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Osmoregulatory physiology and rapid evolution of salinity tolerance in threespine stickleback recently introduced to fresh water

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ABSTRACT

Background: Post-Pleistocene diversification of threespine stickleback in fresh water offers a valuable opportunity to study how changes in environmental salinity shape physiological evolution in fish. In Alaska, the presence of both ancestral oceanic populations and derived landlocked populations, including recent lake introductions, allows us to examine rates and direction of evolution of osmoregulation following halohabitat transition.

Hypotheses: Strong selection for enhanced freshwater tolerance will improve survival of recently lake-introduced stickleback in ion-poor conditions compared with their oceanic ancestors. Trade-offs between osmoregulation in fresh water and seawater will allow members of the ancestral population to survive better in response to seawater challenge, as mediated by upregulating salt-secreting transporters in the gill. Poorer hypo-osmoregulatory performance of derived fish will be marked by higher levels of taurine and other organic osmolytes.

Methods: We reared clutches at a common salinity from an anadromous and a descendant population, Scout Lake, which has been landlocked for only two generations. We challenged 6-week-old juveniles with extreme low and high salinity treatments and sampled fish over 10 days to investigate putative molecular mechanisms underlying differences in halotolerance. We measured whole-body organic osmolyte content as well as gill Na^+/K^+-ATPase (NKA) activity and $Na^+/K^+/2Cl^-$ cotransporter (NKCC) protein abundance. Other juveniles from these populations and also from Cheney Lake, a fourth-generation landlocked descendant, were gradually salt-acclimated to determine maximum halotolerance limits.

Results: Scout Lake stickleback exhibited 67% higher survival in fresh water than the ancestral anadromous population, but individuals from both groups exhibited similar seawater tolerance. Likewise, the gradual salinity threshold for each population was equivalent (71 ppt).

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Gill NKA activity and NKCC abundance were both higher in seawater-challenged fish, but did not differ between populations. Sticklebacks from both populations responded to acute salinity stress by transiently increasing osmolyte levels in seawater and decreasing them in fresh water.

Conclusion: Enhanced freshwater tolerance has evolved rapidly in recently landlocked stickleback compared with their anadromous ancestors (0.569 haldanes), but the former have retained ancestral seawater-osmoregulatory function.

Keywords: comparative physiology, directional selection, free amino acid, ion balance, osmoregulatory divergence, plasticity, threespine stickleback.

INTRODUCTION

The degree to which an organism can physiologically adjust to counter abrupt environmental change has important implications for adaptation. Physiological plasticity (i.e. wide tolerance ranges for factors such as temperature, and nutrient, oxygen and osmotic concentrations) offers the fitness advantages of higher survival in a broader range of conditions and the ability to traverse patches of unsuitable habitat. However, there are constraints on the direction and magnitude of plasticity (Snell-Rood, 2012) and it does not always evolve adaptively (Ghalambor *et al.*, 2015). Plasticity may also be lost in canalized phenotypes that are locally adapted to the new environment (Lande, 2009; Snell-Rood, 2012). These complexities compel us to ask: How rapidly can tolerance thresholds evolve in populations, and under what ecological circumstances will specialization be favoured?

The potential evolutionary outcomes of plasticity can be examined in aquatic organisms that have undergone halohabitat transitions (Schultz and McCormick, 2013; Lee, 2016). Euryhalinity, the ability to survive across a wide salinity spectrum, is an important evolutionary innovation that facilitated range expansion and rapid adaptation of marine or estuarine taxa to freshwater halohabitats (Lee and Bell, 1999; Schultz and McCormick, 2013). Colonization of geographically isolated and heterogeneous freshwater habitats by founding euryhaline fishes that are capable of crossing osmotic barriers has often yielded prolific radiations in freshwater lakes and streams (Bell and Foster, 1994; Betancur-R, 2010; Nakatani *et al.*, 2011). When a marine population invades fresh water, positive selection for enhanced performance in ion-poor conditions is initially intense because survival is contingent on maintaining ion homeostasis. Consequently, freshwater tolerance is expected to improve rapidly as freshwater descendants adapt to local conditions, possibly through a selective sweep of co-adapted freshwater-osmoregulatory gene complexes (DeFaveri *et al.*, 2011; Hohenlohe *et al.*, 2012; Jones *et al.*, 2012b). In contrast, because selection for seawater tolerance is no longer imposed on the derived population, euryhalinity might erode through mutation accumulation in the gene networks underlying hypo-osmoregulation (Snell-Rood *et al.*, 2010). The evolutionary effects of relaxed selection are less clear because they may depend on maintenance costs and pleiotropy of the genes essential for hypo-osmoregulation (Lahti *et al.*, 2009; Brennan *et al.*, 2015). If energetic costs to maintain a functional osmoregulatory response to high salinity are high or the genes involved largely reside in a distinct regulatory module, then loss of these traits should be more rapid than if costs are small or genes are highly integrated within networks necessary for the fish to function in fresh water.

Evidence of local evolution of halotolerance among fish populations from contrasting salinity regimes is extensive (e.g. Whitehead, 2010; Brennan *et al.*, 2015; Velotta *et al.*, 2015). In the majority

of halotolerance studies, however, the populations or species pairs tested diverged thousands of years ago, which precludes characterization of the incipient effects of selection on the osmoregulatory system in real time. The euryhaline threespine stickleback (*Gasterosteus aculeatus*) is an ideal model to capture the pace of physiological evolution because it has a well-documented history of parallel divergence following transitions from marine to freshwater habitats across its circumpolar range. Lab-based manipulations of stickleback have also demonstrated rapid evolution of traits such as trophic morphology and cold tolerance after only a few generations (Wund *et al.*, 2008; Barrett *et al.*, 2011). Freshwater colonization, and subsequent adaptation, have become a prime example of ecological speciation by giving rise to a replicated set of derived freshwater populations whose ages vary from the post-glacial Pleistocene [10,000–20,000 years ago (Bell and Foster, 1994)] to only a few generations (von Hippel and Weigner, 2004; Gelmond *et al.*, 2009; Bell and Aguirre, 2013). The persistence of the seawater-adapted lineage of ancestral marine/anadromous (collectively called oceanic) phenotypes has allowed evolutionary biologists to make direct experimental comparisons between ancestral and derived forms to aid our understanding of the evolutionary consequences of halohabitat transitions. Salinity transfer experiments between oceanic and lake-resident ecotypes have demonstrated salinity-dependent differences in survival that correlate with approximate native salinity, with enhanced freshwater tolerance and/or lower seawater tolerance in derived populations (Heuts, 1947; Marchinko and Schluter, 2007; McCairns and Bernatchez, 2010; DeFaveri and Merilä, 2014). Recent work has shown that ancestral and derived phenotypes exhibit divergence in osmoregulatory genes (DeFaveri *et al.*, 2011; Shimada *et al.*, 2011; Jones *et al.*, 2012a; DeFaveri and Merilä, 2013) as well as osmoregulatory gene transcription (McCairns and Bernatchez, 2010; Taugbøl *et al.*, 2014).

A major site of osmoregulation is the vascularized gill epithelium, where specialized ion transporting cells called ionocytes buffer against osmotic perturbations by taking up ions from the environment (hyper-osmoregulation) or secreting excess ions from circulating plasma (hypo-osmoregulation) to compensate for diffusive ion losses in fresh water or gains in seawater (Edwards and Marshall, 2013). Detailed models of teleost branchial ionocyte function in fresh water and seawater have mapped suites of membrane-bound transporters that control the intake or expulsion of ions. The basolateral Na^+/K^+-ATPase pump (NKA) is required for both functions because it produces electrochemical gradients across the cell membrane that drive the coordinated work of secondary cotransporters and exchangers (Edwards and Marshall, 2013). However, NKA is often upregulated in seawater exposure because it facilitates paracellular extrusion of sodium and also powers the $Na^+/K^+/2Cl^-$ cotransporter (NKCC), which is involved in chloride secretion (for reviews, see McCormick, 2001; Kaneko and Hiroi, 2008).

In addition to actively pumping ions to maintain homeostasis of the extracellular fluid, fishes also regulate cell volume by adjusting intracellular concentrations of organic osmolytes, which are uncharged solutes that increase osmolality without interfering with cellular biochemistry and can also have other cytoprotectant roles (Yancey *et al.*, 1982; Kültz, 2012). Compatible osmolytes, such as taurine and other free amino acids, are upregulated in response to a rise in the osmolality of extracellular fluid during hyperosmotic stress in at least some bony fishes (Fiess *et al.*, 2007). Conversely, osmolytes are downregulated when plasma osmolality drops (Edwards and Marshall, 2013). Therefore, in at least some species, measurements of tissue osmolyte levels can serve as an indicator of plasma osmolality in the extracellular fluid, for instance, where blood samples cannot be acquired.

The precise physiological mechanisms by which euryhaline fishes adjust their osmoregulatory systems have been intensively studied in several diverse groups of teleosts,

including eels, clupeids, salmonids, killifish, and tilapia (for reviews, see Evans *et al.*, 2005; McCormick *et al.*, 2013). Considering the potential importance of environmental salinity for shaping the stickleback radiation, the lack of mechanistic, physiological data on this intensively studied, evolutionary model species is surprising. Moreover, given the diverse mechanisms of ion uptake in fresh water described in other euryhaline models (Hwang and Lin, 2013; Hsu *et al.*, 2014), one cannot assume that osmoregulatory adaptations in Gasterosteiformes have followed the same evolutionary trajectory.

The goals of this study were to measure how rapidly osmoregulatory responses evolve after oceanic stickleback initiate freshwater residency and to characterize the physiological mechanisms underpinning such divergence. In a common lab environment, we reared F_1-generation stickleback from both an anadromous population and its descendants, which had been introduced approximately two generations earlier to nearby, landlocked lakes (Bell *et al.*, 2016). We then compared halotolerance thresholds in members of the ancestral and derived populations by conducting 'direct' and 'gradual' salinity challenge experiments, which measure osmoregulatory capacity in complementary ways (Schultz and McCormick, 2013). Direct salinity transfers assess acute osmoregulatory responses to abrupt osmotic shock. In the gradual design, salinity is changed incrementally, which reveals physiological effects of chronic exposure and also measures absolute halotolerance limits by allowing fish time to acclimate.

Our main objectives were to compare survival between ancestral and derived stickleback subjected to osmotic challenge and to mechanistically link divergence in performance to osmoregulatory machinery at the molecular and tissue levels. We adopted an integrated organismal approach, uniting multiple levels of physiological responses to provide functional explanations for differences in halotolerance. We hypothesized that, compared with juveniles from an anadromous population, lake-introduced stickleback would exhibit a halotolerance shift towards fresh water and away from seawater, and that their diminished hypo-osmoregulatory response would be evidenced by lower gill NKA activity and NKCC abundance in seawater. Furthermore, the signs of osmotic stress would be detectable in organic osmolyte content, with departures from baseline levels indicating osmotic perturbation. Specifically, we predicted the lake-introduced stickleback would have a greater increase in organic osmolytes in seawater to mitigate the effects of higher plasma osmolality. In contrast, anadromous stickleback would downregulate organic osmolytes to offset their relatively lower plasma osmolality when held in ion-poor conditions.

METHODS

Source populations and fish husbandry

Threespine stickleback used in this study were sampled from an anadromous population in Rabbit Slough (RS) of the Matanuska-Susitna Borough, Alaska, and two south-central Alaskan lake populations derived from it (Bell *et al.*, 2016). In 2009 and 2011, respectively, ~3000 anadromous Rabbit Slough adults were captured at a culvert running under the Parks Highway near Palmer (61.534°N, 149.268°W) and released into Cheney Lake (CL; 61.200°N, 149.762°W; conductivity = ~168 $\mu S \cdot cm^{-1}$), located in the Anchorage Municipality, and Scout Lake (SL; 60.535°N, 150.832°W; conductivity = ~35 $\mu S \cdot cm^{-1}$), located to the south on the Kenai Peninsula. Both lakes lack outlet streams and had been treated with rotenone by the Alaska Department of Fish and Game to exterminate

northern pike (*Esox lucius*); this treatment also eradicated the native threespine stickleback populations.

In May–June 2012, breeding stickleback from all three locales were trapped using unbaited, steel minnow traps (0.32-cm mesh) set overnight. Adults were brought to the University of Alaska Anchorage, where we produced an F_1 generation of more than 1700 embryos from each population through 'mass cross' *in vitro* fertilization. Gametes from multiple males and females were mixed prior to fertilization to increase genetic diversity. The number of parents used in the mass cross depended on the size of the female and trapping success (Rabbit Slough = 10 males and 7 females; Scout Lake = 15 males and 15 females; Cheney Lake = 1 male and 10 females). Beginning with the initial cohort that hatched in each of the lakes during the summer of introduction, the experimental progeny from Scout Lake had persisted in fresh water for two generations, and the progeny from Cheney Lake had persisted in fresh water for 3–4 generations, depending on whether they bred after 1 or 2 years post-introduction (Bell *et al.*, 2016).

Fertilized embryos were disinfected with methylene blue and furan (Aquarium Pharmaceuticals, Inc., Chalfont, PA, USA), placed in aerated, 3 ppt water, and shipped to the University of Connecticut's Aquatic Facility, where they developed in a common environment. The fish were raised in reverse osmosis water in which Instant Ocean aquarium salt was dissolved to a salinity of 3 ppt, as measured by a digital salinometer (Yellow Springs Instruments 85, Yellow Springs, OH, USA). Fish were held in replicate 38-litre aquaria, equipped with filters and maintained at 19 ± 1°C with a 14/10 hour light/dark cycle. Larval stickleback were fed brine shrimp nauplii, which had been gut-enriched with Self-Emulsifying Lipid Concentrate (SELCO, Brine Shrimp Direct, Ogden, UT, USA). We transitioned one-month-old juveniles to a pelleted feed [Golden Pearls, Brine Shrimp Direct (Divino and Schultz, 2014)].

The direct salinity transfer

When the F_1 cohort reached 6 weeks post-hatch (standard length = 16.7 ± 1.9 mm; mean ± standard deviation), 410 of the lab-reared individuals from each source population were randomly selected for an abrupt salinity challenge in which they were directly transferred from the rearing salinity into wide-mouth mason jars (Ball brand) containing 1.5 L of water at one of seven salinity treatments at a density of 10 fish per jar (50–60 individuals per salinity per population; Fig. 1). Three hypoosmotic (0, 0.2, 0.4 ppt) and four hyperosmotic salinity treatments (35, 40, 45, 50 ppt) were chosen to encompass anticipated lower and upper tolerance limits. Approximate conductivities for these seven treatments were: 6, 353, and 677 $\mu S \cdot cm^{-1}$ for the low salinity treatments and 50.7, 57.2, 63.7, and 70.3 $mS \cdot cm^{-1}$ for the high salinity treatments. To obtain baseline physiological data, fish were sampled from a control jar (3 ppt; conductivity = 4.9 $mS \cdot cm^{-1}$) on Day 0 of the time course. Because mortality had been zero for stickleback juveniles held in jars at the rearing salinity in numerous salinity trials (J. Divino, unpublished data), we did not collect additional controls at each time point.

Salinity-challenged fish were monitored at least twice per day for 10 days; at each inspection, mortalities were recorded and immediately removed. To capture potential temporal dynamics in physiological responses to salinity stress, acutely challenged stickleback were sampled during the time course on Days 0, 0.25, 1, 3, 7, and 10 (Fig. 1). To increase the sample size necessary for obtaining additional gill samples for molecular analyses after salinity acclimation, additional jars of fish were set up for sampling on Day 7

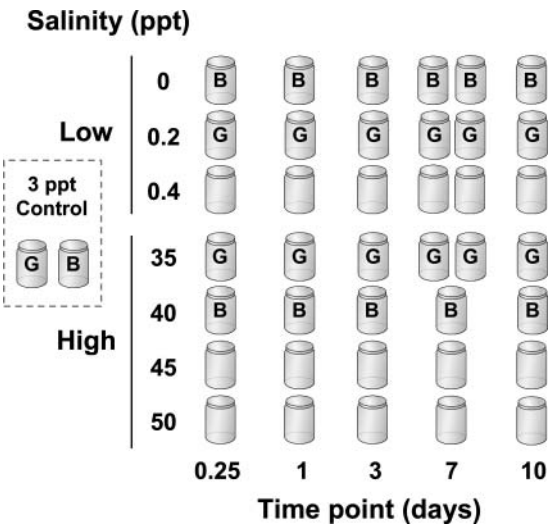

Fig. 1. Experimental design of the acute salinity challenge time course, in which 6-week-old anadromous and lake-introduced stickleback were transferred from the rearing salinity (3 ppt) to one of a range of low (freshwater) or high (seawater and hypersaline) salinity treatments at a density of 10 juveniles of each population per jar (1.5 L water). Mortality was recorded at all salinities for 10 days. During the trial, survivors were sampled from the lettered jars at control (Day 0; 3 ppt) and at low and high salinity treatments for physiological endpoints: gill NKCC abundance (G), gill NKA activity (G, Day 7), and whole-body water content and organic osmolytes (B).

of the trial. We periodically checked ammonium, nitrite, nitrate, and pH levels in jars (Aquarium Pharmaceuticals, Inc.) and maintained water quality throughout the experiment through a combination of debris removal and water changes. Fish were fed pelleted feed beginning on Day 2 of the trial.

Owing to differences in hatch date, this experiment was performed separately on 6-week-old Cheney Lake juveniles. During this period, however, maintenance work was performed on the reverse osmosis water supply, which we believe caused high mortality across the three hypoosmotic treatments, including complete loss of the 0.2 ppt group. Therefore, we have excluded direct transfer results on Cheney Lake fish.

Each Rabbit Slough and Scout Lake fish in the direct transfer experiment was coded as either a mortality or a right-censored event (i.e. sampled fish or final survivors), with time-to-event recorded in either case. A mixed-effects, Cox proportional hazard model [*coxme* package in R (R Development Core Team, 2015)] was used to analyse the effects of population, salinity, and their interaction on survival in the lower and upper halotolerance thresholds (which we determined from the trial to be 0 and 40 ppt; see Results), with experimental jar as a random effect. To further characterize the relationship between population and salinity, we performed log-rank tests on Kaplan-Meier survival curves between each population at each threshold salinity (*survdiff* function in the *survival* package in R), with jar included as a factor. Excluding jar as a factor did not change the statistical outcomes of either the Cox or log-rank tests (data not shown).

For each population–salinity combination, mean proportion survival was calculated at each inspection out of a declining number of fish 'at risk' due to censoring. Just prior to

sampling on Day 3, when most of the mortality had ceased (see Results), we estimated lower- and upper-limit lethal salinity concentrations (LC_x, expressed in ppt, where x is percent survival), using logistic regression. For each population, the proportion of stickleback alive per jar (at 67 hours) was plotted across the three freshwater treatments (0, 0.2, and 0.4 ppt) and the four high salinity treatments (35, 40, 45, and 50 ppt), and LC_x was calculated from fitted curves. It is conventional to report LC_{50}, but because of high survival of Scout Lake juveniles in 0 ppt (see Results), LC_{85} was the lowest common salinity calculable for quantifying freshwater halotolerance across populations.

The gradual seawater challenge

We also performed a gradual salinity challenge experiment on 100 three-week-old, F_1 juveniles from Rabbit Slough, Scout Lake, and Cheney Lake. Fifty fish were transferred into duplicate, 19-litre aquaria initially containing the common rearing salinity (3 ppt water), which we increased by 2 ppt per day. Each tank was equipped with a Penguin 100 power filter with bio-wheel (Marineland Aquarium Products, Cincinnati, OH, USA), and a segment of plastic pipe fitted with plastic plants provided habitat enrichment. Fish were fed gut-enriched nauplii and/or pellets daily. The experiment ended at the salinity in which at least 50% of the current cohort died (the cohort LC_{50}). At the end of the trial, all survivors were sampled either for gill tissue or whole-body water content and osmolyte analysis, as described below.

Molecular assays

At the designated time points of the direct salinity trial, fish were euthanized with an overdose of MS-222 anaesthetic in a salinity approximating that of the challenge treatment. Eight samples were collected per jar, except in a few cases where this target could not be reached due to mortality. Fish were first rinsed in deionized water, blot-dried, and then measured (standard length) using digital calipers. Gill tissue or body samples were collected in pairs of low and high salinity treatments (Fig. 1). Branchial baskets from individuals in the 0.2 ppt and 35 ppt treatments were micro-dissected on ice-cold glass and snap frozen on dry ice, either in empty microcentrifuge tubes for NKCC protein abundance, or in tubes containing 100 μL of sucrose-EDTA-imidazole (SEI) buffer for NKA activity (McCormick, 1993). For body water and osmolyte analysis, we chose stickleback from the 0 and 40 ppt treatments because inclusion of the branchial basket in the sample was necessary for measurement accuracy. These stickleback were eviscerated (to remove the potentially confounding influence of the gut lumen), weighed to 0.01 mg, dried to a constant mass (48 h at 60°C), and reweighed. Percent water content was calculated as the proportional difference between the sample's wet and dry masses multiplied by 100.

Gill NKCC protein abundance was quantified by SDS polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western immunoblotting with infrared (IR) detection. Gill tissue was thawed and homogenized using a Kontes motorized pellet pestle (Kimble-Chase, Vineland, NJ, USA) in a buffer containing 1% Triton X-100 and protease inhibitors (Roche Diagnostics Corp., Indianapolis, IN, USA). Homogenates were then centrifuged at 13,000 rpm for 10 minutes at 4°C to remove insoluble material. Total protein concentration of the supernatant was determined using a Pierce BCA Protein Assay kit (ThermoFisher, Waltham, MA, USA). Samples were mixed with a 4x-concentrated Laemmli buffer

containing 8% SDS. Ten micrograms of protein was loaded into each of 11 sample lanes in 12-well pre-cast, 7.5% acrylamide TGX gels (Bio-Rad, Hercules, CA, USA). A molecular weight ladder was loaded into the first lane of each gel, and to control for potential blot-to-blot variation in signal intensity, a common calibrator sample was also added into this lane, which we prepared by pooling gill homogenates from several 19-week-old juveniles that had been exposed to 35 ppt for 7 days. Proteins separated through the gel at 250 V for 45 minutes and were then wet-transferred onto nitrocellulose membranes by submerging both in ice-cold Tris-glycine sample buffer and applying a 400 mA current for 90 minutes. We probed blots for NKCC with the monoclonal T4 antibody (mouse IgG1; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), which binds to the conserved carboxy-terminus region of the protein (MET902-SER1212). This antibody detects both NKCC1 and NKCC2 isoforms, but only the former isoform is present in gill tissue (Hiroi *et al.*, 2008). Immunoblots were blocked in 0.05% Tween in phosphate-buffered saline (PBS-T) containing 1% bovine serum albumin (BSA) for 1 hour at room temperature and then incubated in T4 (1:1000) overnight at 4°C. Following three washes in PBS-T, blots were incubated with a conjugated secondary antibody (1:10,000; goat anti-mouse IgG; IR-Dye 800CW, LI-COR Biosciences, Lincoln, NE, USA) for 1 hour at room temperature. Blots were washed again and wet-pressed with protein side down onto a dual-channel IR laser scanner (LI-COR Odyssey Classic). Blots were scanned at 169 μm resolution at two wavelengths: the secondary antibody (800 channel, green) and the molecular weight ladder (700 channel, red). Pixel intensity of the NKCC bands was quantified from digital images using Image Studio Lite (v4.0, LI-COR). NKCC was visualized as multiple bands on the Western blots, presumably due to the presence of glycosylated and multimeric forms of the cotransporter (www.evolutionary-ecology.com/data/2982Appendix.pdf). NKCC multimers resisted cleavage even when dosed with high concentrations of reducing agents, and so we quantified all bands to yield total NKCC abundance. Potential blot-to-blot variation in signal strength was accounted for by normalizing the signal from the sample to that of the calibrator.

Additional gill tissue in 0.2 and 35 ppt treatments was examined on Day 7 of the abrupt challenge to compare NKA activity relative to controls, following a 96-well microplate spectrophotometric assay that enzymatically couples ADP production with NADH oxidation (McCormick, 1993). Briefly, gills stored in the SEI buffer were thawed on ice, homogenized, and centrifuged to pellet insoluble material. Supernatants were then loaded into duplicate wells containing the assay mixture in the presence or absence of 0.5 mM ouabain, a potent NKA inhibitor. Kinetic oxidation of NADH was measured by repeated 340-nm absorbance readings taken for 10 minutes on a plate reader at 25°C using Gen5 software (BioTek Instruments, Winooski, VT, USA). For each sample, NKA activity was quantified as the difference between the mean NADH decay slope of the reaction with and without ouabain, normalized to the sample's total protein concentration, as determined by the Pierce BCA method.

Organic osmolyte levels were quantified in 0 and 40 ppt-treated fish using high-performance liquid chromatography [HPLC (Wolff *et al.*, 1989)]. The dried carcasses were first reweighed to determine appropriate dilution volumes. We dissolved the samples in ice-cold 7% perchloric acid and homogenized them using a tapered glass tissue grinder. Protein was precipitated and removed from homogenates by incubating them for 4 hours at 4°C, followed by centrifugation at 15,000 rpm for 30 minutes (at 4°C). The supernatant was neutralized with 2 M KOH, passed through a C_{18} cartridge (Sep-Pak, Waters Corp., Milford,

MA, USA) to remove lipids, and then a 0.22-μm filter. We separated small carbohydrates, methylamines, amino acids, and other small organic solutes by injecting the supernatant (Series 200 pump, PerkinElmer, Waltham, MA, USA) through a heated (80°C), Sugar-Pak I column (Waters Corp.). Samples were run for 60 minutes with peak detection by a refractive index detector (Bio-Rad #1755) and chromatogram peaks were integrated using eDAQ Powerchrom software. Sensitivity limitations of the assay necessitated within-group pooling of the smallest individuals to achieve a minimum dry mass of 10 mg per sample, which typically reduced sample size by at least half.

Pilot tests in juvenile stickleback tissue identified the following composition of organic osmolytes: taurine, myo-inositol, alanine, glycine, and creatine. The osmolyte content of each of these molecules was determined in sample homogenates in relation to 1-mM standards. The five values were summed to yield the total osmolyte content and normalized to the sample wet mass to more closely approximate a physiological concentration. Removal from the osmolyte panel of creatine, which has a separate role in creatine-phosphate energy storage, did not appreciably alter patterns.

For the direct transfer experiment, we statistically analysed physiological endpoints using three-way analysis of variance (ANOVA), testing for effects of population (pop), salinity (salt), experimental day, and associated interactions. Two-way models were also performed on the time-course data separately for the freshwater and seawater challenge salinities, which incorporated control groups as Day 0. Models were re-evaluated with non-significant terms removed, but this did not affect interpretation. Multiple-comparison tests were performed on significant effects ($P < 0.05$): Tukey's HSD tests were used to examine mean differences between the freshwater and seawater treatments at each time point, whereas Dunnett's tests compared means of each freshwater or seawater time point to pre-transfer controls. To quantify a per-generation rate of phenotypic divergence between the Rabbit Slough and Scout Lake populations, all response variables measured in the direct salinity transfer were converted into haldanes (h_p; Table 1) (Hendry and Kinnison, 1999; Bell and Aguirre, 2013). For the gradual seawater challenge, percent water content and osmolyte content were compared across populations using non-parametric Kruskal-Wallis (K-W) tests due to small sample sizes.

RESULTS

The direct salinity transfer

The majority of stickleback mortality occurred within 3 days following direct transfer to extreme salinities and typically happened sooner in the seawater treatments (Fig. 2). Most of the mortality in the 45 and 50 ppt hypersaline treatments, in which all fish perished, occurred within 12 hours. At least 90% of stickleback survived in 0.2 ppt or 35 ppt. Survival of Scout Lake juveniles was 97% in the 0.4 ppt treatment, but for their Rabbit Slough counterparts, two additional deaths in the Day 10 jar depressed mean survival subsequent to the Day 7 sampling, when it became the last remaining jar in the trial (Fig. 2).

At the threshold salinities, population differences in survival emerged just prior to the Day 3 sampling: compared with Rabbit Slough stickleback, Scout Lake fish had 89% higher survival in 0 ppt (0.85 ± 0.10 vs. 0.45 ± 0.06; mean $\pm$ standard deviation), but had 12.5% lower survival in 40 ppt (0.47 ± 0.06 vs. 0.53 ± 0.06). These patterns persisted through Day 10, albeit without replication at this final time point, when the Scout Lake fish had 67%

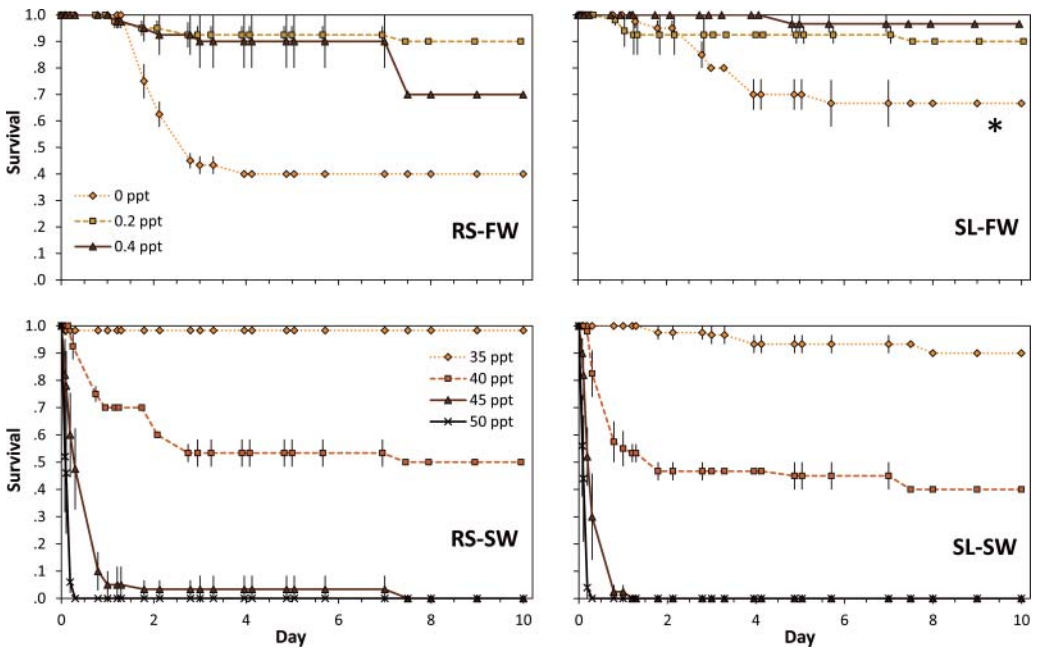

Fig. 2. Survival of 6-week-old ancestral anadromous (Rabbit Slough, RS) and lake-introduced stickleback (Scout Lake, SL) held at low (freshwater, FW) and high salinities (seawater, SW) for 10 days. Fish were held in replicate jars and were systematically censored from the trial at designated sampling times (Fig. 1). Each data point represents the mean proportion (± standard error) of fish alive during a census. We performed a survival analysis at the threshold halotolerances, 0 ppt and 40 ppt. The asterisk denotes a significant difference in the 0 ppt survival curves between the two populations according to log-rank tests.

higher survival in fresh water (0.67 vs. 0.40), but had 20% lower survival in seawater (0.40 vs. 0.50; Fig. 2). At 0 ppt, total mortality was 2.4-fold higher in Rabbit Slough fish than in Scout Lake fish (24/60 vs. 10/60), but at 40 ppt, mortality of Rabbit Slough fish was slightly (26%) lower (17/50 vs. 23/50). The salinity-dependent change in mortality contributed to sharply different hazard function ratios for each population in the Cox proportional hazards model: risk of dying in 40 ppt, relative to 0 ppt, did not change in Rabbit Slough stickleback ('exp(coef)' = $e^{\beta} = 1.01$), but increased almost five-fold in Scout Lake fish ($e^{\beta} = 4.86$). Consequently, the model yielded highly significant pop and pop*salt effects (Wald tests: pop $P = 0.0043$; salt $P = 0.97$; pop*salt $P = 0.0014$). Log-rank tests performed at the threshold salinities revealed that survival differed between the two populations only in fresh water (0 ppt challenge: $\chi^2 = 14.7$, d.f. = 7, $P = 0.0401$; 40 ppt: $\chi^2 = 8.9$, d.f. = 9, $P = 0.45$). The high survival of Scout Lake stickleback in 0 ppt relative to Rabbit Slough fish influenced the low-salinity LC_{85} (Scout Lake: 0.015 ± 0.205 ppt; Rabbit Slough: 0.208 ± 0.133 ppt). For two generations of development under different salinity regimes, this amounts to a population divergence rate of 0.5689 h_p (Table 1).

Although the Rabbit Slough and Scout Lake populations did not differ statistically in the molecular physiological endpoints we measured in the direct transfer experiment, we report phenotypic haldanes for all response variables in Table 1, as recommended by Hendry and

Table 1. Per-generation rate of phenotypic divergence (haldanes, h_p) for all physiological responses measured in juvenile anadromous (Rabbit Slough, RS) and lake-introduced (Scout Lake, SL) stickleback in the 10-day direct salinity transfer experiment

	Measurement	Salinity (ppt)	Trial day	RS mean (SD, *N*)	SL mean (SD, *N*)	h_p [a]
Freshwater halotolerance	LC_{50} [b]	0, 0.2, 0.4	3	0.004 (0.123, 12)	NA[c]	NA
	LC_{85}	0, 0.2, 0.4	3	0.208 (0.133, 12)	0.015 (0.205, 12)	**0.569**
Seawater halotolerance	LC_{50}	35, 40, 45, 50	3	40.3 (1.5, 13)	39.7 (1.5, 13)	0.190
	LC_{85}	35, 40, 45, 50	3	38.3 (2.1, 13)	37.7 (2.3, 13)	0.121
Gill NKCC abundance	normalized fold change	0.2	10	2.99 (1.91, 8)	1.98 (1.49, 8)	0.296
		35	10	4.61 (2.45, 8)	3.58 (1.31, 8)	0.274
Gill NKA activity	μmol ADP·mg protein^{-1}·h^{-1}	0.2	7	10.2 (1.8, 8)	11.0 (3.1, 8)	0.159
		35	7	12.6 (2.4, 8)	15.0 (1.8, 8)	0.573
Total organic osmolyte content	mmol·kg^{-1} wet mass [d]	0	3	28.5 (5.8, 2)	29.7 (3.3, 5)	0.160
		40	0.25	61.3 (5.9, 4)	54.1 (7.6, 2)	0.576
Water content	percent	0	3	84.3 (2.2, 4)	83.2 (1.8, 8)	0.298
		40	0.25	78.3 (1.4, 8)	81.1 (3.3, 6)	0.621

[a] Phenotypic haldanes were calculated using the following equation: $h_p = |(x_1 - x_2)/SD_{pooled}/g|$, where x is the mean trait value for a population, SD_{pooled} is the pooled standard deviation, and g is the generation time since divergence (= 2 in this comparison). **Bold** values denote significance at the population level as determined by inferential statistics.

[b] Lethal salinity concentrations were determined using logistic regression models, fitted for proportion survival (per experimental jar) by salinity.

[c] The LC_{50} could not be discerned for Scout Lake due to high survival in fresh water.

[d] Osmolyte content was measured on pooled samples. Because salinity had a transient effect on osmolyte and water content, populations were compared at time points of maximal hypo- and hyperosmotic stress.

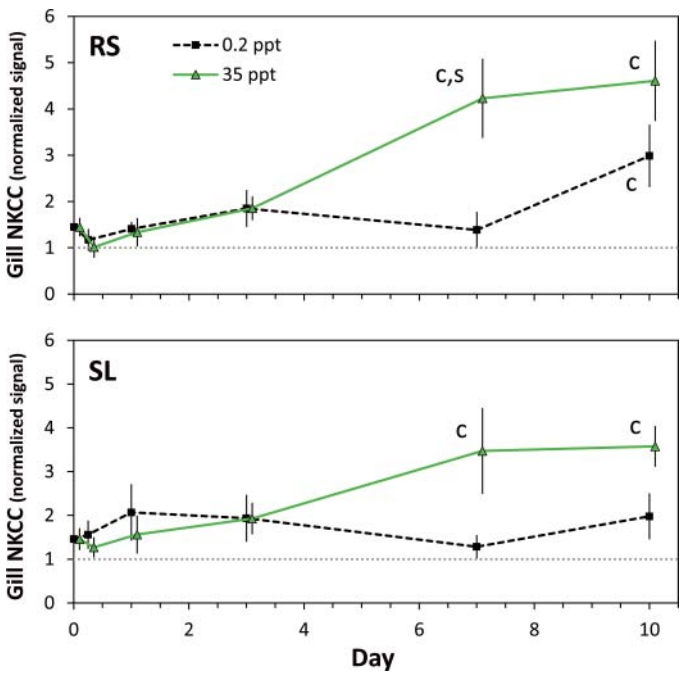

Fig. 3. Protein abundance of gill NKCC in anadromous (Rabbit Slough, RS) and lake-descendant (Scout Lake, SL) stickleback challenged in 0.2 ppt or 35 ppt for 10 days. Total NKCC was determined by IR fluorescence immunoblotting using the T4 antibody. Protein expression was normalized by dividing sample signal by that of a calibrator run in each blot. The horizontal line represents the calibrator's signal, set to one. Letters denote time points when protein expression differed significantly from control levels ('C') and/or between salinities ('S'), according to Dunnett and Tukey *post hoc* tests, respectively. Each data point represents the mean (± standard error) of eight individuals.

Kinnison (1999). Relative to freshwater and control levels (3 ppt rearing salinity), abundance of gill NKCC increased 2.6-fold in stickleback challenged with 35 ppt for one week, and was almost three-fold higher by Day 10 (Fig. 3). In freshwater-challenged stickleback, gill NKCC abundance remained at or slightly above control levels (Fig. 3). No population differences in NKCC protein expression were detected (three-way ANOVA: salt $P = 0.002$; day $P < 0.0001$; salt*day $P < 0.0001$; pop $P = 0.60$; salt*pop $P = 0.58$). Gill NKA activity increased by about 50% after 7 days in seawater (13.8 vs. 9.3 μmol ADP per mg protein per hour) compared with control fish (3 ppt), but it did not differ between the populations (two-way ANOVA: salt $P < 0.00001$; pop $P = 0.17$; salt*pop $P = 0.17$) (Fig. 4).

Organic osmolytes fluctuated transiently in response to acute salinity challenge in a manner consistent with the pattern of mortality in fresh water and seawater (three-way ANOVA: salt $P < 0.0001$; day $P < 0.0001$; salt*day $P < 0.0001$) (Fig. 5). Across both populations, osmolyte content spiked more than 51% after 6 hours in 40 ppt to 58.9 ± 6.8 mmol $\cdot$ kg^{-1} wet mass (mean ± standard deviation) and then returned to baseline levels (38.9 ± 5.1 mmol $\cdot$ kg^{-1}) by Day 3 of the time course, doing so more quickly in Rabbit Slough fish. In contrast, the drop in osmolyte content in fish exposed to deionized water was slower, reaching minimum values in both populations by Day 3. In Rabbit Slough fish, the freshwater decrease in osmolyte content was significantly (31%) lower than control levels (Fig. 5).

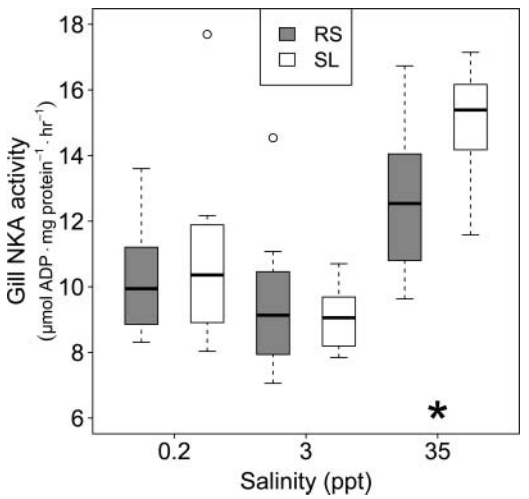

Fig. 4. Gill NKA activity in anadromous (Rabbit Slough, RS) and lake-descendant (Scout Lake, SL) stickleback held in the rearing salinity (3 ppt) or challenged for 7 days in fresh water (0.2 ppt) or seawater (35 ppt). The asterisk denotes that NKA activity was higher in seawater compared with the other salinities, as indicated by a two-way ANOVA.

Compared with Rabbit Slough fish, osmolyte content in the Scout Lake group was lower overall, but all interactions involving population were non-significant (three-way ANOVA: pop $P = 0.028$; pop*salt $P = 0.62$; pop*day $P = 0.11$; pop*salt*day $P = 0.24$).

The composition of osmolytes, in descending order, was as follows: taurine (47.8%), alanine (21.6%), creatine (20.0%), glycine (9.4%), and myo-inositol (1.2%). Water content of eviscerated carcasses ranged from 80 to 83% during the time course, typically increased to maximum at Day 3, and then returned to control levels by Day 10, irrespective of salinity. Water content in all fish held at 40 ppt was about 1% lower than those held at 0 ppt, but it did not differ between the two populations ($n_{pop} = 70–72$; three-way ANOVA: salt $P = 0.0056$; day $P = 0.0006$; salt*day $P = 0.0089$; pop $P = 0.17$).

The gradual seawater challenge

The upper threshold of salinity tolerance increased greatly when juvenile stickleback were gradually acclimated to hyperosmotic conditions, achieving an LC_{50} of 71 ppt for all three populations (Rabbit Slough, Scout Lake, Cheney Lake), when the fish were 8 weeks old (Fig. 6). During this 35-day experiment, essentially no mortality was observed until salinity exceeded 61 ppt (Day 29), at which point feeding was reduced. At 69 ppt, fish stopped eating and the daily survival rate began to decline rapidly.

The five organic osmolytes that were observed in abruptly challenged stickleback were also detected in gradually challenged fish, with a proportional composition, in descending order, of taurine (38.4%), alanine (34.2%), glycine (14.3%), creatine (10.1%), and myo-inositol (3.0%). Average osmolyte content in the 71 ppt survivors was 60.5 $mmol \cdot kg^{-1}$ and did not differ among populations (K-W: $P = 0.06$). This was similar to maximal levels recorded in the 40 ppt challenge and 72% higher than levels in the Week 6 control fish. The whole-body 71 ppt samples displayed an additional HPLC peak that did not match known

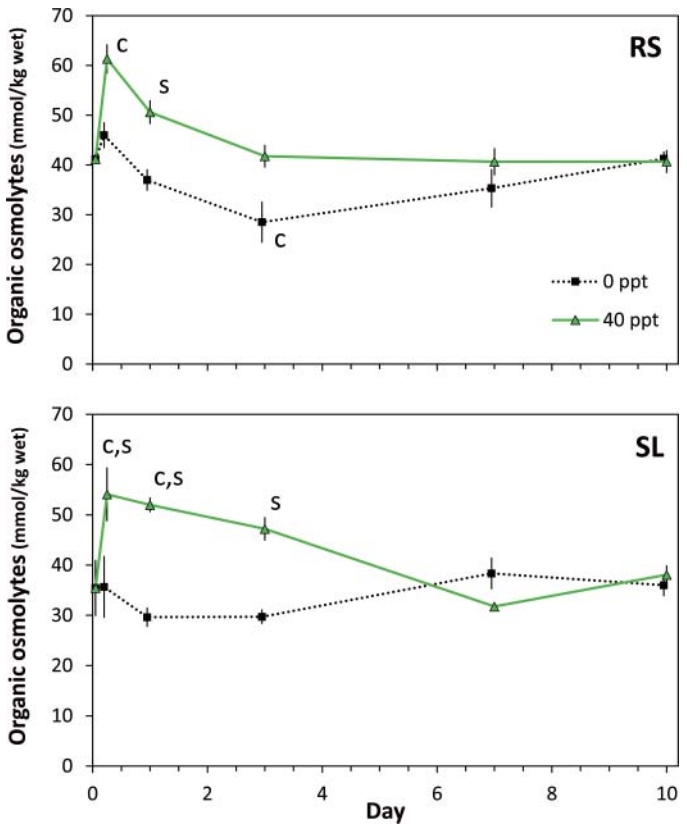

Fig. 5. Total organic osmolyte content in 6-week-old anadromous (Rabbit Slough, RS) and lake-descendant (Scout Lake, SL) stickleback subjected to extreme low (0 ppt) and high (40 ppt) salinities for 10 days. Total osmolyte content for each population is expressed per unit wet mass. Letters denote time points when osmolyte content differed significantly from control levels ('C') and/or between salinities ('S'), according to Dunnett and Tukey *post hoc* tests, respectively. Each data point represents the mean (± standard error) of individual and pooled samples; only one pooled Scout Lake sample was measured in the Day 7, 40 ppt group.

standards and was considerably lower in the 40 ppt samples. We did not account for the contribution of this unidentified solute in our calculation of osmolyte content, which may make our estimate of osmolyte content for the 71 ppt group conservative. Water content in the gradual seawater challenge survivors was higher in Scout Lake fish than in samples from the other two populations (n_{pop} = 4–6; Rabbit Slough = 76.8%; Cheney Lake = 76.2%; Scout Lake = 79.1%; K-W: $P = 0.01$).

DISCUSSION

After only two generations in fresh water, lake-introduced stickleback (Scout Lake) had enhanced freshwater tolerance relative to their anadromous ancestor (Rabbit Slough), suggesting rapid physiological evolution. Indeed, the divergence rate of 0.569 h_p we calculated for acute, low-salinity LC_{85} is much faster than evolutionary rates reported for many morphological traits in stickleback populations (Bell and Aguirre, 2013). However, the

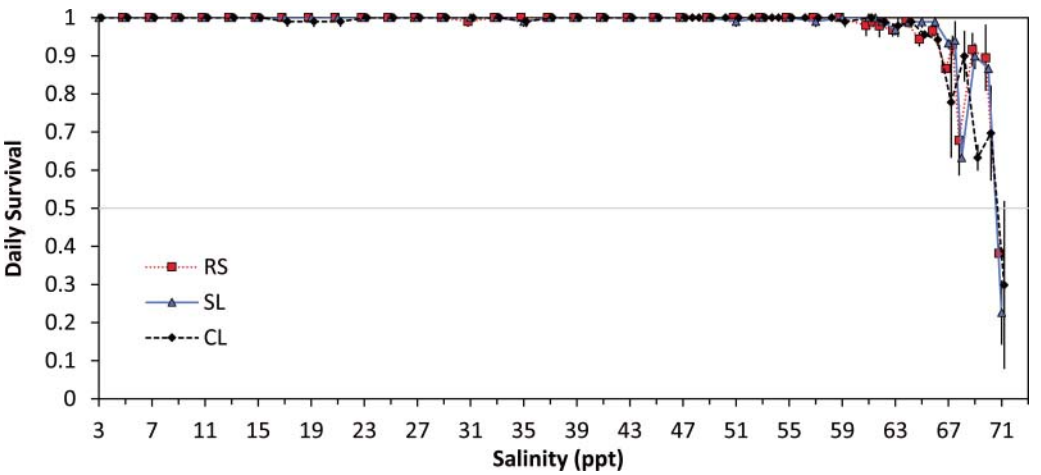

Fig. 6. Salinity tolerance over time in 3-week-old threespine stickleback gradually exposed to increasingly hyperosmotic conditions at a rate of +2 ppt per day. Juveniles from the anadromous ancestor (Rabbit Slough, RS) and two introduced-lake descendants (Scout Lake, SL; Cheney Lake, CL) were initially held at the common rearing salinity of 3 ppt. Survival is plotted as the mean daily proportion alive over time (± standard deviation). The trial ended when half or more of the surviving cohort died (defined as the LC_{50}), which was at 71 ppt for all groups (Day 35).

freshwater halotolerance rate is similar to the 0.63 h_p measured for cold tolerance adaptation in an experimentally manipulated marine population (Barrett *et al.*, 2011), which suggests that selection on physiological traits required to survive in seasonally cold, ion-poor lakes is very strong. However, we did not detect a trade-off in halotolerance: Scout Lake juveniles maintained a high seawater tolerance, as evidenced by their similar performance in hypersaline conditions and hypo-osmoregulatory responses compared with Rabbit Slough fish. Nor did we identify a clear physiological mechanism for the observed population-level difference in performance under low-ion conditions: salinity affected gill NKCC, NKA activity, and whole-body organic osmolytes, but did so similarly for each population.

Our salinity challenge experiments were designed to characterize the physiological limits and temporal dynamics of halotolerance in an evolutionary context by comparing an anadromous population with descendant populations that had only recently become freshwater-restricted. All F_1-generation fish used in this study had lived in a common rearing salinity since fertilization, thus we cannot rule out the potential influence of maternal or epigenetic effects. Our panel of freshwater and seawater treatments included salinities that were ecologically relevant to anadromous threespine stickleback. Deionized and hypersaline treatments were also included to expose potentially hidden variation among populations in reaction norms, which may only appear at extreme salinities (Whitehead, 2010). We focused our halotolerance tests on early ontogeny because juveniles in this anadromous population typically emigrate from Rabbit Slough to the Cook Inlet marine environment when they are 6–10 weeks old (John Baker, Clark University, personal communication). Thus, it is possible that anadromous juveniles could lose hyper-osmoregulatory capacity as they develop, which has been shown in anadromous American shad, *Alosa sapidissima* (Zydlewski and McCormick, 1997).

To assess divergence in freshwater and seawater halotolerance, survival was analysed at extreme salinity thresholds. Population-level performance was similar in 40 ppt, but differences in deionized water indicate rapid evolution of the osmoregulatory system in a manner consistent with local adaptation to low-ion conditions. Enhanced freshwater tolerance has been documented in embryos and adults from other derived stickleback populations (Heuts, 1947; Schaarschmidt *et al.*, 1999). In contrast, others found no ecotypic-level differences in freshwater survival, but reported that derived populations had lower seawater tolerance compared with anadromous fish (Marchinko and Schluter, 2007; McCairns and Bernatchez, 2010; DeFaveri and Merilä, 2014). These seemingly conflicting survival reaction norms are difficult to reconcile because the salinities selected as the 'freshwater' and 'seawater' treatments are typically singular and differ widely across studies, yet patterns may reflect different colonization histories of derived populations in evolutionary time.

In other fishes that have transitioned from seawater to freshwater halohabitats, improved freshwater performance can be associated with loss of seawater tolerance. Compared with ancestral anadromous alewives (*Alosa pseudoharengus*), derived landlocked populations have lower survival and higher osmotic imbalance when challenged with seawater (Velotta *et al.*, 2014, 2015). Similarly for a killifish species pair, the euryhaline rainwater killifish (*Lucania parva*) and the landlocked bluefin killifish (*L. goodei*) each display higher survival in their native salinity and the latter has a much lower halotolerance limit (Fuller *et al.*, 2007; Whitehead, 2010). Salinity-stratified populations of Atlantic killifish (*Fundulus heteroclitus*) inhabiting the Chesapeake Bay watershed differ in their physiological and transcriptomic responses to osmotic stress (Whitehead *et al.*, 2011; Brennan *et al.*, 2015). These examples suggest a trade-off between freshwater and seawater osmoregulatory ability, which may arise from antagonistic pleiotropies between gene complexes that govern each pathway (Snell-Rood *et al.*, 2010; Hohenlohe *et al.*, 2012).

A freshwater–seawater trade-off was not detected in this study: survival in the 40 ppt hypersaline treatment was statistically comparable between Rabbit Slough and Scout Lake fish, which indicates that the derived stickleback have retained full hypo-osmoregulatory capability after two generations of freshwater residency. Similarly, in the gradual seawater challenge the ancestral and both derived populations (Scout Lake and Cheney Lake, landlocked for 2 and ~4 generations, respectively) had the same LC_{50}, remarkably withstanding up to 71 ppt. Future salinity challenges conducted on the lake-introduced populations may yet reveal a decline of seawater tolerance after more time has elapsed in the freshwater environment. Gradual loss of seawater tolerance in lake stickleback has been used as a 'physiological clock' to estimate timing of freshwater colonization. For example, in British Columbia lakes containing benthic–limnetic species pairs, decreased hatching success and survival in seawater of benthic, but not limnetic, stickleback was used to argue that the benthic stickleback had invaded fresh water before the limnetic ecotype (Kassen *et al.*, 1995).

Application of general theory on rates of adaptation following environmental change suggests that following seawater–freshwater transitions, the pace of evolution of osmoregulatory plasticity is expected to be slow in situations where negative pleiotropies are absent and there is little or no cost to maintain seawater tolerance in the derived habitat. Specifically, relaxed selection on seawater tolerance experienced by derived, freshwater-residents, will slow the decay of hyper-osmoregulatory capacity if it is governed strictly by neutral processes (Lahti *et al.*, 2009). Likewise, genetic assimilation of specialized freshwater phenotypes from euryhaline ancestors will be gradual in the predictable lake

environment (Lande, 2009). Such mechanisms could explain why non-anadromous sockeye salmon (*Oncorhynchus nerka*, called kokanee) landlocked for ~10,000 years still exhibit an increase in seawater tolerance during the seasonal period of smoltification undergone by anadromous conspecifics (Foote *et al.*, 1992, 1994).

Euryhaline fishes can plastically switch from hypo- to hyper-osmoregulation by remodelling their ion-exchanging epithelia upon acclimating to new halohabitats (McCormick, 2001; Kaneko and Hiroi, 2008; Evans, 2010; Christensen *et al.*, 2012). Hyperosmotic challenge caused both Rabbit Slough and Scout Lake stickleback to increase salt-secreting branchial ionocytes, as evidenced by increases in both NKCC and NKA during the 10-day time course. Increases in these proteins during acclimation coincided with return of tissue osmolytes to resting levels and no further mortality, suggesting that gill epithelia successfully remodelled to excrete excess ions and restore internal osmolality within one week. Upregulation of NKCC and/or NKA activity following seawater transfer has been reported in many euryhaline teleosts, such as Atlantic salmon [*Salmo salar* (Pelis *et al.*, 2001; McCormick *et al.*, 2009)], anadromous alewife (Christensen *et al.*, 2012; Velotta *et al.*, 2015), Mozambique tilapia [*Oreochromis mossambicus* (Hiroi *et al.*, 2008)], killifish (Berdan and Fuller, 2012), and Japanese medaka [*Oryzias latipes* (Hsu *et al.*, 2014)].

Although there were not enough survivors of the gradual seawater challenge to perform the planned molecular assays on gill tissue at desired sample sizes, gill NKCC abundance at 71 ppt appears to be strongly elevated in both Rabbit Slough and Scout Lake stickleback, relative to levels found at lower salinities (S. Lim and M. Monette, unpublished data). This preliminary finding invites further examination of gradual osmoregulatory responses to extreme hypersaline challenge, with the aim of comparing the magnitude of salinity-dependent upregulation of seawater transporters across populations.

The increase of gill NKCC over time in 0.2 ppt-challenged stickleback was unexpected and may be attributable to cross-reactivity of the T4 antibody with a structurally similar apical transporter responsible for ion uptake in freshwater ionocytes, sodium chloride cotransporter (NCC). Apical NCC has been detected immunohistochemically with T4 in branchial freshwater ionocytes in other teleosts (Hiroi *et al.*, 2008; Hsu *et al.*, 2014), and an apical NCC-like protein has been found in stickleback using this antibody (Makoto Kusakabe, University of Tokyo, personal communication). Upregulation of NCC would be expected during freshwater acclimation, which may have increased the NKCC signal in this treatment due to their shared epitope in the C-terminus. However, in seawater the presence of NCC would be minimal (Hiroi *et al.*, 2008), so it is unlikely that NKCC levels in the 35 ppt treatment were confounded by NCC-bound T4. In fact, because NKCC abundance in fresh water may be overestimated, true salinity-dependent differences in NKCC may be greater than we have reported. Future immunoblotting work using stickleback-specific antibodies to each transporter will be necessary to resolve changes in stickleback NCC-like proteins during acclimation to hypoosmotic conditions.

The fluctuation in whole-body organic osmolytes during the 10-day time course revealed short-term osmotic shock and later acclimation of the survivors. Furthermore, the asymmetry in the deviation from baseline osmolyte levels between the freshwater (0 ppt) and seawater (40 ppt) treatments matched the observed pattern of mortality. The initial slopes of the survival curves dropped steeply in the seawater treatments, but the decline was more gradual and delayed in freshwater conditions. Correspondingly, a rapid spike in osmolyte content 6 hours after seawater transfer indicated that internal osmolality was elevated almost immediately. In deionized water, however, diffusive loss of salts to the environment may have been slower, as suggested by the slower decline in osmolyte content

(more strongly seen in the Rabbit Slough samples), which again matched the period of mortality in fresh water. Few stickleback died after Day 3, which coincided with organic osmolyte values returning to near baseline levels. Water content in the acutely challenged stickleback did not fluctuate widely and did not differ between populations, but compared with freshwater samples, the seawater treatment had a slight dehydrating effect. In contrast to the transient response of osmolyte content in the direct transfer experiment, juveniles gradually seawater-challenged for 35 days matched the maximum osmolyte content recorded in fish abruptly challenged at 40 ppt, which suggests chronic hyperosmotic stress and ultimately osmoregulatory failure as the cause of death. Water content was lower in stickleback surviving the 71 ppt gradual challenge than in those directly transferred to 40 ppt, in accord with expectations of greater dehydration resulting from increasingly hyperosmotic conditions. However, the pattern of population differences in water content in the gradual seawater challenge was opposite the prediction that anadromous stickleback would be superior hypo-osmoregulators. Scout Lake fish were significantly less dehydrated than the other populations, albeit sample size was small.

Analysis of multiple organic osmolytes and the sensitivity of osmolyte content to transient salinity responses via HPLC is a promising alternative (or addition) to measuring osmotic stress, particularly when ion concentrations cannot be measured from blood plasma. A wider use of this technique could enrich comparative studies of osmoregulation by providing an additional metric of physiological performance. In both our direct-transfer and gradual salinity challenges, taurine was the predominant osmolyte, approaching 50% of osmolyte content, with lesser contributions from alanine and glycine. Similar relative proportions of these free amino acids were detected in tissues from adults from anadromous and stream populations in Germany (Schaarschmidt *et al.*, 1999). Myo-inositol, a major osmolyte in some Mozambique tilapia tissues (Fiess *et al.*, 2007), composed no more than a few percent of osmolyte content in our samples. The relative proportions of osmolytes did not change between the direct and gradual experiments, with the exception of creatine. Creatine in the 71 ppt survivors was at half the level found in abruptly challenged fish (10% vs. 20%), which might be due to cessation of feeding in the former group as the rising salinity became intolerable. These chronically, hyperosmotically stressed stickleback may have also upregulated an additional osmolyte, which we could not identify in our HPLC analysis.

The physiological endpoints we measured did not provide mechanistic explanations for differences in survival between the anadromous and landlocked populations. Osmoregulatory divergence may be operating at other molecular levels and/or on other ion transporters. Genome scanning of oceanic and freshwater stickleback has identified many osmoregulatory genes under strong directional selection between these environments, including *atp1a1* (NKA), *aqp3* (aquaporin 3; a water channel), and *kir2.2* (a potassium channel) (Shimada *et al.*, 2011; DeFaveri *et al.*, 2011). Because performance differed in fresh water, researchers should consider examining the expression or abundance of transporters associated with ion uptake [e.g. NHE, VHA, NCC (Evans, 2011)], as well as tight junction and mucous proteins (mucins), which help reduce diffusive ion loss across the gill epithelium (Jones *et al.*, 2012a; Bossus *et al.*, 2015). These proteins may differ among salinity-divergent stickleback populations with respect to their gene expression, abundance, or isoform variants. Transcriptomic analysis may reveal regulatory networks and transcriptional pathways that have evolved and could account for improved freshwater tolerance in derived stickleback. Another possibility is that population-level differences in osmoregulatory responses may manifest further downstream in protein function via transporter localization to the cell

membrane or their rapid activation. For example, an increase in the proportion of phosphorylated NKCC in response to osmotic stimuli would suggest more functionally active protein, and hence, a higher capacity for salt secretion (Flemmer *et al.*, 2010). The signalling pathways responsible for phospho-activation of ion transporters (e.g. cAMP-dependent protein kinase A in the case of NKCC) may thus be targeted by selection rather than the transporters themselves. At the tissue level, branchial ionocyte topology along gill filaments could be compared among populations, salinities, and exposure times using scanning electron microscopy (Whitehead *et al.*, 2012).

The recent stocking of Rabbit Slough stickleback into Cheney and Scout Lakes simulated colonization events that have occurred repeatedly since the last ice age (Bell and Foster, 1994). Our examination of osmoregulatory performance in these ancestral and derived populations provides a baseline for future studies of physiological evolution in these lakes and other young, freshwater isolates (see Gelmond *et al.*, 2009; Bell and Aguirre, 2013; Lescak *et al.*, 2015; Bell *et al.*, 2016). The rate at which osmoregulatory plasticity evolves will be better understood by examining a time series of derived stickleback populations that have older, known dates of freshwater invasion. Comparisons between populations that had established freshwater-residency ~10,000 years ago to others less than a century old could reveal the degree, rate, and physiological basis of evolutionary change of halotolerance breadth and plasticity. It may be of value for future studies to incorporate additional developmental stages (e.g. pre- and post-outmigration juveniles, as well as adults), since osmoregulatory capacity changes with ontogeny (Zydlewski and Wilkie, 2013). Complementary work examining evolution of salinity preference behaviour (e.g. Baggerman, 1957; Audet *et al.*, 1986) may yield important insights into the evolution of halotolerance, especially in freshwater populations of threespine stickleback whose migratory routes to the sea are intact.

The stickleback radiation in fresh water is an exemplar of salinity's role in phenotypic divergence and ecological speciation (Bell and Foster, 1994; McKinnon and Rundle, 2002). The availability of ancestral oceanic populations throughout coastal regions of the northern hemisphere, coupled with the adaptive radiation of derived freshwater populations, many of which have a unique history of isolation, makes this species complex ideal for characterizing the evolution of euryhalinity. Our work underscores the advantages of using this model fish to investigate divergence of osmoregulatory responses and their mechanisms following halohabitat transition.

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and J. Velotta. All experimentation on animals was approved by the University of Connecticut's IACUC (Protocols A10-013 and A13-019). Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the US Government.

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