Trade-offs in osmoregulation and parallel shifts in molecular function follow ecological transitions to freshwater...

Article *in* Evolution · September 2015

DOI: 10.1111/evo.12774

CITATIONS
4

READS
145

3 authors:

Jonathan P Velotta
University of Montana
10 PUBLICATIONS **32** CITATIONS
SEE PROFILE

Stephen D. McCormick
USGS, Conte Anadromous Fish Research Center
173 PUBLICATIONS **9,836** CITATIONS
SEE PROFILE

Eric T Schultz
University of Connecticut
91 PUBLICATIONS **2,803** CITATIONS
SEE PROFILE

doi:10.1111/evo.12774

Trade-offs in osmoregulation and parallel shifts in molecular function follow ecological transitions to freshwater in the Alewife

Jonathan P. Velotta,[1,2] Stephen D. McCormick,[3] and Eric T. Schultz[1]

[1] *Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, Connecticut 06269-3043*

[2] *E-mail: jonathan.velotta@gmail.com*

[3] *USGS, Conte Anadromous Fish Research Center, Turners Falls, Massachusetts 01376*

Received January 17, 2015
Accepted September 4, 2015

Adaptation to freshwater may be expected to reduce performance in seawater because these environments represent opposing selective regimes. We tested for such a trade-off in populations of the Alewife (*Alosa pseudoharengus*). Alewives are ancestrally anadromous, and multiple populations have been independently restricted to freshwater (landlocked). We conducted salinity challenge experiments, whereby juvenile Alewives from one anadromous and multiple landlocked populations were exposed to freshwater and seawater on acute and acclimation timescales. In response to acute salinity challenge trials, independently derived landlocked populations varied in the degree to which seawater tolerance has been lost. In laboratory-acclimation experiments, landlocked Alewives exhibited improved freshwater tolerance, which was correlated with reductions in seawater tolerance and hypo-osmotic balance, suggesting that trade-offs in osmoregulation may be associated with local adaptation to freshwater. We detected differentiation between life-history forms in the expression of an ion-uptake gene (*NHE3*), and in gill Na^+/K^+-ATPase activity. Trade-offs in osmoregulation, therefore, may be mediated by differentiation in ion-uptake and salt-secreting pathways.

KEY WORDS: *Alosa pseudoharengus*, anadromy, Na^+/K^+-ATPase activity, real-time PCR, salinity tolerance.

Spatial variability in selection promotes adaptation to the local environment, which may reduce an organism's fitness in alternative environments. Such trade-offs in fitness maintain genetic variation by promoting functional specialization (Futuyma and Moreno 1988), phenotypic diversification (Schluter 2000), and ecological speciation (Rundle and Nosil 2005). However, a recent analysis indicates that trade-offs associated with local adaptation may be weak (Hereford 2009). The strength of a trade-off appears to be greatest when heterogeneity between local and foreign environments is large (assuming this translates into heterogeneous selection pressures; Hereford 2009).

For osmoregulating aquatic animals, freshwater and seawater represent strongly contrasting environments that potentially impose large trade-offs. Although the concentration of salts in freshwater and seawater differs by more than two orders of magnitude, the body fluids of bony fishes must be maintained at an intermediate concentration of roughly one-third seawater. To maintain osmotic balance, freshwater animals take in ions from a dilute environment (hyper-osmoregulation), whereas seawater animals secrete excess ions back into a concentrated environment (hypo-osmoregulation; Evans et al. 2005). Because hyper- and hypo-osmoregulation are accomplished by opposing physiological processes they may compete for resources within an individual. For example, in the fish gill, ion uptake and secretion are powered by different forms of highly specialized cells known as ionocytes (Hwang and Lee 2007; Hiroi and McCormick 2012), which potentially compete for space along gill epithelia. In accordance with the expectation of a trade-off, fishes tend to specialize on either freshwater or seawater, and few species inhabit environments with fluctuating salinity (estuarine and diadromous

EVOLUTION
INTERNATIONAL JOURNAL OF ORGANIC EVOLUTION

fishes; Schultz and McCormick 2013). Despite this expectation, there are surprisingly few empirical demonstrations of trade-offs in osmoregulatory function (Schultz and McCormick 2013).

Colonization of freshwater by marine or anadromous fish has been linked to improvements in freshwater osmoregulatory performance and ion-uptake capacity (Scott et al. 2004; Whitehead et al. 2011, 2012), or a reduction of seawater tolerance and ion secretion capacity (Foote et al. 1992; Staurnes et al. 1992; Nilsen et al. 2003; Bystriansky et al. 2007; Fuller, 2008, 2009; McCairns and Bernatchez 2010; DeFaveri and Merilä 2014; Velotta et al. 2014). Few studies have demonstrated reciprocal trade-offs, in which enhanced osmoregulatory function in one environment corresponds to a reduction in the other (sensu Kawecki and Ebert 2004). Fitness trade-offs are clearly evident in the freshwater-invading copepod *Eurytemora affinis* (Lee et al. 2007, 2011). In contrast, studies of fish have discerned performance trade-offs without demonstrable fitness consequences (Marchinko and Schluter 2007; Brennan et al. 2015). Previous work on Alewives (*Alosa pseudoharengus*; Velotta et al. 2014) indicates that colonization of freshwater is associated with reduced seawater tolerance, but whether there is a trade-off with freshwater tolerance and osmoregulatory function, and whether candidate molecular pathways have evolved in parallel along with shifts in osmoregulation, is not known.

Alewives are native to the coastal waters of eastern North America and are ancestrally anadromous, migrating to spawn in coastal streams and ponds (Scott and Crossman 1973; Fay et al. 1983). In Connecticut, multiple populations of Alewives have been independently and recently (300–400 years) restricted to freshwater (landlocked), most likely as the result of damming (Palkovacs et al. 2008). Population genetic analyses indicate that landlocked populations in Connecticut were independently derived from a common ancestral anadromous stock (Palkovacs et al. 2008). Landlocked Alewife populations vary in the degree to which they have genetically diverged from the anadromous ancestor, suggesting that they may vary in the degree of adaptation to freshwater. Variation in salinity tolerance among landlocked populations allowed us to test whether freshwater and seawater tolerances trade-off across multiple populations. To examine the mechanistic basis for these trade-offs, we tested for corresponding changes in expression and activity of genes and enzymes with known roles in gill ion regulation. Parallel changes in candidate loci among independently derived populations may indicate the influence of natural selection rather than stochastic processes such as genetic drift (Kawecki and Ebert 2004).

Adaptation to novel salinity environments involves changes in enzyme activity and gene expression of ion transport pathways (Scott et al. 2004; Scott and Schulte 2005; Nilsen et al. 2007; Lee et al. 2011; Whitehead et al. 2011, 2012; Czesny et al. 2012; Velotta et al. 2014); and sequence changes in regulatory or coding portions of osmoregulation loci (DeFaveri et al. 2011; Shimada et al. 2011; Jones et al. 2012; Michalak et al. 2014). Ion exchange in fishes occurs via coordination of several ion transport proteins at gill ionocytes. In both freshwater and seawater, Na^+/K^+-ATPase (NKA) generates an electrochemical gradient that drives all ion exchange (Evans et al. 2005). Na^+ uptake in freshwater occurs via: (1) Na^+/H^+ exchanger member 3 (NHE3; Watanabe et al. 2008; Inokuchi et al. 2009); (2) a putative epithelial channel coupled to a V-type proton ATPase (VATP; Evans et al. 2005); and (3) an apical Na^+/Cl^- cotransporter (NCC). Alewives lack apical NCC (Hiroi and McCormick 2012), which was not considered in this study. In seawater, $Na^+/K^+/2Cl^-$ cotransporter (NKCC) transports cations from extracellular fluid into ionocytes down an electrochemical gradient generated by NKA. Cl^- is secreted through an apical Cl^- channel (cystic fibrosis transmembrane conductance regulator), and Na^+ ions are secreted through junctions between ionocytes and accessory cells (Evans et al. 2005; Hwang and Lee 2007). We investigated two candidate pathways for hyper-osmoregulation (expression of NHE3 and VATP), and two for hypo-osmoregulation (NKA activity and expression of NKCC).

The objectives of this study were to determine whether the transition to freshwater has incurred trade-offs in osmoregulatory function and fitness, and to identify potential mechanisms of underlying molecular control. We compared anadromous Alewives to those from independently derived landlocked populations in two experiments: (1) an acute (24 h) seawater challenge designed to determine whether seawater tolerance varies among landlocked populations and how it is related to genetic divergence from the anadromous ancestor; (2) a two-week freshwater and seawater challenge on laboratory-acclimated fish designed to test for a trade-off between seawater and freshwater tolerance. We measured survival and plasma osmolality (an indicator of osmoregulatory performance) after exposure to freshwater and seawater. We then determined whether gill NKA activity, and expression of genes for ion uptake (*NHE3*, *VATP*) and ion secretion (*NKCC*) evolved along with osmoregulatory performance, and whether these changes occurred in parallel. We predicted that landlocked Alewives would exhibit higher expression of *NHE3* and *VATP*, and reduced NKA activity and *NKCC* expression.

Methods and Materials

ACUTE SEAWATER CHALLENGE

Young-of-the-year (YOY) Alewives from one anadromous and five landlocked sites (Fig. 1) were captured by purse seine from their natal lakes in Connecticut in August and September 2011 (Table S1). We determined salinity and conductivity at each site (Table S1) using a YSI Model 85 (Yellow Springs Instruments, Yellow Springs, OH). We transported Alewives from each site to the University of Connecticut in 19-L buckets with aerated

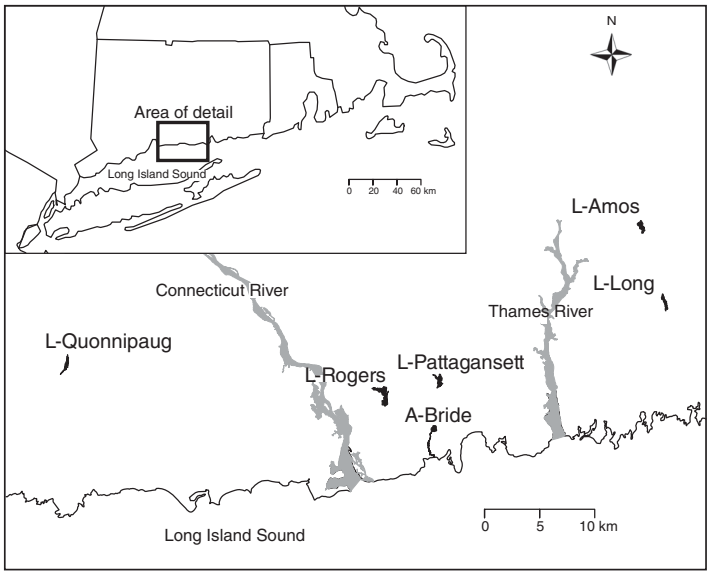

Figure 1. **Study sites in Connecticut, United States. Site details are listed in Table S1. The L prefix denotes landlocked sites, and the A denotes the anadromous site.**

lake water, to which we added sea salt (Instant Ocean, Spectrum Brands, Madison, WI) to 1 ppt to reduce handling stress and mortality (Stanley and Colby 1971; Johnson and Metcalf 1982; Nikinmaa et al. 1983; Carneiro and Urbinati 2001). We held fish overnight at 1 ppt in 150-L oval tanks with aeration. Approximately 25 Alewives per population were then transferred to replicate tanks containing conditioned, de-chlorinated tap water with sea salt at 1 ppt freshwater (control treatment) or full-strength seawater (35 ppt) for 24 h, following a direct acute onset design that is widely used in salinity tolerance experiments (Schultz and McCormick 2013). A total of two tanks per population per salinity were used. We checked each tank for mortality hourly for the first 12 h, and then again at 24 h. We conducted one test per population on separate days (see Table S2). All animals were handled in accordance with the University of Connecticut's Institutional Animal Care and Use Committee (protocol A09-024).

LABORATORY-ACCLIMATION SALINITY CHALLENGE

Based on the results of the acute seawater challenge experiment, we chose two landlocked populations with fish that differed in their seawater tolerance (one relatively intolerant and one moderately tolerant) and from which we could readily collect YOY of similar size for additional analysis of differentiation (L-Pattagansett and L-Rogers; Fig. 2). Anadromous and landlocked YOY were captured by purse seine in October 2011 and immediately transported to the Conte Anadromous Fish Research Center in Turners Falls, Massachusetts in aerated 190-L cylindrical containers containing natal lake water and sea salt at 1 ppt. Once in the laboratory, fish were held at 1 ppt salinity for one day, after which salinity was decreased to the rearing salinity of 0.5 ppt (Crystal Sea Marine Mix, Marine Enterprises International, Baltimore, MD mixed with filtered de-chlorinated tap water). We segregated fish by site in separate 1200-L re-circulating oval tanks fitted with charcoal filtration for one month prior to experimentation. Fish were maintained at 14.5–16°C with an ambient photoperiod, and fed to satiation once daily (Biotrout, Bio-Oregon, Westbrook, ME).

We subjected laboratory-acclimated anadromous and landlocked Alewives to 15-day challenges at one of four salinity levels. Facilities constraints required dividing challenges into two rounds. Trial 0/30, imposing a 0 ppt freshwater treatment at a lower conductivity (mean conductivity $= 19.9 \pm 6.8$ μS) than is present at any YOY habitat (Table S1), and 30 ppt seawater treatments, was conducted 16 November–1 December 2011. Trial 35/40, imposing full-strength seawater and hyper-saline treatments (35 ppt and 40 ppt, respectively), was conducted 20 December 2011–3 January 2012. Salinity treatments were prepared from filtered de-chlorinated tap water using a resin exchange cartridge (low-ion freshwater: Culligan International Company, Rosemont, IL) or by adding artificial sea salt. Low-ion freshwater tanks were buffered with 0.2 ppm calcium carbonate (mean pH 6.4 ± 0.4). We transferred approximately 25–35 Alewives from each site to one of two replicate tanks per salinity. Each tank was 250 L and equipped with charcoal filtration. In daily checks, any dead fish were immediately removed and measured for length.

Salinity treatments used in this study span and exceed values that would be found in the species' habitat. Conductivity in the low-ion freshwater treatment was lower than that in any of the YOY habitats (Table S1), and was designed to constitute a significant hypotonic osmoregulatory challenge. Similarly,

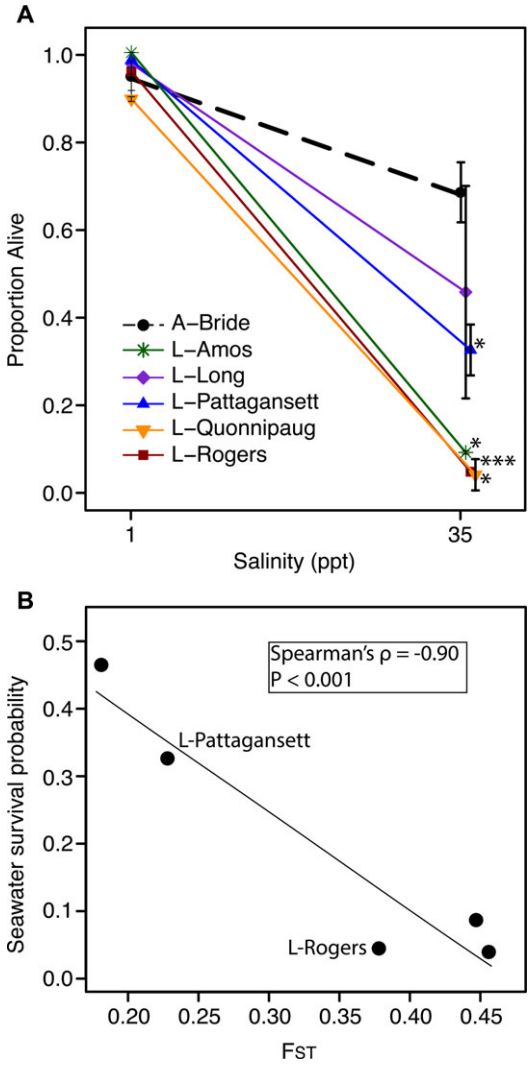

Figure 2. **(A) Survival of anadromous and landlocked Alewives after 24-h acute challenge at 1 ppt and 35 ppt. The L prefix denotes landlocked, the A denotes anadromous. Each point is the mean value ± standard error of the mean. Asterisks indicate a significant site × salinity interaction according to a generalized linear mixed effects model (GLMM). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Full results of GLMMs including all main effects are presented in Table 1. (B) Seawater survival probability among landlocked Alewife populations versus genetic differentiation (pairwise F_{ST}). Seawater survival probability is based on results of acute seawater challenge experiment (see text and Fig. 2A). Pairwise F_{ST} values between each landlocked site and A-Bride were based on mitochondrial control region CR1 obtained from Palkovacs et al. (2008). Values for two landlocked populations used in laboratory-acclimation challenge experiments are labeled.**

seawater treatments were chosen to represent a range of natural (30 ppt, 35 ppt) and extreme (40 ppt) salinity conditions.

Blood and gill tissue were sampled before (pretransfer) and at several times after transfer to treatment salinities. In trial 0/30, we sampled Alewives at days 1, 2, 5, and 14, and in trial 35/40 we sampled at days 2 and 14. We selected times at which we expected to observe survival differences, perturbations in osmotic balance, and responses of ion transporters to salinity. At each sampling, we euthanized fish in 250 mg·L^{-1} tricaine methanesulfonate (MS-222; Argent, Redmond, WA) and measured length and mass. We then severed the caudal peduncle and collected blood from each fish in a heparinized microhematocrit tube. Following centrifugation at 3200 × *g* for 5 min, plasma was transferred to 0.5-mL tubes and stored at −80°C. Plasma osmolality was subsequently measured on a vapor pressure osmometer (Wescor Inc., Logan, UT). Immediately after blood collection, we excised gill arches. Gill filaments were trimmed from the left gill arches and incubated at 4°C overnight in 1 mL of RNAlater solution (Ambion, Life Technologies, Grand Island, NY) and then stored at −20°C for gene expression assays. The first right gill arch was placed in 100 μL of ice-cold SEI buffer (150 mmol·L^{-1} sucrose, 10 mmol·L^{-1} EDTA, 50 mmol·L^{-1} imidazole, pH 7.3) and stored at −80°C for NKA activity assay.

ASSAYS

Determination of gill NKA activity followed the method of McCormick (1993), in which ATPase activity was measured by the production of ADP to NADH using lactic dehydrogenase and pyruvate kinase in the presence and absence of 0.5 mmol·L^{-1} ouabain. Gill tissue homogenates were run in two technical replicates in 96-well microplates at 25°C. Samples were read at 340 nm for 10 min on a THERMOmax microplate reader using SOFTmax software (Molecular Devices, Menlo Park, CA). Total protein content of the homogenate was determined using a bicinchoninic acid assay (Pierce, Rockford, IL). We calculated NKA activity as the ouabain-induced reduction in ATP hydrolysis, expressed as micromoles of ADP per milligram of protein per hour.

Gene expression was measured using quantitative real-time PCR (qPCR). For this assay, we analyzed samples from trial 0/30 on days 0, 1, 2, and 14. We limited analysis of gene expression to trial 0/30 for two reasons: (1) the 30 ppt treatment was run simultaneously to the 0 ppt treatment, providing the most direct seawater/freshwater contrast; (2) at 30 ppt, osmoregulatory function in landlocked Alewives is reduced, but not to the point of incurring mortality over the two weeks of the experiment (in contrast to trial 35/40; see Results). This enabled a comparison of gene expression differences related to osmoregulation that is not biased by selective mortality.

Total RNA was extracted from approximately 30 mg of homogenized gill tissue using the RNeasy Mini Kit (Qiagen, Valencia, CA) following manufacturer instructions. We quantified RNA spectrophotometrically and assessed the purity (260/280 > 1.8) of each sample. Purified RNA was treated with DNase using the TURBO DNA-free kit (Life Technologies). We verified the integrity of a subset (15%) of purified, DNase-treated RNA samples on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano

Kit (Agilent Technologies, Inc., Santa Clara, CA) following manufacturer instructions. First strand cDNA was synthesized from 500 ng RNA as template using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies). Target cDNA was amplified by qPCR on a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA) and Bio-Rad's iTaq Universal SYBR Green Supermix. Primers (Table S3) for candidate genes *NKCC*, *VATP, NHE,* and a reference gene (*elongation factor 1*α [*EF1*α]) were designed using sequences generated from a gill transcriptome of wild-caught juvenile Alewives (J. Velotta). Three technical replicates were included for each sample. Reaction conditions for qPCR were 10 min at 95°C, 45 cycles of 95°C for 20 sec, and 60°C for 50 sec. Melt curve analysis was performed following each reaction to ensure that a single qPCR product was produced. We prepared a standard sample, referred to as the calibrator, by combining gill RNA from acclimated, pretransfer samples from the three populations. Standard curves derived from triplicate dilutions of the calibrator yielded estimates of amplification efficiency (*E*-value) that were near the ideal value of 2.0 (*EF1*α: 1.93; *NKCC*: 2.08; *VATP*: 1.97; *NHE*: 1.93). Three technical replicate wells were devoted to the calibrator on each qPCR plate to account for variance among plates. Placement of technical replicates of all samples was randomized. Relative expression was calculated as $\Delta\Delta C_T$ (Pfaffl 2001):

$$\Delta\Delta C_T = \frac{E_{tar}^{\Delta C_T tar(calibrator-test)}}{E_{ref}^{\Delta C_T ref(calibrator-test)}}, \quad (1)$$

where E_{tar} is the amplification efficiency of the primer for the gene of interest, E_{ref} is the amplification efficiency of the primer for the reference gene *EF1*α, ΔC_T *tar* (target) is the difference in cycle threshold value between calibrator and test sample for the gene of interest, and ΔC_T *ref* (reference) is the difference in C_T between calibrator and test sample for the reference gene.

STATISTICAL ANALYSES

Survival was analyzed using a generalized linear mixed effects model (GLMM; GLMM with a binomial distribution; *lmer* function in the *lme4* package in R version 3.1.0). For the acute salinity challenge, the proportion of individuals alive at the end of the experiment was the response variable, tank was included as a random effect, and site (A-Bride, L-Amos, L-Long, L-Pattagansett, L-Quonnipaug, L-Rogers) and salinity (1 ppt, 35 ppt) were fixed effects. Survival did not vary with individual length ($P > 0.05$). For the laboratory-acclimation experiment, we coded survival as a binary response variable, site (A-Bride, L-Pattagansett, L-Rogers) and salinity (0 ppt, 30 ppt, 35 ppt, 40 ppt) as fixed effects, tank as a random effect, and length as a covariate. GLMMs were conducted to test for interactions between population and environment, a diagnostic test for local adaptation. For consistency with a previous study (Velotta et al. 2014), and a more nuanced analysis of survival differences, we also used Cox proportional hazards models (*coxph* function in the *survival* package in R version 3.1.0) to estimate effects of site and salinity in the laboratory-acclimation experiment. The Cox method models death rate as a log-linear function of predictors, computing a baseline hazard function that is modified multiplicatively by the covariates (Venables and Ripley 2002).

To determine whether seawater tolerance in landlocked populations is related to genetic divergence from the anadromous ancestor, we correlated seawater survival in the acute salinity challenge with pairwise F_{ST} using Spearman's rank correlation in R version 3.1.0 (*cor.test* function). Pairwise F_{ST} was calculated from a mitochondrial locus (control region [CR1]) and seven microsatellite loci, separately. F_{ST} values were obtained from Palkovacs et al. 2008). We also tested the correlation between seawater survival and the conductivity of the natal lake.

Linear mixed effects models were used to assess differences in mean plasma osmolality ($n = 12$ per site per salinity per time point), NKA activity ($n = 12$ per site per salinity per time point), and candidate gene expression ($n = 8$ per site per salinity per time point) in the laboratory-acclimation experiment. Response variables were log transformed for normality and homoscedasticity. Full models included site (A-Bride, L-Pattagansett, or L-Rogers), salinity (0 ppt, 30 ppt, 35 ppt, and 40 ppt), and time (pretransfer and all sampling time-points) as fixed effects, as well as all possible interactions between the terms. All models included length as a covariate and tank as a random effect. *P*-values were calculated with the *summary* function in the *LmerTest* package (R version 3.1.0) using restricted maximum likelihood and Satterthwaite estimation for denominator degrees of freedom. We reduced full models by eliminating nonsignificant interaction terms ($P < 0.05$).

Results

SURVIVAL

Response to 24-h acute seawater challenge differed between landlocked and anadromous Alewives (Fig. 2). Survival at 1 ppt was relatively high, ranging from 89 to 100%. Seawater survival was considerably lower among landlocked populations, varying from 4% to 45% compared to nearly 70% for A-Bride. We detected a significant population by salinity interaction for each landlocked site versus A-Bride (GLMM; Table 1), with the exception of L-Long. We found that seawater tolerance among landlocked Alewife populations varied inversely with genetic divergence from A-Bride (Fig. 2B): F_{ST} based on CR1 mitochondrial locus was negatively correlated with seawater survival (Fig. 2B). Note that a correlation between seawater survival and F_{ST} based on microsatellite loci (Palkovacs et al. 2008) yielded similar results (data not shown). There was no correlation between seawater survival and natal lake conductivity

Table 1. Results of generalized linear mixed effects models testing for variation in survival probability for acute challenge experiment.

Fixed effect	Estimate	z-Value
Site		
L-Amos	0.08	0.09
L-Long	0.08	0.11
L-Pattagansett	0.41	0.55
L-Quonnipaug	−0.93	−1.31
L-Rogers	−0.04	−0.06
Salinity		
35 ppt	−1.79	-3.47^{***}
Site × salinity		
L-Amos × 35 ppt	−2.53	-2.39^{*}
L-Long × 35 ppt	−0.87	−1.02
L-Pattagansett × 35 ppt	−1.75	-2.05^{*}
L-Quonnipaug × 35 ppt	−2.00	-2.05^{*}
L-Rogers × 35 ppt	−3.10	-3.41^{***}

A-Bride and 1 ppt were used as references for site and salinity effect, respectively. Tank was included as a random effect in the model (see Methods). $^{*}P < 0.05$; $^{***}P < 0.001$.

($P > 0.05$). The results of the acute challenge experiment identified L-Rogers and L-Pattagansett as populations of landlocked fish that are intolerant and moderately tolerant of seawater, respectively, and we selected these populations for further study in laboratory-acclimation challenges.

Survivorship in freshwater and seawater differed considerably between anadromous and both landlocked populations in laboratory-acclimation challenges (Fig. 3). GLMMs revealed a significant site by salinity interaction between A-Bride and L-Rogers at 35 ppt and 40 ppt compared to 0 ppt (Table 2), but not between A-Bride and L-Pattagansett (Table 2). We also detected a significant effect of length, in that smaller fish were more likely to die (Table 2). Low-ion freshwater survival was lower in anadromous Alewives (56%) than landlocked L-Rogers Alewives (99%; Cox proportional hazard model, significant effect of site, $z = -2.39$, $P = 0.016$), but not lower than landlocked L-Pattagansett Alewives (66%; $z = 1.53$, $P = 0.13$). We detected negligible mortality in landlocked and anadromous Alewives at 30 ppt (Cox proportional hazards model; $P > 0.05$). Differences in survivorship between life-history forms were detected for full-strength seawater (35 ppt) and hyper-saline (40 ppt) treatments. Survival of anadromous Alewives (96%) was higher than landlocked Alewives from L-Pattagansett (92%; $z = 2.91$, $P = 0.004$), and L-Rogers (72%; $z = 4.17$, $P < 0.001$) in 35 ppt seawater. At 40 ppt, no fish from L-Rogers survived ($z = 8.65$, $P < 0.0001$ for site effect), and survival of L-Pattagansett Alewives was lower than for A-Bride (46% vs. 82%, respectively; $z = 4.17$, $P < 0.001$). For completeness, we analyzed survival for both acute and acclimation experiments using a generalized linear model (with a binomial distribution) without the inclusions of "tank" as a random effect. This analysis yielded identical patterns of statistical significance.

PLASMA OSMOLALITY

Low-ion freshwater and seawater treatments differentially altered plasma osmolality in anadromous and landlocked Alewives over the two-week laboratory-acclimation challenges (Fig. 4). A full model with site, salinity, and time revealed a significant three-way interaction (GLMM, $P < 0.001$), so separate analyses were conducted for each salinity treatment (results presented in Table S4). Low-ion freshwater treatment caused a steady decline of plasma osmolality (significant day effect; Table S4), and there were no differences among populations detected ($P > 0.05$). At 30 ppt, plasma osmolality immediately increased sharply for Alewives from L-Pattagansett and L-Rogers, whereas fish from A-Bride maintained steadier levels (significant site by time interactions; Table S4; Fig. 4). Plasma osmolality

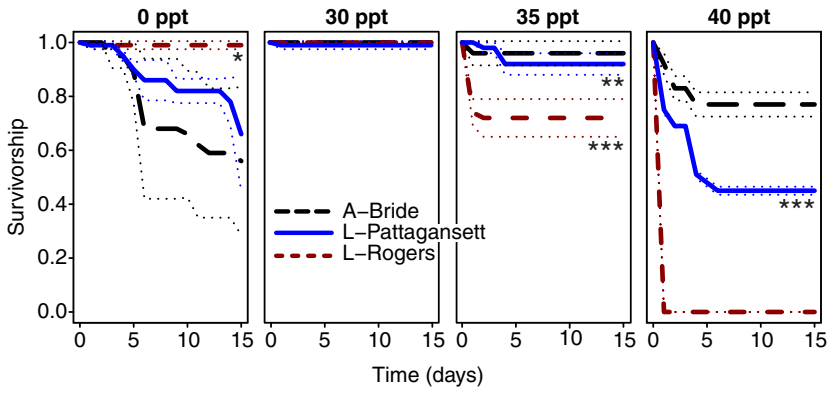

Figure 3. Survival of anadromous and landlocked Alewives in low-ion freshwater (0 ppt) and seawater (30 ppt, 35 ppt, and 40 ppt) during two-week laboratory-acclimation experiments. Dotted lines represent standard error of the mean. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ indicating significant differences in survivorship compared to A-Bride, according to a Cox proportional hazards model. Results of generalized linear mixed effects models are in Table 2.

Table 2. Results of generalized linear mixed effects models testing for variation in survival probability for laboratory-acclimation salinity challenge experiment.

Fixed effect	Estimate	z-Value
Site		
L-Pattagansett	2.54	4.40^{***}
L-Rogers	−2.36	-2.21^{*}
Salinity		
30 ppt	−3.13	-2.97^{**}
35 ppt	−1.66	-2.07^{*}
40 ppt	0.05	0.10
Site × salinity		
L-Pattagansett × 30 ppt	0.53	0.40
L-Pattagansett × 35 ppt	0.66	0.63
L-Pattagansett × 40 ppt	1.24	1.95#
L-Rogers × 30 ppt	3.26	1.82
L-Rogers × 35 ppt	5.83	4.27^{***}
L-Rogers × 40 ppt	9.87	5.89^{***}
Covariate		
Length	−1.38	-6.09^{***}

A-Bride and 0 ppt were used as references for site and salinity effect, respectively. Tank was included as a random effect in the model (see Methods). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; #$P = 0.051$.

varied positively with length at 30 ppt. Full-strength seawater and hyper-saline treatments dramatically increased plasma osmolality in landlocked Alewives (significant site by time interaction for L-Pattagansett at 35 ppt and 40 ppt, and for L-Rogers at 35 ppt, compared to A-Bride; Table S4). By day 15, plasma osmolality in surviving landlocked Alewives did not differ from that of anadromous Alewives (Table S4).

GILL NKA ACTIVITY

Gill NKA activity increased in response to all salinities, most strongly after seawater challenge (Fig. 5). The increase in gill NKA activity in response to salinity was more pronounced in anadromous Alewives, which had higher pre- and post-transfer levels. The full GLMM model revealed a significant three-way population by salinity by time interaction ($P < 0.05$), so we analyzed each salinity treatment separately (Table S4). In low-ion freshwater, gill NKA activity was higher by day 15 (significant time effect; Table S4), and was higher among A-Bride Alewives than Alewives from either landlocked population (significant site effect for L-Pattagansett and L-Rogers compared to A-Bride; Table S4). At 30 ppt, gill NKA also increased and was higher among A-Bride Alewives than L-Pattagansett Alewives (significant site effect; Table S4), but not L-Rogers Alewives ($P > 0.05$). Transfer to full-strength seawater and hyper-saline treatment resulted in upregulation of gill NKA activity that was more pronounced in anadromous Alewives than in all landlocked Alewives (significant site by time interactions; Table S4).

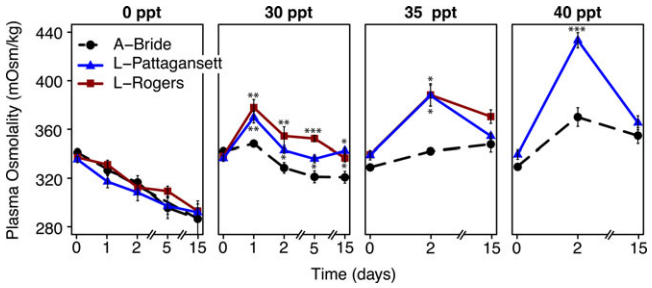

Figure 4. Plasma osmolality of anadromous and landlocked Alewives in low-ion freshwater (0 ppt) and seawater (30 ppt, 35 ppt, and 40 ppt) during two-week laboratory-acclimation challenges ($n = 12$ individuals per site per salinity treatment per time point). Each point is the mean value ± standard error of the mean. Asterisks indicate a significant site × time interaction according to a linear mixed effects model. Day 0 (pretransfer) and A-Bride were used as reference levels. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. All model results are presented in Table S4.

GENE EXPRESSION

Gene expression was modified by salinity, but there were few overall differences between anadromous and landlocked Alewives. In low-ion freshwater, *NHE3* increased by day 15 and was not significantly different between life-history forms (GLMM, Table S4; Fig. 6A). In 30 ppt seawater, *NHE3* was higher in landlocked Alewives than in anadromous Alewives (significant site effect; Table S4), and was significantly upregulated by day 2 (Table S4). *VATP* was not affected by salinity, but was generally lower in anadromous than landlocked Alewives in seawater (significant site effect; Fig. 6B; Table S4). *NKCC* expression was transiently downregulated in response to freshwater and transiently upregulated in response to seawater (significant time effect at each salinity; Table S4; Fig. 6C). Landlocked Alewives from L-Pattagansett exhibited higher *NKCC* than fish from L-Rogers or fish from A-Bride in seawater (significant site effect; Table S4).

Discussion

This study adds to a growing body of literature suggesting that the ecological transition to an exclusively freshwater environment results in an evolutionary trade-off in osmoregulatory function. The majority of studies indicate that fully freshwater forms have evolved reduced performance in seawater relative to an ancestral seawater or migratory form (Foote et al. 1992; Staurnes et al. 1992; Nilsen et al. 2003; Bystriansky et al. 2007; Lee et al. 2007; Fuller 2008, 2009; McCairns and Bernatchez 2010; Whitehead 2010; DeFaveri and Merila 2014; Velotta et al. 2014), whereas several studies in Mummichog suggest that the transition to freshwater also leads to enhanced hyper-osmoregulatory performance (Scott et al. 2005; Whitehead et al. 2011, 2012; Brennan et al. 2015). Here, we demonstrate that seawater survival and

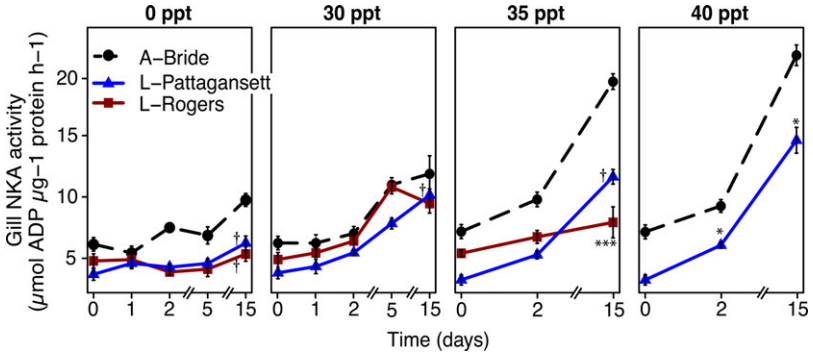

Figure 5. Na^+/K^+-A TPase activity of anadromous and landlocked Alewives in low-ion freshwater (0 ppt) and seawater (30 ppt, 35 ppt, and 40 ppt) during two-week laboratory-acclimation challenges (n = 12 individuals per site per salinity treatment per time point). Each point is the mean value ± standard error of the mean. Asterisks indicate a significant site × time interaction according to a linear mixed effects model. Day 0 (pretransfer) and A-Bride were used as reference levels. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Daggers indicate a significant main effect of site ($P < 0.05$) where no significant interactions were found. Results of generalized linear mixed effects models are in Table S4.

hypo-osmotic balance are inversely related to freshwater survival across independent populations of landlocked Alewife. Such a reciprocal trade-off may indicate local adaptation because it satisfies the "local versus foreign" criterion of Kawecki and Ebert (2004); satisfying this criterion indicates divergent selection in either habitat (i.e., freshwater vs. seawater). Few studies in fish provide evidence of a reciprocal trade-off (Marchinko and Schluter 2007 in stickleback; Brennan et al. 2015 in killifish) and none across multiple, independently derived populations. Finally, we show that activity of gill NKA, a major component of ion exchange, is consistently reduced among landlocked forms at all salinities, suggesting a potential mechanism of adaptation to freshwater.

We cannot rule out the possibility that life-history form divergence in osmoregulation is the result of environmental or maternal effects, although environmental effects that would be most likely to cause the population differences we observed are likely to be minimal or nonexistent. To minimize environmental influences, we acclimated Alewives to a common salinity, which acts to limit physiological differences due to differences in the environment (Whitehead and Crawford 2006; Whitehead et al. 2011, 2012). Furthermore, the lakes used in this study differ little in solute levels (Table S1), which is the environmental variable that is expected to influence osmoregulatory ability.

DIVERGENCE IN OSMOREGULATORY FUNCTION AND ASSOCIATED TRADE-OFFS

Acute seawater challenges revealed that landlocked Alewives exhibit reduced and variable seawater tolerance compared to anadromous Alewives (Fig. 2). Variation in tolerance is associated with genetic distance (F_{ST} based on mitochondrial CR1) from the anadromous population (Fig. 2B). To the extent that F_{ST} measures neutral genetic differentiation, the degree of seawater tolerance loss among landlocked populations appears to be influenced by divergence time, which would be consistent with a selective or neutral (e.g., accumulation of loss of function mutations or differences in effective population size) explanation. More extensive genetic analysis will be required to discern the relative contributions of these evolutionary processes in explaining variation in seawater tolerance. A deeper analysis of genetic differentiation that quantifies diversity at other loci will also be needed. We confined our analysis to values of F_{ST} at the CR1 locus, and omitted analysis using F_{ST} of microsatellites (Palkovacs et al. 2008); F_{ST} at microsatellites and other regions subject to relatively high mutation rates is regarded as an unreliable indicator of population differences (Meirmans and Hedrick 2011; Whitlock 2011).

We chose two landlocked populations (L-Pattagansett and L-Rogers) that represented the range of responses to seawater to test for a trade-off in osmoregulatory function. In particular, we predicted that the loss of seawater tolerance would be associated with a proportional gain in freshwater tolerance in laboratory-acclimation experiments. This prediction was borne out in the results. First, we found a significant site by salinity interaction for survival between A-Bride and L-Rogers Alewives (Table 1), which is diagnostic of local adaptation (Kawecki and Ebert 2004). Second, our prediction that the degree of seawater sensitivity would be inversely related to the degree of freshwater tolerance was supported: the more seawater-sensitive population (L-Rogers) was more tolerant of freshwater, whereas tolerance of seawater and freshwater was intermediate in L-Pattagansett (Fig. 3). This provides evidence that trade-offs in osmotic tolerance may reflect local adaptation in Alewives.

We acknowledge the possibility of other evolutionary explanations for this pattern. For example, whether enhanced freshwater tolerance leads directly to lowered seawater tolerance (a

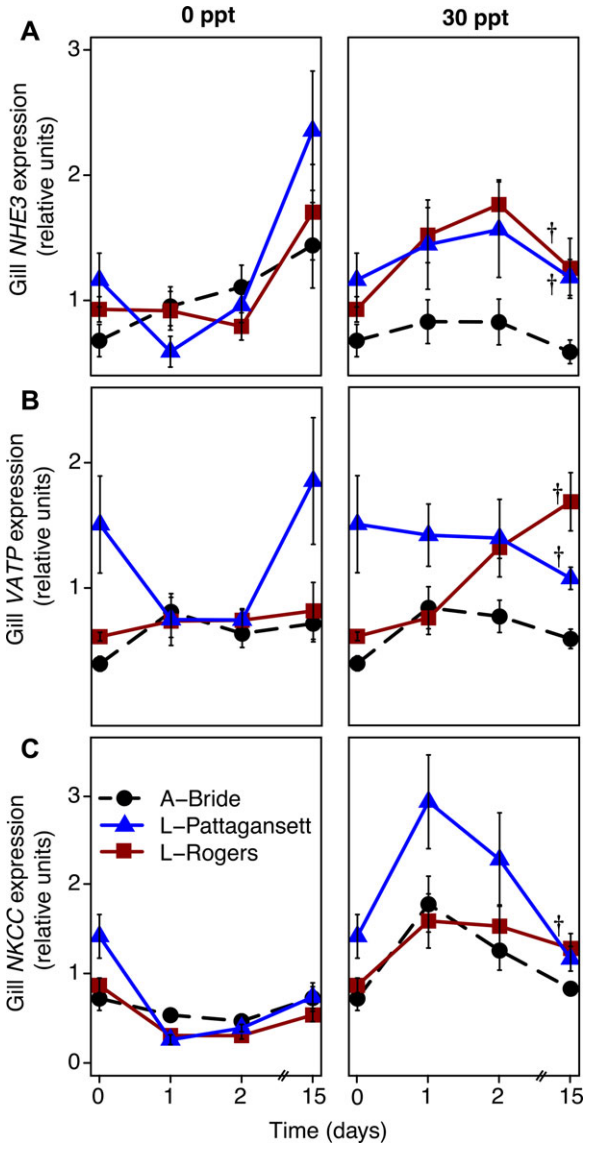

Figure 6. Gill gene expression of anadromous and landlocked Alewives in low-ion freshwater (0 ppt) and seawater (30 ppt) for (A) *NHE3*, (B) *VATP*, and (C) *NKCC*. Values were normalized to the expression of a reference gene (*EF1α*). $n = 8$ individuals per site per salinity treatment per time point. Each point is the mean value ± standard error of the mean. Daggers indicate a significant main effect of site ($P < 0.05$) where no significant interactions were found. Results of generalized linear mixed effects models are in Table S4.

requirement of a trade-off) cannot be explicitly deduced without examining the underlying mechanism. It remains possible that selection acts on different and uncoupled mechanisms in either salinity environment. The loss of seawater osmoregulatory function among landlocked forms may also reflect the slow deterioration of function by neutral genetic processes (i.e., relaxed selection; Lahti et al. 2009). However, natural selection is the most plausible explanation, considering that divergence has occurred relatively recently (likely 300–400 years; Palkovacs et al. 2008), that changes in tolerance are reciprocal, and that they occur in parallel.

Enhanced freshwater tolerance in landlocked forms is likely linked to loss of the physiological changes that accompany diadromous migration. In American Shad (*Alosa sapidissima*, a congener of Alewife), declining temperatures at the onset of the migratory season correspond with a reduced hyper-osmoregulatory capacity, a response that may serve as a proximate cue for migration (Zydlewski and McCormick 1997). Conversely, seawater tolerance develops at the larval–juvenile transition in Shad (well before the peak of migration; Zydlewski and McCormick 1997) and perhaps even earlier in Alewives (Yako 1998). Our results indicate that freshwater tolerance is reduced in anadromous forms around the end of the migration season (Gahagan et al. 2010). As such, challenging anadromous Alewives in low-ion freshwater prior to migration may not result in freshwater mortality, although this remains to be tested. Among landlocked Alewives, the loss of a putative physiological response to migratory cues may facilitate the prolongation of hyper-osmoregulatory capacity throughout the year. The existence of such a mechanism in Alewives, and whether it may subsequently limit osmoregulatory performance in seawater (via a trade-off), should be the focus of future investigations.

Osmotic homeostasis is reduced in response to seawater among independently derived landlocked populations, providing further evidence that adaptation to freshwater results in lowered hypo-osmoregulatory performance. When challenged with high salinity, landlocked Alewives lost osmotic balance more severely and for significantly longer than anadromous Alewives. In contrast, anadromous Alewives maintained a near-constant plasma osmolality, remaining close to pretransfer levels of 338 mOsmol/kg (Fig. 4). After two weeks at 35 ppt, anadromous Alewife plasma osmolality (average = 345 mOsm/kg) was comparable to that of seawater-acclimated American Shad (335 ± 4 mOsm/kg; Zydlewski and McCormick 1997). Landlocked Alewife osmolality in seawater had moderated from higher earlier levels but remained higher (L-Pattagansett, ~355 mOsm/kg; L-Rogers, ~370 mOsm/kg) than anadromous Alewife values, suggesting that surviving landlocked Alewives had not fully acclimated to seawater.

Alewives from the anadromous and landlocked populations steadily lost plasma osmolality in low-ion freshwater over the time course of the experiment (Fig. 4), demonstrating that this salinity represents a significant hypotonic challenge. These results did not fit our predictions; we expected landlocked Alewives to maintain osmotic balance in low-ion freshwater to a greater degree than anadromous Alewives. Freshwater populations of Mummichog (*Fundulus heteroclitus*) maintain osmotic balance after seawater-to-freshwater transfer better than seawater-derived populations

(Scott et al. 2004; Whitehead et al. 2011, 2012; Brennan et al. 2015). Alewives did not acclimate to low-ion freshwater even after two weeks of exposure; plasma osmolality was on average 291 mOsmol/kg for surviving Alewives, which is lower than values for American Shad in freshwater (318 ± 4.8 mOsmol/kg; Zydlewski and McCormick 1997) and the average for all diadromous fishes in freshwater for which data are available (311 ± 6.5 mOsmol/kg; Nordlie 2009). The steady decline in osmolality in freshwater may have contributed to mortality among A-Bride and L-Pattagansett Alewives. The high survival of L-Rogers in low-ion freshwater (Fig. 3), despite the decline of plasma osmolality, suggests that the tolerance of low plasma ion levels may be an adaptive response to an exclusively freshwater life history.

MOLECULAR MECHANISMS OF DIVERGENCE IN OSMOREGULATORY FUNCTION

Gill NKA activity is reduced in independently derived populations of landlocked Alewives in response to freshwater and seawater compared to the anadromous population (Fig. 5). Reductions in the upregulation of gill NKA activity in response to seawater likely contribute to reduced seawater tolerance and hypo-osmoregulatory performance among landlocked Alewives because NKA is the primary driver of ion secretion at the gill (Evans et al. 2005). This result is consistent with our previous findings (Velotta et al. 2014); in the present study, we show that the patterns of differentiation of seawater NKA activity are the same when animals are acclimated to a common environment, and that this pattern is similar for two independently derived populations.

Our NKA activity results are generally consistent with studies from other species. Freshwater populations of copepods have reduced NKA activity at any salinity compared to their seawater ancestor (Lee et al. 2011), and landlocked salmonids cannot upregulate gill NKA activity to the same degree that anadromous forms can (Bystriansky et al. 2007; Nilsen et al. 2007). However, a study in Mummichog demonstrated that gill NKA activity increased more strongly after freshwater or brackish water transfer in northern (primarily freshwater) compared to southern (primarily brackish water) populations (Scott et al. 2005). Evolutionary responses to independent freshwater invasions may be species specific and reflect past or present differences in selective pressures or evolutionary constraints. Nevertheless, that multiple freshwater forms of unrelated taxa have differentiated in gill NKA activity relative to a seawater ancestor suggests that changes to the function of NKA may be adaptive. Additional evidence is provided by population genetic studies demonstrating that NKA is under selection in freshwater populations of Threespine Stickleback (Hohenlohe et al. 2010; DeFaveri et al. 2011; Shimada et al. 2011; Jones et al. 2012), and that NKA mRNA expression is lowered among landlocked populations of Steelhead trout (Aykanat et al. 2011; but see McCairns and Bernatchez 2010 who did not find evidence of divergence).

Several explanations for lowered gill NKA activity among landlocked forms may be possible. First, although landlocked Alewife would not be exposed to seawater and thus would not experience costs of plasticity per se (Dewitt et al. 1998; Auld et al. 2010), there may be energetic costs or trade-offs associated with the maintenance of sensory-response systems for responding to salinity (Lessels 2008; McCormick 2009). It is also possible that genetic drift has resulted in neutral deterioration of this response, either at the level of seawater detection (i.e., osmosensing; Evans 2010; Kultz 2012), or at a regulatory pathway that leads to its upregulation once seawater is sensed. Finally, lowered gill NKA activity may be an adaptive response to living in a low productivity environment, as suggested by Aykanat et al. (2011) for anadromous Rainbow Trout. Because powering NKA is energetically expensive (Tseng and Hwang 2008), and because freshwater tends to be less productive than seawater, selection may favor individuals with reduced activity. This explanation is supported by the fact that NKA activity is reduced across all salinity environments (Fig. 5). Additional support for this hypothesis is that growth rates are lower for landlocked Alewives than for anadromous Alewives both in the wild (Scott and Crossman 1973) and in the laboratory (J. Velotta). Data from Threespine Stickleback, however, indicate that low-plated (but not fully plated) freshwater forms have higher growth in freshwater than seawater forms (Marchinko and Schluter 2007). Future work should test hypotheses regarding lowered energy expenditure in freshwater forms and its effects on gill NKA activity.

Among anadromous Alewives, expression of *NHE3* was upregulated in response to freshwater, but remained relatively constant in response to seawater (Fig. 6A), consistent with its putative role in gill Na^+ uptake (Fig. 6A; Scott et al. 2005; Hiroi et al. 2008; Wanatabe et al. 2008; Inokuchi et al. 2009). In contrast, expression of *NHE3* in landlocked Alewives was upregulated in freshwater and in seawater, where it remained high throughout the experiment. This result differs slightly from a previous study of Alewife by Christensen et al. (2012), who demonstrated that the abundance of NHE3 indicated in immunohistochemical preparations is similar in the freshwater and seawater gill. Inconsistencies between this study and Christensen et al. (2012) may indicate that differences in gill transcription of *NHE3* do not yield differences in protein abundance. Future studies should address whether population differentiation in mRNA transcription is mirrored by differentiation in protein abundance and localization. Finally, our results suggest that landlocked Alewives do not downregulate *NHE3* transcription in response to seawater. The parallel evolution among landlocked populations of high *NHE3* expression across salinity environments is consistent with the expectation that landlocked Alewives increase the regulation of

transporters involved in ion uptake as an adaptive response to a fully freshwater life history. To our knowledge, this is the first documentation of population-level divergence of *NHE3* in any fish species.

Transcription of *VATP* was not increased by freshwater exposure in landlocked or anadromous Alewives (Fig. 6B), which suggests that it plays a minimal role in ion uptake in the Alewife gill. This was an unexpected finding, given suggestions that electrogenic apical *VATP* drives passive Na^+ uptake in the fish gill (Katoh et al. 2003; Evans et al. 2005). We did, however, observe a variable pattern of *VATP* transcription in response to seawater between Alewife life-history forms (Fig. 6B); transcription of *VATP* was not responsive to seawater for anadromous Alewives, but was for L-Rogers Alewives. L-Pattagansett Alewives had greater pretransfer expression than any other population, and expression remained high throughout the time course. Our results differ from those of Lee et al. (2011), who showed that freshwater-adapted copepods have evolved elevated VATP activity and transcription in response to freshwater. This may reflect taxon-specific differences in the role of VATP in acclimation to freshwater on physiological timescales, and/or in adaptation to freshwater on an evolutionary scale.

In a previous study, Alewives from A-Bride showed stronger upregulation of *NKCC* in response to 30 ppt seawater than Alewives from L-Pattagansett (Velotta et al. 2014), reflecting its role in gill Cl^- secretion. However, although *NKCC* appears to be upregulated after seawater exposure in the laboratory-acclimation experiment, its transcription was highest among L-Pattagansett fish, in contrast to our prediction and previous results. High expression of *NKCC* among L-Pattagansett fish may be a compensatory response to seawater challenge; greater osmotic imbalance at 30 ppt may lead to the recruitment of more NKCC transcripts. It remains unclear then why transcription of *NKCC* among L-Rogers fish is not comparably upregulated. More work is needed to clarify the role of *NKCC* expression in differentiation in osmoregulatory capacity. These contradictory results may also reflect differences in experimental design in which acclimation to a common laboratory environment influenced the transcriptional response to salinity differently than when animals are not acclimated.

CONCLUSIONS

The results of this study expand our current understanding of osmoregulatory trade-offs associated with the ecological transition into novel freshwater habitats. Our study uniquely demonstrates that high tolerance of freshwater is correlated with reductions in osmoregulatory function in seawater among several freshwater populations of fish that are independently derived. The reciprocal nature of performance differences is suggestive of local adaptation (sensu Kawecki and Ebert 2004), although relaxation of selective constraint on ionoregulatory processes, accompanied by neutral drift, could also yield these patterns. Recent divergence of landlocked Alewives from the anadromous ancestor (likely 300–400 years ago; Palkovacs et al. 2008) suggests that differentiation in osmoregulation may occur rapidly. Furthermore, these results contribute to the growing body of literature suggesting that changes in NKA—one of the most important enzymes involved in ion regulation—underlies adaptation to freshwater (Bystriansky et al. 2007; Nilsen et al. 2007; McCairns and Bernatchez 2010; DeFaveri et al. 2011; Lee et al. 2011; Jones et al. 2012). Finally, we provide the first account of evolutionary shifts in the transcriptional response of *NHE3* to salinity, although the significance of this shift will require further attention. Future work should be aimed at clarifying the role of gene expression in adaptation to freshwater, including the role of genome-wide transcriptional changes, which will uncover novel pathways involved in adaptation to salinity.

ACKNOWLEDGMENTS

We thank G. Casselberry, J. Divino, T. Duffy, E. Funk, N. Murphy, M. F. O'Dea (deceased), J. Rack, and A. Regish for assistance with field and laboratory work. We thank C. Schlichting, M. Urban, and three anonymous reviewers for providing insightful comments on earlier versions of this manuscript. Funding provided by University of Connecticut's Department of Ecology and Evolutionary Biology and Center for Conservation and Biodiversity, as well as MVAO. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

DATA ARCHIVING

All data are archived in supplementary information.

LITERATURE CITED

Auld, J. R., A. A. Agrawal, and R. A. Relyea. 2010. Re-evaluating the costs and limits of adaptive phenotypic plasticity. Proc. R. Soc. B 277:503–511.

Aykanat, T., F. P. Thrower, and D. D. Heath. 2011. Rapid evolution of osmoregulatory function by modification of gene transcription in steelhead trout. Genetica 139:233–242.

Brennan, R. S., F. Galvez, and A. Whitehead. 2015. Reciprocal osmotic challenges reveal mechanisms of divergence in phenotypic plasticity in the killifish *Fundulus heteroclitus*. J. Exp. Biol. 218:1212–1222.

Bystriansky, J. S., N. T. Frick, J. G. Richards, P. M. Schulte, and J. S. Ballantyne. 2007. Failure to up-regulate gill Na+, K+-ATPase α-subunit isoform α1b may limit seawater tolerance of land-locked Arctic char (*Salvelinus alpinus*). Comp. Biochem. Phys. A 148:332–338.

Carneiro, P. C. F., and E. C. Urbinati. 2001. Salt as a stress response mitigator of matrinxã, *Brycon cephalus* (Günther), during transport. Aquac. Res. 32:297–304.

Christensen, A. K., J. Hiroi, E. T. Schultz, and S. D. McCormick. 2012. Branchial ionocyte organization and ion-transport protein expression in juvenile alewives acclimated to freshwater or seawater. J. Exp. Biol. 215:642–652.

Czesny, S., J. Epifanio, and P. Michalak. 2012. Genetic divergence between freshwater and marine morphs of alewife (*Alosa pseudoharengus*): a "next-generation" sequencing analysis. PLoS ONE 7:e31803.

DeFaveri, J., and J. Merilä. 2014. Local adaptation to salinity in the three-spined stickleback? J. Evol. Biol. 27:290–302.

DeFaveri, J., T. Shikano, Y. Shimada, A. Goto, and J. Merilä. 2011. Global analysis of genes involved in freshwater adaptation in threespine sticklebacks (*Gasterosteus aculeatus*). Evolution 65:1800–1807.

DeWitt, T. J., A. Sih, and D. S. Wilson. 1998. Costs and limits of phenotypic plasticity. Trends Ecol. Evol. 13:77–81.

Evans, T. G. 2010. Co-ordination of osmotic stress responses through osmosensing and signal transduction events in fishes. J. Fish Biol. 76:1903–1925.

Evans, D. H., P. M. Piermarini, and K. P. Choe. 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85:97–177.

Fay, C. W., R. J. Neves, and G. B. Pardue. 1983. Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (Mid-Atlantic). Alewife/Blueback Herring. Biological Report - United States Fish and Wildlife Service FWS/OBS-82/11.9. USFW publication:1–20.

Foote, C. J., C. C. Wood, W. C. Clarke, and J. Blackburn. 1992. Circannual cycle of seawater adaptability in *Oncorhynchus nerka*: genetic differences between sympatric Sockeye Salmon and Kokanee. Can. J. Fish. Aquat. Sci. 49:99–109.

Fuller, R. C. 2008. Genetic incompatibilities in killifish and the role of environment. Evolution 62:3056–3068.

———. 2009. A test for a trade-off in salinity tolerance in early life-history stages in *Lucania goodei* and *L. parva*. Copeia 2008:154–157.

Futuyma, D. J., and G. Moreno. 1988. The evolution of ecological specialization. Ann. Rev. Ecol. Syst. 19:207–233.

Gahagan, B. I., K. E. Gherard, and E. T. Schultz. 2010. Environmental and endogenous factors influencing emigration in juvenile anadromous alewives. Trans. Am. Fish. Soc. 139:1069–1082.

Hereford, J. 2009. A quantitative survey of local adaptation and fitness tradeoffs. Am. Nat. 173:579–588.

Hiroi, J., and S. D. McCormick. 2012. New insights into gill ionocyte and ion transporter function in euryhaline and diadromous fish. Resp. Physiol. Neurobi. 184:257–268.

Hiroi, J., S. Yasumasu, S. D. McCormick, P.-P. Hwang, and T. Kaneko. 2008. Evidence for an apical Na–Cl cotransporter involved in ion uptake in a teleost fish. J. Exp. Biol. 211:2584–2599.

Hohenlohe, P. A., S. Bassham, P. D. Etter, N. Stiffler, E. A. Johnson, and W. A. Cresko. 2010. Population genomics of parallel adaptation in threespine stickleback using sequenced RAD tags. PLoS Genet. 6:e1000862.

Hwang, P.-P., and T.-H. Lee. 2007. New insights into fish ion regulation and mitochondrion-rich cells. Comp. Biochem. Phys. A 148:479–497.

Inokuchi, M., J. Hiroi, S. Watanabe, P.-P. Hwang, and T. Kaneko. 2009. Morphological and functional classification of ion-absorbing mitochondria-rich cells in the gills of Mozambique tilapia. J. Exp. Biol 212:1003–1010.

Johnson, D. L., and M. T. Metcalf. 1982. Causes and controls of freshwater drum mortality during transportation. Trans. Am. Fish. Soc. 111:58–62.

Jones, F. C., M. G. Grabherr, Y. F. Chan, P. Russell, E. Mauceli, J. Johnson, R. Swofford, M. Pirun, M. C. Zody, S. White, et al. 2012. The genomic basis of adaptive evolution in threespine sticklebacks. Nature 484:55–61.

Katoh, F., S. Hyodo, and T. Kaneko. 2003. Vacuolar-type proton pump in the basolateral plasma membrane energizes ion uptake in branchial mitochondria-rich cells of killifish *Fundulus heteroclitus*, adapted to a low ion environment. J. Exp. Biol. 206:793–803.

Kawecki, T. J., and D. Ebert. 2004. Conceptual issues in local adaptation. Ecol. Lett. 7:1225–1241.

Kültz, D. 2012. The combinatorial nature of osmosensing in fishes. Physiology 27:259–275.

Lahti, D. C., N. A. Johnson, B. C. Ajie, S. P. Otto, A. P. Hendry, D. T. Blumstein, R. G. Coss, K. Donohue, and S. A. Foster. 2009. Relaxed selection in the wild. Trends Ecol. Evol. 24:487–496.

Lee, C. E., J. L. Remfert, and Y.-M. Chang. 2007. Response to selection and evolvability of invasive populations. Genetica 129:179–192.

Lee, C. E., M. Kiergaard, G. W. Gelembiuk, B. D. Eads, and M. Posavi. 2011. Pumping ions: rapid parallel evolution of ionic regulation following habitat invasions. Evolution 65:2229–2244.

Lessells, C. M. 2008. Neuroendocrine control of life histories: what do we need to know to understand the evolution of phenotypic plasticity? Phil. Trans. R. Soc. B 363:1589–1598.

Marchinko, K. B., and D. Schluter. 2007. Parallel evolution by correlated response: lateral plate reduction in threespine stickleback. Evolution 61:1084–1090.

McCairns, R. J. S., and L. Bernatchez. 2010. Adaptive divergence between freshwater and marine sticklebacks: insights into the role of phenotypic plasticity from an integrated analysis of candidate gene expression. Evolution 64:1029–1047.

McCormick, S. D. 1993. Methods for nonlethal gill biopsy and measurement of Na^+, K^+-ATPase activity. Can. J. Fish. Aquat. Sci. 50:656–658.

———. 2009. Evolution of the hormonal control of animal performance: insights from the seaward migration of salmon. Integr. Comp. Biol. 49:408–422.

Meirmans, P. G., and P. W. Hedrick. 2011. Assessing population structure: F_{ST} and related measures. Mol. Ecol. Resour. 11:5–18.

Michalak, K., S. Czesny, J. Epifanio, R. J. Snyder, E. T. Schultz, J. P. Velotta, S. D. McCormick, B. L. Brown, G. Santopietro, and P. Michalak. 2014. Beta-thymosin gene polymorphism associated with freshwater invasiveness of alewife (*Alosa pseudoharengus*). J. Exp. Zool 321:233–240.

Nikinmaa, M., A. Soivio, T. Nakari, and S. Lindgren. 1983. Hauling stress in brown trout (*Salmo trutta*): physiological responses to transport in fresh water or salt water, and recovery in natural brackish water. Aquaculture 34:93–99.

Nilsen, T. O., L. O. E. Ebbesson, and S. O. Stefansson. 2003. Smolting in anadromous and landlocked strains of Atlantic salmon (*Salmo salar*). Aquaculture 222:71–82.

Nilsen, T. O., L. O. E. Ebbesson, S. S. Madsen, S. D. McCormick, E. Andersson, B. T. Björnsson, P. Prunet, and S. O. Stefansson. 2007. Differential expression of gill Na^+,K^+-ATPaseα - and β-subunits, Na^+,K^+,$2Cl^-$ cotransporter and CFTR anion channel in juvenile anadromous and landlocked Atlantic salmon *Salmo salar*. J. Exp. Biol. 210:2885–2896.

Nordlie, F. G. 2009. Environmental influences on regulation of blood plasma/serum components in teleost fishes: a review. Rev. Fish. Biol. Fish. 19:481–564.

Palkovacs, E. P., K. B. Dion, D. M. Post, and A. Caccone. 2008. Independent evolutionary origins of landlocked alewife populations and rapid parallel evolution of phenotypic traits. Mol. Ecol. 17:582–597.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucl. Acids Res. 29:e45–e45.

Rundle, H. D., and P. Nosil. 2005. Ecological speciation. Ecol. Lett. 8:336–352.

Schluter, D. 2000. The ecology of adaptive radiation. Oxford Univ. Press, Oxford, U. K .

Schultz, E. T., and S. D. McCormick. 2013. Euryhalinity in an evolutionary context. *In* S. D. McCormick, A. P. Farrell, C. J. Brauner, eds. Fish physiology. Vol. 32. Academic Press, Oxford. 477–534.

Scott, G. R., Claiborne, J. B., Edwards, S. L., Schulte, P. M., & Wood, C. M. (2005). Gene expression after freshwater transfer in gills and opercular epithelia of killifish: insight into divergent mechanisms of ion transport. J. Exp. Biol., 208(14), 2719–2729.

Scott, G. R., and P. M. Schulte. 2005. Intraspecific variation in gene expression after seawater transfer in gills of the euryhaline killifish *Fundulus heteroclitus*. Comp. Biochem. Phys. A 141:176–182.

Scott, W., and E. Crossman. 1973. Freshwater fishes of Canada. Fish. Res. Board Can. Bull. 184, New York, NY.

Scott, G. R., J. T. Rogers, J. G. Richards, C. M. Wood, and P. M. Schulte. 2004. Intraspecific divergence of ionoregulatory physiology in the euryhaline teleost *Fundulus heteroclitus*: possible mechanisms of freshwater adaptation. J. Exp. Biol. 207:3399–3410.

Shimada, Y., T. Shikano, and J. Merilä. 2011. A high incidence of selection on physiologically important genes in the three-spined stickleback, *Gasterosteus aculeatus*. Mol. Biol. Evol. 28:181–193.

Stanley, J. G., and P. J. Colby. 1971. Effects of temperature on electrolyte balance and osmoregulation in the alewife (*Alosa pseudoharengus*) in fresh and sea water. Trans. Am. Fish. Soc. 100:624–638.

Staurnes, M., T. Sigholt, G. Lysfjord, and O. A. Gulseth. 1992. Difference in the seawater tolerance of anadromous and landlocked populations of arctic char (*Salvelinus alpinus*). Can. J. Fish. Aquat. Sci. 49:443–447.

Tseng, Y.-C., and P.-P. Hwang. 2008. Some insights into energy metabolism for osmoregulation in fish. Comp. Biochem. Phys. C 148: 419–429.

Velotta, J. P., S. D. McCormick, R. J. O'Neill, and E. T. Schultz. 2014. Relaxed selection causes microevolution of seawater osmoregulation and gene expression in landlocked Alewives. Oecologia 175:1081–1092.

Venables, W. N., and B. D. Ripley. 2002. Modern applied statistics using S. Springer Science & Business Media, New York, NY.

Watanabe, S., M. Niida, T. Maruyama, and T. Kaneko. 2008. Na^+/H^+ exchanger isoform 3 expressed in apical membrane of gill mitochondrion-rich cells in Mozambique tilapia *Oreochromis mossambicus*. Fish. Sci. 74:813–821.

Whitehead, A. 2010. The evolutionary radiation of diverse osmotolerant physiologies in Killifish (*Fundulus sp.*). Evolution 64:2070–2085.

Whitehead, A., and D. L. Crawford. 2006. Neutral and adaptive variation in gene expression. Proc. Natl. Acad. Sci. USA 103:5425–5430.

Whitehead, A., J. L. Roach, S. Zhang, and F. Galvez. 2011. Genomic mechanisms of evolved physiological plasticity in killifish distributed along an environmental salinity gradient. Proc. Natl. Acad. Sci. USA 108:6193–6198.

Whitehead, A., J. L. Roach, S. Zhang, and F. Galvez. 2012. Salinity- and population-dependent genome regulatory response during osmotic acclimation in the killifish (*Fundulus heteroclitus*) gill. J. Exp. Biol. 215:1293–1305.

Whitlock, M. C. 2011. G'_{ST} and D do not replace F_{ST}. Mol. Ecol. 20:1083–1091.

Yako, L. A. 1998. Community interactions influencing juvenile anadromous herring in freshwater: migration cues and predation. M.S. Thesis, University of Massachusetts Amherst, Amherst, Massachusetts.

Zydlewski, J., and S. D. McCormick. 1997. The loss of hyperosmoregulatory ability in migrating juvenile American shad, *Alosa sapidissima*. Can. J. Fish. Aquat. Sci. 54:2377–2387.

Associate Editor: J. Storz
Handling Editor: J. Conner

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Study site details.
Table S2. Dates of acute salinity exposure experiments.
Table S3. Primer sequences (F: forward; R: reverse) for candidate osmoregulation genes (*NHE3*, *NKCC*, *VATP*) and a reference gene (*EF1*α).
Table S4. Results of linear mixed effects models for plasma osmolality, gill NKA activity, and candidate gene expression data.