal lakes were intermediate in these variables (Fig. S2).

Lake/river sampling

For *C. asper* sampled directly from their lake or river sites, there were significant population-within-habitat-type differences in plasma Na^+ among the coastal river populations and in plasma Cl^- among the coastal river and interior lake populations; however, nested ANOVA results revealed a significant effect of habitat type on plasma Na^+ and Cl^- (Fig. 1A,B). Coastal river fish had significantly higher plasma Na^+ and Cl^- compared with fish from the other two habitat types (Fig. 1A,B). For both gill Na^+/K^+-ATPase and H^+-ATPase activity, there were significant population-within-habitat effects in all habitat types except for gill Na^+/K^+-ATPase in fish sampled from interior lakes (Fig. 1C,D). Despite the population-within-habitat differences, gill Na^+/K^+-ATPase activity was found to vary significantly across habitat types, with fish from coastal lakes having higher activity than fish from coastal rivers. Fish from interior lakes had intermediate gill Na^+/K^+-ATPase activity (Fig. 1C). For gill H^+-ATPase, nested ANOVA revealed that fish from the interior lakes had significantly higher activity compared with fish from the other two habitat types, which were similar to each other (Fig. 1D).

ALW and IPW treatments

Body mass and survival

In fish that had been acclimated to ALW for ∼4 weeks and subsequently transferred to ALW and held for 2 weeks, there was no effect of the 2 weeks in ALW on body mass within any population (P>0.05; Table S2). In ALW-acclimated fish that were transferred to IPW and held for 2 weeks, there was no change of body mass except for fish from the Fraser River population (coastal river), where body mass decreased by 36% (P=0.03; Table S2). No fish died as a result of the ALW or IPW treatments, but several fish from various treatments died as a result of cannibalism.

Plasma ions

For *C. asper* sampled after transfer to ALW and IPW, three-factor nested ANOVA revealed a significant difference in plasma Na^+ between the two treatments and among populations (Table S3A). For *C. asper* sampled after transfer to ALW, *post hoc* analysis revealed no statistical differences among habitats or population-within-habitat for plasma Na^+ (Fig. 2A). Transfer to IPW resulted a significant increase in plasma Na^+ compared with that in ALW for the populations from the coast lake habitats (Table S3B), but not in the other habitats. In IPW, there was a significant population-within-habitat difference in plasma Na^+ in the interior lake habitat, but no significant differences were detected among the three habitat types (Fig. 2A).

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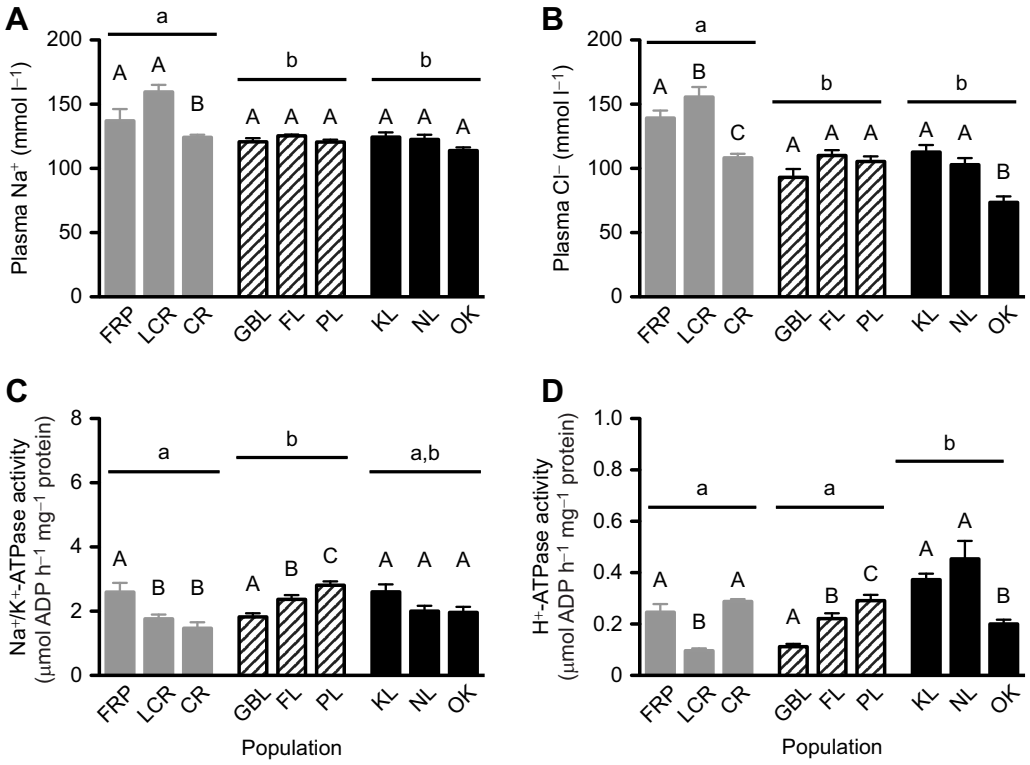

Fig. 1. Plasma ion concentration and Na^+/K^+-ATPase and H^+-ATPase activity of prickly sculpins from lake/river populations. Plasma Na^+ (A) and Cl^- (B) concentration, Na^+/K^+-ATPase activity (C) and H^+-ATPase activity (D) for the different populations (means+s.e.m., *N*=5–10). Grey bars represent coastal river populations, striped bars represent coastal lake populations and black bars represent interior lake populations. Uppercase letters above each bar indicate significant intra-habitat differences. Lowercase letters above each line indicate significant inter-habitat differences; plasma Na^+: $P<0.001$ for coastal rivers–coastal lakes, $P<0.001$ for coastal rivers–interior lakes; plasma Cl^-: $P<0.001$ for coastal rivers–coastal lakes, $P<0.001$ for coastal rivers–interior lakes; Na^+/K^+-ATPase activity: $P=0.02$ for coastal rivers–coastal lakes; H^+-ATPase activity: $P<0.001$ for coastal rivers–interior lakes, $P<0.001$ for coastal lakes–interior lakes. FRP, Fraser River Park; LCR, Little Campbell River; CR, Cowichan River; GBL, Garden Bay Lake; FL, Frederick Lake; PL, Pachena Lake; KL, Kamloops Lake; NL, Nicola Lake; OK, Okanagan Lake.

In fish sampled after transfer to ALW and IPW, three-factor nested ANOVA revealed significant differences in plasma Cl^- between the two treatments, among habitats as well as among populations. In ALW, there were population-within-habitat differences in the coastal river habitat, but no differences among habitats were detected (Fig. 2B). Transfer to IPW resulted in a significant decrease in plasma Cl^- in the coastal river habitat compared with that in ALW (Table S3B) and within IPW, plasma Cl^- was also significantly lower in fish from the coastal river populations than in those from the other two habitat types. There were also significant population-within-habitat differences among populations from the coastal lake and interior lake habitats (Fig. 2B).

Gill Na^+/K^+-ATPase

For gill Na^+/K^+-ATPase activity, three-factor ANOVA revealed significant differences between the ALW and IPW treatments, and among habitat types and populations. There was also a significant interaction between treatment and habitat type (Table S3A). In ALW, *post hoc* analysis revealed significant population-within-habitat differences among the coastal lakes, but not in the coastal river or interior lake habitats (Fig. 3A). Among habitats, the prickly sculpin from coastal lake populations exposed ALW had significantly higher gill Na^+/K^+-ATPase activity compared with fish from the coastal river populations, with fish from the interior lakes having intermediate gill Na^+/K^+-ATPase activity (Fig. 3A). In comparison to ALW, fish from all three habitats increased gill Na^+/K^+-ATPase activity in IPW (Table S3B), but the significant interaction between habitat and salinity suggests that populations from some habitats upregulate to a greater degree than others. Unfortunately, *post hoc* analysis did not identify the specific nature of the interaction. In IPW, there were significant population-within-habitat differences in gill Na^+/K^+-ATPase activity within all habitats, but despite these differences, *post hoc* analysis revealed that fish from the coastal lake and interior lake habitats had significantly higher gill Na^+/K^+-ATPase activity compared with fish from the coastal river habitat (Fig. 3A).

For gill Na^+/K^+-ATPase α-subunit protein content, immunoreactivity appeared as one major band at a molecular mass of 112 kDa and occasionally a second less intense band at approximately 68 kDa, but these less intense bands were not quantified as Na^+/K^+-ATPase (Fig. S3A,B). Three-factor ANOVA revealed significant differences in gill Na^+/K^+-ATPase α-subunit relative content between the two treatments, among habitat types as well as among population-within-habitat type (Table S3A). In ALW, *post hoc* analysis revealed population-within-habitat differences in the interior lake habitat (Fig. 3B). Among habitats, the prickly sculpin from coastal river and interior lake habitats had significantly higher gill Na^+/K^+-ATPase α-subunit content compared with those from the coastal lake habitat. In comparison to ALW, transfer to IPW resulted in a significant increase in the expression of gill Na^+/K^+-ATPase α-subunit in fish from the coastal lake habitat, but not in those from the other two habitats (Table S3B). In IPW, significant population-within-habitat

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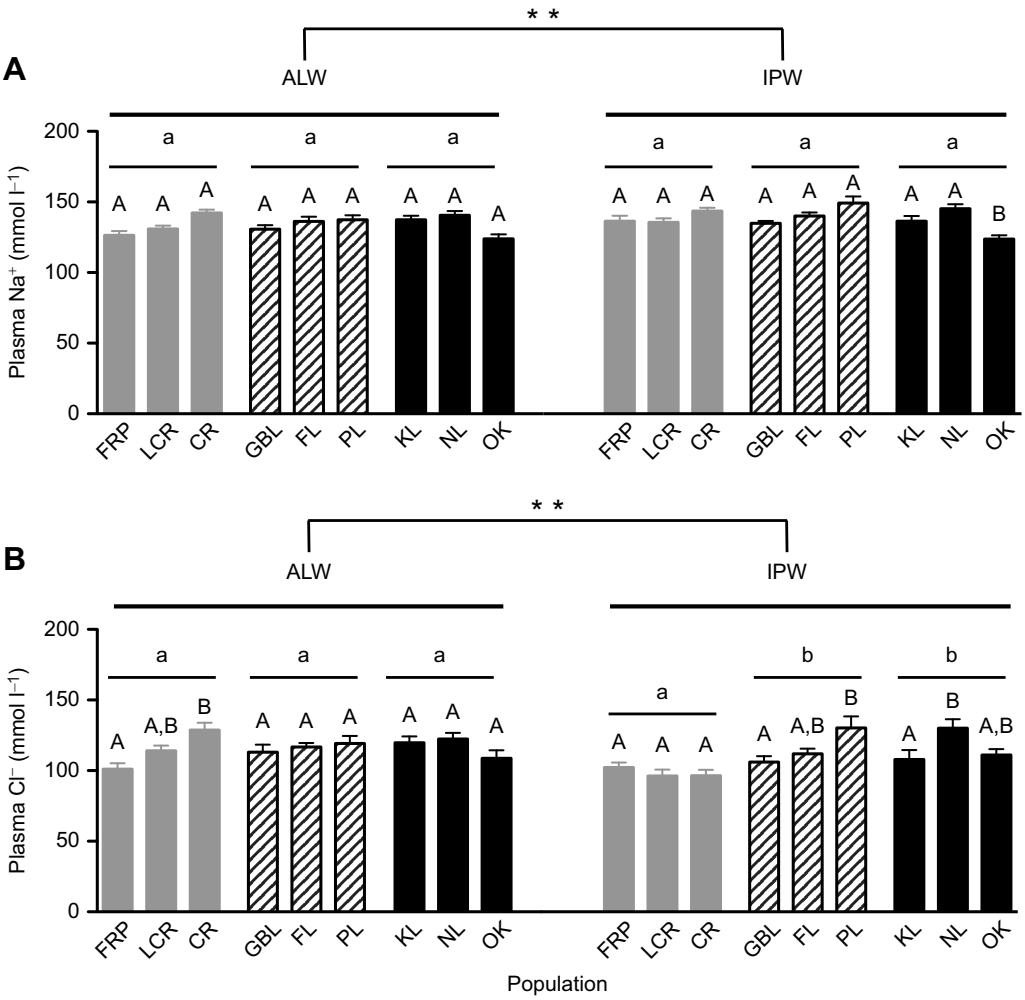

Fig. 2. Plasma Na^+ and Cl^- of prickly sculpins in the two water treatments. (A) Artificial lake water (ALW) and (B) ion-poor water (IPW) treatments (means+s.e.m., *N*=7–10). Grey bars represent coastal river populations, striped bars represent coastal lake populations and black bars represent interior lake populations. Uppercase letters above each bar indicate significant intra-habitat differences. Lowercase letters above each line indicate significant inter-habitat differences; plasma Cl^- (IPW): *P*=0.0003 for coastal rivers–coastal lakes, *P*=0.05 for coastal rivers–interior lakes. Asterisks indicate a significant difference between the two treatments (**P<0.01).

differences were detected from the coastal river populations (Fig. 3B). Among habitats, *post hoc* analysis revealed that fish from the coastal lake habitat had a significantly lower level of gill Na^+/K^+-ATPase α-subunit expression compared with those from the coastal river habitat. Interior lake habitat had an intermediate expression level. To investigate whether there is a relationship between Na^+/K^+-ATPase activity and α-isoform content, we conducted association analysis across populations in ALW and IPW, but no significant relationships were detected.

Gill H^+-ATPase

For gill H^+-ATPase activity, three-factor ANOVA revealed significant differences between ALW and IPW treatments, among habitat types and populations (Table S3A). In ALW, *post hoc* analysis revealed a significant population-within-habitat difference in the two coastal habitats (Fig. 4A), and among habitats, the interior lake populations had significantly higher gill H^+-ATPase activity than the coastal river and coastal lake populations (Fig. 4A). Transfer to IPW resulted in an increase in gill H^+-ATPase activity compared with that in ALW in populations from all three habitats (Table S3B). In IPW, despite population-within-habitat differences in coastal lakes and interior lakes, fish from interior lakes had the highest overall gill H^+-ATPase activity compared with fish from the coastal river and coastal lake habitats, which were similar to each other (Fig. 4A).

Gill H^+-ATPase B-subunit immunoreactivity was detected as a band at the expected molecular mass of 56 kDa. Occasionally, bands around 130 and 70 kDa were also observed, but these were not quantified as H^+-ATPase (Fig. S3C.D). For gill H^+-ATPase B-subunit relative abundance, three-factor ANOVA revealed significant differences between the two treatments, and among population-within-habitat type in the fish transferred to ALW and IPW (Table S3A). In ALW, no significant differences were detected among populations or among habitats (Fig. 4B). Transfer to IPW resulted in a significant upregulation of gill H^+-ATPase B-subunit expression in all habitats (Table S3B). In IPW, significant population-within-habitat differences were detected in the two coastal habitats, but there were no differences among the habitats (Fig. 4B). Both in ALW and IPW treatments, gill H^+-ATPase protein content and activity were positively correlated with each other among the populations (ALW r=0.68, P=0.045; IPW r=0.69, P=0.04; Fig. 5).

Arterial pH

Three-factor nested ANOVA revealed significant differences in arterial pH between the two treatments, among habitats and among populations (Table S3A). There was also a significant interaction between treatment and habitat type (Table S3A). In ALW, *post hoc* analysis revealed significant population-within-habitat differences among the coastal lake populations, but not among the coastal river or interior lake populations. Among habitats, fish from the interior lakes had a significantly lower arterial pH compared with those from the two coastal habitats, which had similar arterial pH (Fig. 6). Compared with ALW, transfer to IPW resulted in a significant

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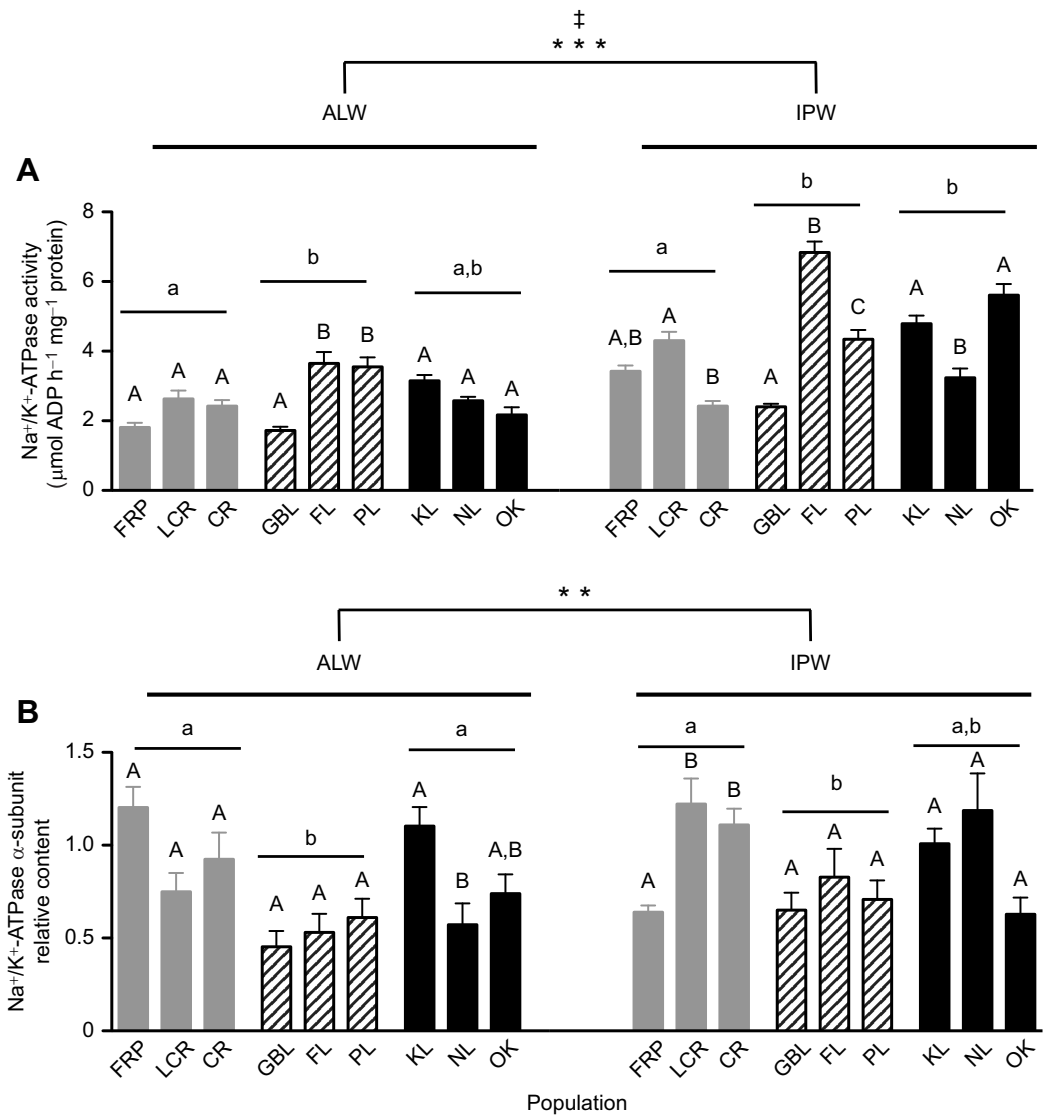

Fig. 3. Gill Na^+/K^+-ATPase in prickly sculpins in the two water treatments. (A) Activity and (B) α-subunit relative content in ALW and IPW fish (means+s.e.m., *N*=5–10). Grey bars represent coastal river populations, striped bars represent coastal lake populations and black bars represent interior lake populations. Uppercase letters above each bar indicate significant intra-habitat differences. Lowercase letters above each line indicate significant inter-habitat differences; Na^+/K^+-ATPase activity in ALW: P=0.0008 for coastal rivers–coastal lakes; Na^+/K^+-ATPase activity in IPW: P<0.0001 for coastal rivers–coastal lakes, P<0.0001 for coastal rivers–interior lakes; Na^+/K^+-ATPase α-subunit relative amount in ALW: P<0.0001 for coastal rivers–coastal lakes, P=0.0093 for coastal lakes–interior lakes; Na^+/K^+-ATPase α-subunit relative amount in IPW: P=0.0033 for coastal rivers–coastal lakes. Asterisks indicate a significant difference between the two treatments (**P<0.01, ***P<0.001). Double-daggers indicate a significant habitat and treatment interaction effect (‡P<0.05).

decrease in arterial pH in fish from both coastal lake and interior lake habitats (Table S3B). In IPW, significant population-within-habitat differences were detected in coastal lake and interior lake habitats. Among habitats, populations from coastal river habitats had significantly higher arterial pH than populations from the coastal and interior lakes, which were similar to each other (Fig. 6).

Associations between physiological characteristics and lake/river water chemistry

Across all populations, plasma Na^+ and Cl^- from fish sampled from the lake/river were positively correlated with water Na^+ and conductivity of their natural habitats (Fig. 7). Further, gill H^+-ATPase activity from *C. asper* from the lake/river and following ALW transfer was positively correlated with PC2 scores of water chemistry (e.g. PC2 scores shown in Fig. S2). Here, increasing environmental pH and decreasing Na^+ concentration were associated with higher gill H^+-ATPase activity (lake/river r=0.69, P=0.04; ALW r=0.83, P=0.01; Fig. 8). In addition, there was a significant negative association between blood pH and H^+-ATPase activity in the ALW transfer experiment (r=0.78, P=0.02, Fig. 9).

DISCUSSION

Interspecific variation in ionoregulation has been extensively studied in fishes from a variety of contrasting environments with the aim of understanding the ecological implications of variation in physiology (Gonzalez and McDonald, 1994; Henriksson et al., 2008; Matey et al., 2011; Scott et al., 2004); however, intraspecific comparisons are not as common (Velotta et al., 2015). Postglacial recolonization of aquatic habitats of BC by *C. asper* provides a unique system to assess the association between environment and physiological performance with respect to ionoregulation. Previous studies had demonstrated phenotypic (McAllister and Lindsey,

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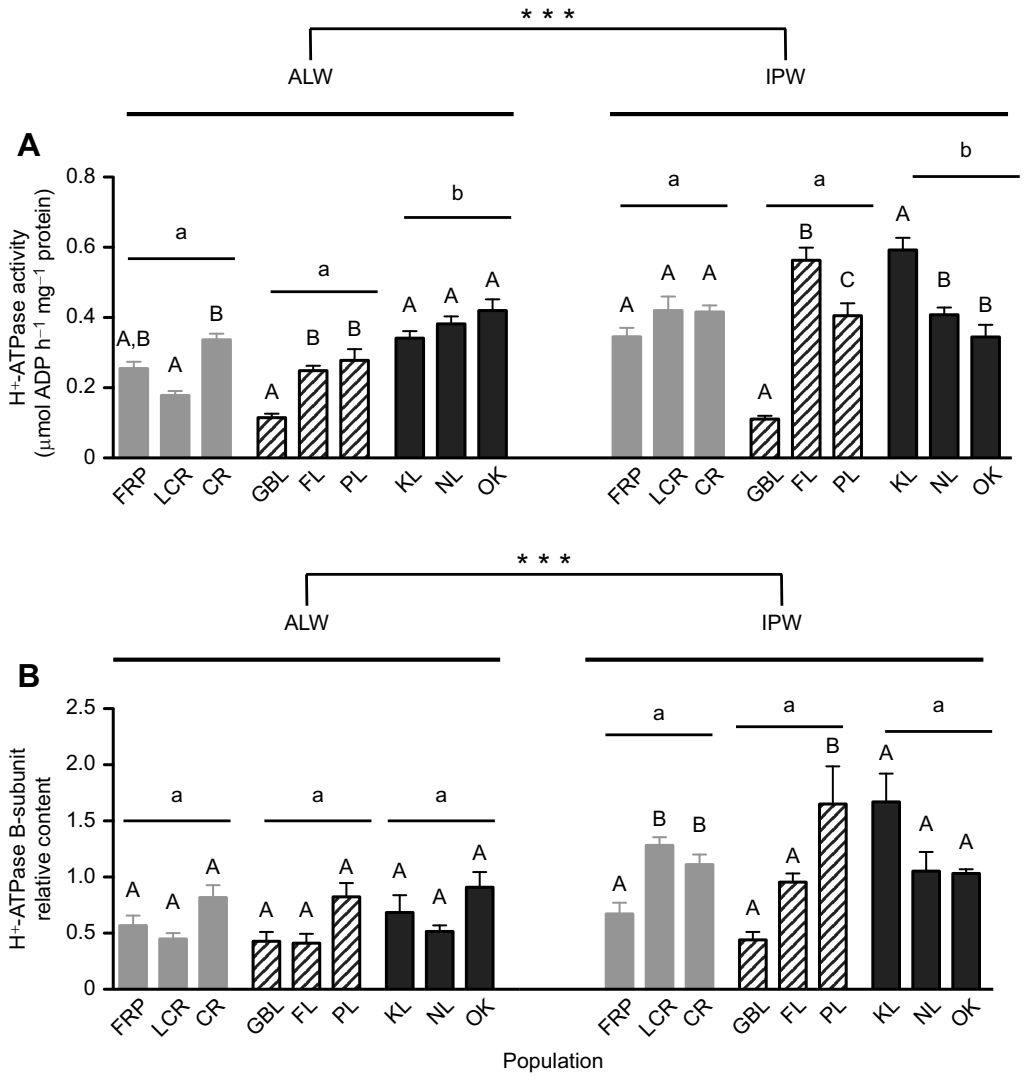

Fig. 4. Gill H$^+$-ATPase in prickly sculpins in the two water treatments. (A) Activity and (B) B-subunit relative content in ALW and IPW fish (means+s.e.m., *N*=5–10). Grey bars represent coastal river populations, striped bars represent coastal lake populations and black bars represent interior lake populations. Uppercase letters above each bar indicate significant intra-habitat differences. Lowercase letters above each line indicate significant inter-habitat differences; H$^+$-ATPase activity in ALW: $P<0.001$ for coastal rivers–interior lakes, $P<0.001$ for coastal lakes–interior lakes; H$^+$-ATPase activity in IPW: $P<0.0001$ for coastal rivers–interior lakes, $P<0.0001$ for coastal lakes–interior lakes. Asterisks indicate a significant difference between the two treatments (***$P<0.001$).

1959; Bohn and Hoar, 1965) and genetic (Dennenmoser et al., 2014; 2017) diversification in morphology and physiology among populations of *C. asper* and this diversification has been associated with differences in the local aquatic habitat. In particular, allele frequencies at loci related to Na^+ and Cl^- regulation (Na^+/K^+-ATPase and Na^+/Cl^--co-transporter) and gill permeability (claudins) are divergent between populations of *C. asper* sampled from freshwater creeks and estuaries (Dennenmoser et al., 2017). Our study builds upon these findings and is the first to document intraspecific variation in the physiology of ion regulation among populations of *C. asper* that inhabit water systems that vary in water chemistry and post-glacial history. Based upon our sampling of *C. asper* populations from distinct habitat types and after exposure to ALW and IPW, our study reveals four key findings which support the hypotheses outlined in the Introduction. First, the habitat-specific differences in plasma Na^+ and Cl^- observed in fish sampled directly from the lake/river environments were eliminated upon ALW acclimation; however, the habitat-based differences in gill Na^+/K^+-ATPase and H^+-ATPase activity observed in fish sampled from the lake/river were preserved following acclimation to ALW. Second, exposure to IPW resulted in an upregulation of gill Na^+/K^+-ATPase and H^+-ATPase activity compared with that in ALW in fish from all habitats, but in IPW, fish from the coastal and inland lake habitats had higher gill Na^+/K^+-ATPase activity and they were better able to maintain plasma Cl^- than fish from the coastal river habitats, which lost plasma Cl^- in IPW. Third, gill H^+-ATPase activity consistently differentiated the interior lake populations from the other two habitats in that it was higher in lake/river populations, and following transfer to ALW and IPW. Furthermore, the positive correlation between gill H^+-ATPase activity and water Na^+ and pH across all populations suggests that natural selection may favour high gill H^+-ATPase activity in fish from environments with high Na^+ and pH. Lastly, across populations, there was a positive relationship between gill H^+-ATPase activity and gill H^+-ATPase B-subunit protein abundance, which suggests that the variation in H^+-ATPase activity is related to the amount of the functional protein expressed in the gill.

Associations between ion regulation and habitat types

The study by Bohn and Hoar (1965) was one of the first to suggest that populations of *C. asper* that encounter estuarine conditions have a reduced capacity to retain electrolytes in fresh water. More recent genomics analyses corroborate these findings and reveal differences in allelic frequency at loci relevant to ion regulation between populations of *C. asper* that reside in environments that differ in osmolality (Dennenmoser et al., 2017). We hypothesized that ion regulation would differ among fish from different habitats based upon historical and current connections to estuarine areas.

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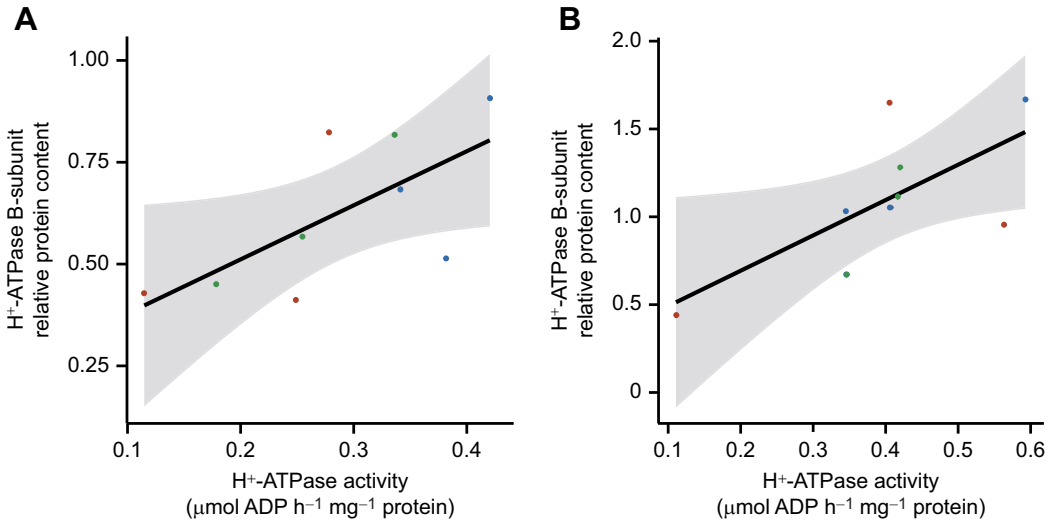

Fig. 5. Correlation analyses between H$^+$-ATPase B-subunit relative protein content and activity in prickly sculpins in the two water treatments. Correlation under (A) ALW treatment (r=0.68, P=0.045) and (B) IPW treatment (r=0.69, P=0.04). Each point represents the mean for each of 9 populations. Green dots represent populations collected from coastal rivers, red dots represent populations collected from coastal lakes and blue dots represent populations collected from inland lakes. Shading represents 95% confidence limits of the regression.

Our data are generally consistent with this hypothesis. For instance, our studies showed that the habitat-based differences in gill Na^+/K^+-ATPase and H^+-ATPase activity observed in the lake/river populations were preserved after 6 weeks acclimation to the common ALW condition, during which plasma ions were similar across all populations and habitats. Indeed, the coastal river populations consistently had the lowest gill Na^+/K^+-ATPase and H^+-ATPase activity, with the coastal lake populations having the highest gill Na^+/K^+-ATPase and the interior lake populations having the highest H^+-ATPase in both LRS and ALW. These data clearly separate the coastal river populations as unique from the coastal and interior lake populations, and the lower gill ion transporter activity in the river populations is likely related to the higher ion content of the coastal river, which may ease the pressure on gill ion transporters in terms of maintaining plasma ion balance. In fact, in the field-sampled fish, plasma Na^+ and Cl^- levels were higher in the coastal river population than in the two lake populations, but these habitat-based effects on plasma ion balance were eliminated upon acclimation to ALW. This suggests that although the coastal river populations have consistently lower gill Na^+/K^+-ATPase and H^+-ATPase activity in ALW, it is still sufficient to maintain plasma ion balance.

Habitat-based differences in ion regulation were further illuminated upon exposure to IPW. In IPW, the metabolic costs

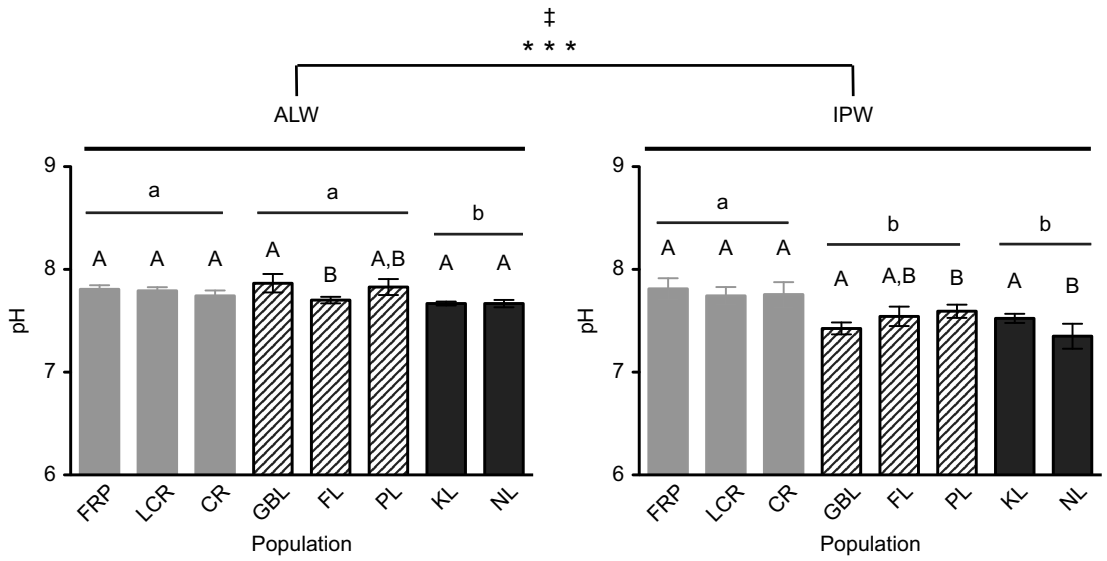

Fig. 6. Arterial blood pH in prickly sculpins in the two water treatments. Data are for ALW and IPW treatments (means+s.e.m., N=5–10). Grey bars represent coastal river populations, striped bars represent coastal lake populations and black bars represent interior lake populations. Uppercase letters above each bar indicate significant intra-habitat differences. Lowercase letters above each line indicate significant inter-habitat differences; ALW: P=0.0129 for coastal rivers–interior lakes, P=0.0081 for coastal lakes–interior lakes; IPW: P<0.0001 for coastal rivers–coastal lakes, P<0.0001 for coastal rivers–interior lakes. Asterisks indicate a significant difference between the two treatments (***P<0.001). Double-daggers indicate a significant habitat and treatment interaction effect (‡P<0.05).

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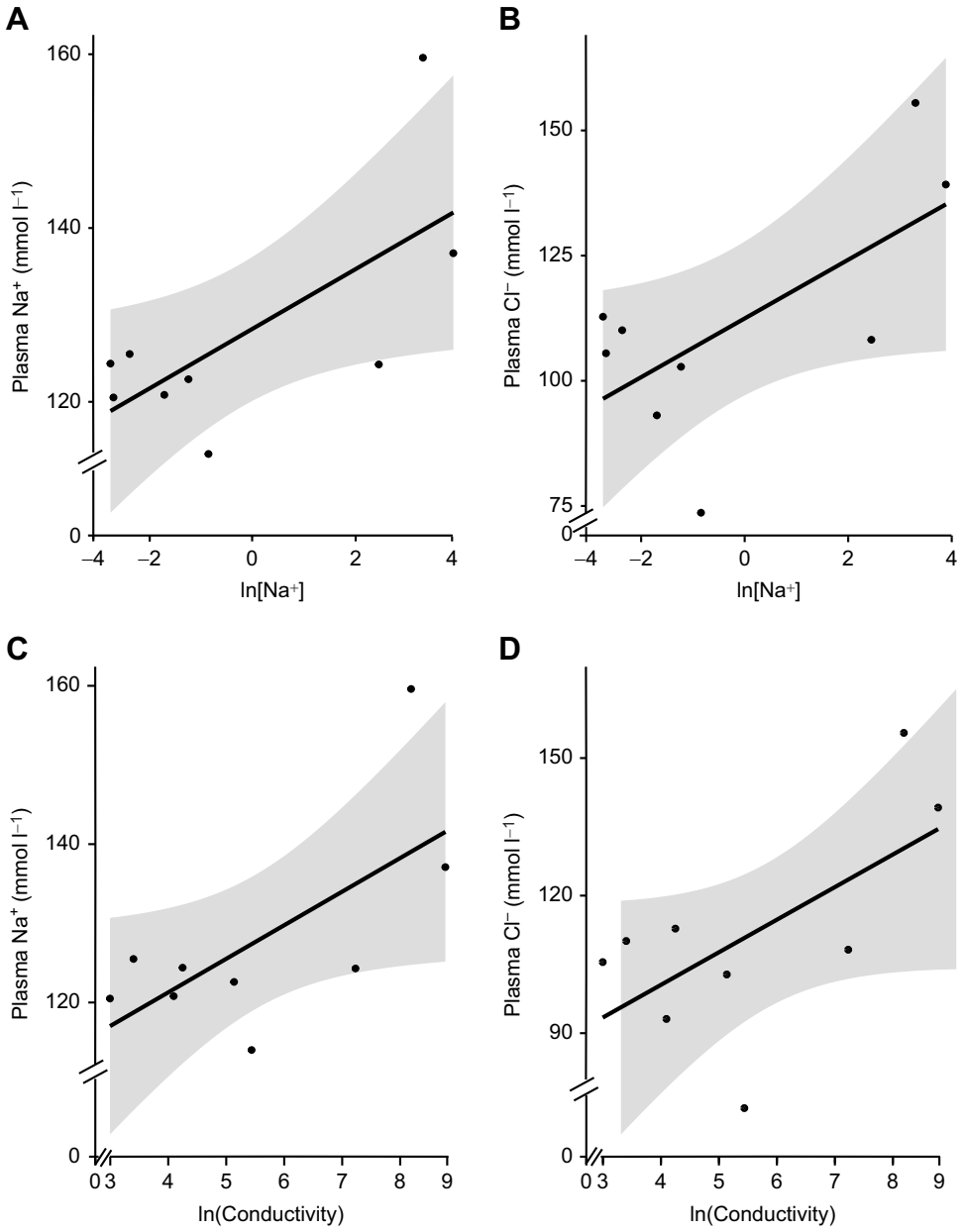

Fig. 7. Correlation analyses between plasma ion concentrations in prickly sculpins from lake/river populations and water chemistry. Correlation between (A) plasma Na^+ concentration and ln water Na^+ concentration (r=0.69, P=0.04), (B) plasma Cl^- concentration and ln water Na^+ concentration (r=0.76, P=0.02), (C) plasma Na^+ concentration and ln water conductivity (r=0.69, P=0.04) and (D) plasma Cl^- concentration and ln water conductivity (r=0.75, P=0.02). Each point represents the mean for each of 9 populations. Shading represents 95% confidence limits of the regression.

associated with ion regulation increase as a result of the upregulation of ion transporter proteins to counteract the unfavourable ion gradient between the plasma and the IPW (Greco et al., 1995). Overall, all populations of *C. asper* from all habitats increased gill Na^+/K^+-ATPase and H^+-ATPase activity in response to IPW, with differences between habitat types. For gill Na^+/K^+-ATPase, we detected a statistical interaction between the ALW/IPW treatment and habitat type effect, indicating that the populations from the different habitat types responded to IPW differently. In IPW, fish from coastal river habitats had lower gill Na^+/K^+-ATPase activity when compared with fish from both the coastal lake and interior lake habitats. This suggests that fish from coastal river populations (all of which have a direct connection with estuarine habitats where salinities of up 20 ppt have been measured; Hagen, 1967; Krejsa, 1967; Ward, 1976; Levy and Northcote, 1982) have a lower capacity to upregulate gill Na^+/K^+-ATPase activity compared with both lake populations.

The reduced responsiveness of the gill Na^+/K^+-ATPase activity to IPW exposure in the coastal river populations also appears to be associated with a reduced capacity for ion regulation, particularly for Cl^-. Indeed, compared with the coastal lake and interior lake populations, the coastal river populations were the only populations to lose plasma Cl^- after transfer to IPW (compared with ALW), resulting in them having lower plasma Cl^- concentrations in IPW. These results suggest that the coastal river populations have an overall reduce capacity for ion regulation in freshwater, particularly IPW. Our results are thus consistent with studies showing that fish with a greater regulatory ability in hyperosmotic environments tend to exhibit a reduced ionoregulatory ability in hypo-osmotic environments. For instance, freshwater-resident alewives (*Alosa pseudoharengus*) exhibited greater freshwater tolerance compared with anadromous alewives (Velotta et al., 2014; 2015). A similar phenomenon was also detected among populations of seawater-adapted Atlantic killifish (*Fundulus heteroclitus*) sampled across a

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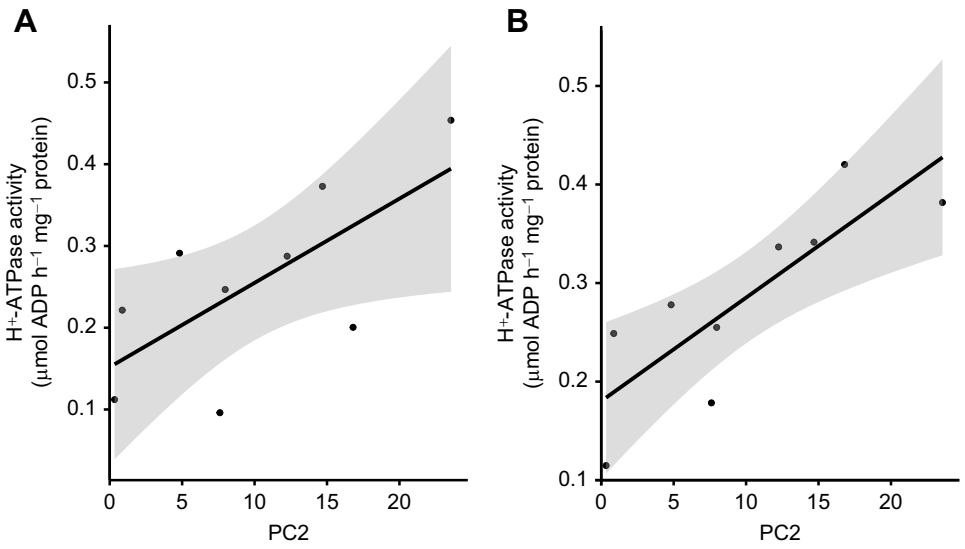

Fig. 8. Correlation analyses between H^+-ATPase activity and the second principal component (PC2) in prickly sculpins from the lake/river and following ALW transfer. Correlation in fish (A) from the lake/river (r=0.69, P=0.04) and (B) following ALW transfer (r=0.83, P=0.01). Each point represents the mean for each of 9 populations. Shading represents 95% confidence limits of the regression.

latitudinal gradient. Populations of Atlantic killifish from the southern USA had higher mortality in fresh water and showed a more rapid decrease in plasma Cl^-, which remained low following abrupt transfer to fresh water, than was observed in populations from the northern USA (Scott et al., 2004). The elevated plasma Cl^- loss in southern Atlantic killifish was thought to be due to high paracellular permeability and a high density of apical crypts compared with Atlantic killifish sampled from northern parts of their range (Scott et al., 2004). The mechanisms underlying the lower capacity for plasma Cl^- regulation in coastal river populations of *C. asper* compared with freshwater-resident sculpins (coastal lakes and interior lakes), however, remains unknown, but it may involve a low affinity for Cl^- uptake in the coastal river fish

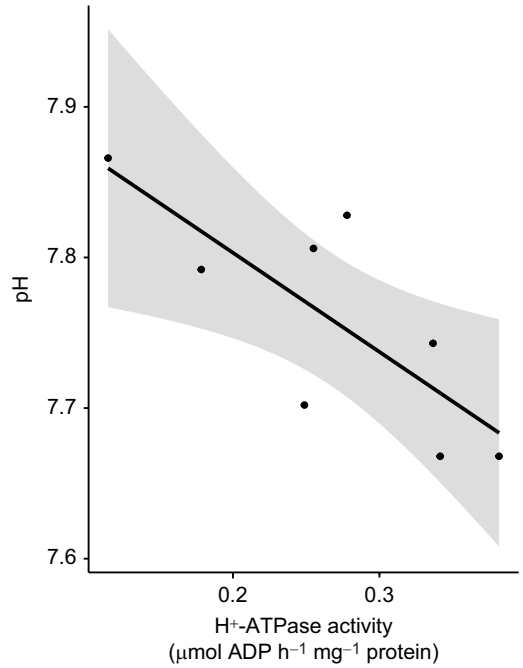

Fig. 9. Correlation analysis between arterial blood pH and H^+-ATPase activity in prickly sculpins following ALW transfer. Each point represents the mean for each of 9 populations (r=0.78, P=0.02). Shading represents 95% confidence limits of the regression.

especially when challenged by IPW, which would be consistent with the low Cl^- influx observed in southern populations of killifish in extreme freshwater environments (~1 mmol l^{-1} external NaCl concentration; Wood and Marshall, 1994; Patrick and Wood, 1999).

In contrast to the patterns of differentiation in gill Na^+/K^+-ATPase activity, which separated the coastal river populations from the coastal and interior lake populations, gill H^+-ATPase appears to strongly differentiate the interior lake populations from the others. Indeed, under all conditions examined, including LRS, ALW and IPW, the interior lake populations had higher gill H^+-ATPase activity than the coastal lake and river populations. Furthermore, gill H^+-ATPase activity increased in IPW compared with ALW in all populations, but the highest activity was observed in the interior populations. This differentiation of gill H^+-ATPase across habitats aligns with the previously described morphological forms that separate the coastal lake and river populations into a 'coastal form' and the interior lakes into the long-term freshwater resident 'inland form' (McAllister and Lindsey, 1959; Krejsa, 1967). Moreover, the differentiation of gill H^+-ATPase activity across the coastal and inland forms suggests that long-term isolation in fresh water (perhaps dating to the Miocene; Krejsa, 1965) may have resulted in the evolution of higher gill H^+-ATPase activity and an enhanced ability to ionoregulate in fresh water, particularly under the stressful IPW conditions. The ionoregulatory differences observed between the coastal and inland forms of prickly sculpin is consistent with studies on the coastal copepod (*Eurytemora affinis*), which invaded fresh water from marine environments along both coasts of the USA and the Great Lakes region multiple times during the last century. Here, the freshwater-adapted population had higher H^+-ATPase activity than the marine-adapted population under freshwater conditions (Lee et al., 2011). More generally, our results are consistent with profound differences in biogeoclimatic regions between coastal and interior regions of BC, and with divergence in genetics, behaviour and physiology between coastal and interior populations in coho salmon (*Oncorhynchus kisutch*; Taylor and McPhail, 1985; Wehrhahn and Powell, 1987), grey wolf (*Canis lupus*; Muñoz-Fuentes et al., 2009) and Douglas fir (*Pseudosuga menziesii var. menziesii* and *P. menziesii var. glauca*; Campbell and Sugano, 1979; Rehfeldt, 1989).

The activity of gill ion transporters, such as H^+-ATPase, can be regulated by a variety of mechanisms including changes in gene/protein expression, isoform switching, differential cellular

localization and various post-translational modifications (Feng and Forgac, 1992, 1994; Guffey et al., 2011; Lee and Bell, 1999; Lin and Randall, 1993). To gain insight into one mechanism of regulation, we measured protein amount using immunoblots to address the hypothesis that differences in protein content contribute to the regulation of enzyme activity. For gill Na^+/K^+-ATPase, we did not observe an association between activity and relative protein expression in either ALW or IPW, as would be expected if total protein content contributed as a mechanism of regulating enzyme activity. Indeed, in several instances, the populations with the highest activity (coastal lake population in both ALW and IPW) had the lowest Na^+/K^+-ATPase protein content, suggesting that other mechanisms may be responsible for regulating Na^+/K^+-ATPase activity across populations from the different habitats. Furthermore, gill Na^+/K^+-ATPase protein content did not increase in IPW relative to that in ALW in two out of the three habitats examined, despite Na^+/K^+-ATPase activity increasing in all habitats. The activity of Na^+/K^+-ATPase is known to be regulated, at least in part, by post-translational modifications such as phosphorylation (e.g. MacDonald and Storey, 1999), which may explain the variation in activity with no discernible differences in total protein. For H^+-ATPase, however, we found an association between enzyme activity and protein content. In IPW compared with ALW, the increase in gill H^+-ATPase activity was mirrored by increases in H^+-ATPase protein content, suggesting a mechanistic link underlying plasticity. In addition, although there were no habitat-based differences in gill H^+-ATPase protein content that matched gill H^+-ATPase activity in either ALW or IPW, across all populations we detected a strong, positive correlation between gill H^+-ATPase protein activity and protein abundance among populations. These associations between gill H^+-ATPase activity and protein suggest that the activity of H^+-ATPase is, at least in part, related to the amount of the protein presented in the gill.

Ecophysiology of ionoregulation

Fish ionoregulation has been extensively studied in closely related species from contrasting environments in terms of salinity, O_2 or pH, with the aim of understanding the environmental factors that may drive natural selection involved in local adaptation (Matey et al., 2011; DeFaveri and Merilä, 2014; Velotta et al., 2015). In our study, we found that despite the observed habitat-based differences in both gill Na^+/K^+-ATPase and H^+-ATPase from lake/river populations being preserved in ALW, the habitat-based differences in plasma Na^+ and Cl^- were eliminated. This, together with earlier findings, led us test for an association between plasma ion concentrations and water ion content, i.e. water Na^+ and conductivity (a general measure of total ion concentration; Gray, 2004), in the field-collected samples. The results revealed a positive correlation between plasma ions and water Na^+ and conductivity. These observations from field-sampled tissues are consistent with findings that plasma ion concentrations in Atlantic killifish and alewives were higher in fish that were acclimated to higher salinities compared with levels in fish acclimated to lower salinities (Scott et al., 2004; Velotta et al., 2014; Kelly et al., 1999).

H^+-ATPase in the freshwater gill is well known to play important roles in Na^+ uptake and pH regulation (Al-Fifi, 2006; Craig et al., 2007; Galvez et al., 2002; Sullivan et al., 1995; Yan et al., 2007). Upon our discovery that gill H^+-ATPase activity clearly and consistently differentiates the coastal and inland forms of *C. asper*, we wished to further investigate the association between gill H^+-ATPase activity and water chemistry in the native environment, particularly Na^+ and pH, which largely differentiates the habitats along PC2 in our PCA of water chemistry. Through correlation analysis, we discovered a significant association between PC2 and gill H^+-ATPase activity in fish from the lake/river population and after acclimation to ALW. These results suggest that environmental pH and Na^+ concentration have a predictable effect on gill H^+-ATPase activity and that these effects persist even when animals are held under common conditions. The association between gill H^+-ATPase activity and environmental pH and Na^+ is consistent with the accepted role of gill H^+-ATPase in the freshwater fish gill, which excretes protons and promotes Na^+ uptake, thus contributing to whole-animal Na^+ and acid–base regulation. Furthermore, after fish were transferred to ALW, we observed a negative association between gill H^+-ATPase activity (high) and fish arterial blood pH (low), suggesting that the environmentally induced changes in gill H^+-ATPase activity do indeed affect whole animal acid–base status. In combination with previous functional studies, our results therefore suggest that water Na^+ and pH might play an important role in driving ecophysiological differentiation among fish from distinct ionic habitats.

In conclusion, our study has made novel contributions to the study of freshwater ionoregulatory physiology among post-glacial populations of a euryhaline fish. First, we examined the ionoregulation physiology of forms known to have diverged at loci related to ionoregulation and revealed activity differences in the enzymatic products of one of the loci, gill Na^+/K^+-ATPase, which was previously shown to have gene sequence divergence among populations sampled from coastal river and lake environments (Dennenmoser et al., 2017). When we expanded our analyses to include *C. asper* from interior lakes located much farther from the sea and from a distinct biogeoclimatic zone, however, the functional differences in gill Na^+/K^+-ATPase were resolved between the two forms (populations from coastal river and interior lake habitats) only under the most ionically challenging IPW conditions. Second, we found that interior lake populations always had significantly higher gill H^+-ATPase activity compared with fish from coastal habitats and the variation in gill H^+-ATPase activity was, at least in part, explained mechanistically by variation in gill protein content. The high gill H^+-ATPase activity under LRS and ALW experimental conditions was positively correlated with Na^+ and pH levels of the natal habitats of *C. asper*. In addition, there was a negative correlation between gill H^+-ATPase activity and arterial pH under ALW in fish from interior lakes and adapted to fresh water since postglacial times, and thus gill H^+-ATPase could be a key indicator of freshwater adaptation. Moreover, the combined effect of water Na^+ and pH variation in native habitats may have played a key role in physiological adaptation in fishes during post-glacial freshwater colonization.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Methodology: S.L., J.M.W., E.T., J.R.; Formal analysis: S.L.; Investigation: S.L.; Resources: J.M.W.; Data curation: S.L.; Writing - original draft: S.L.; Writing - review & editing: J.M.W., E.T., J.R.; Visualization: S.L.; Supervision: E.T., J.R.; Project administration: E.T., J.R.; Funding acquisition: E.T., J.R.

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Data availability

Data are available from figshare: doi:10.6084/m9.figshare.21086575

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