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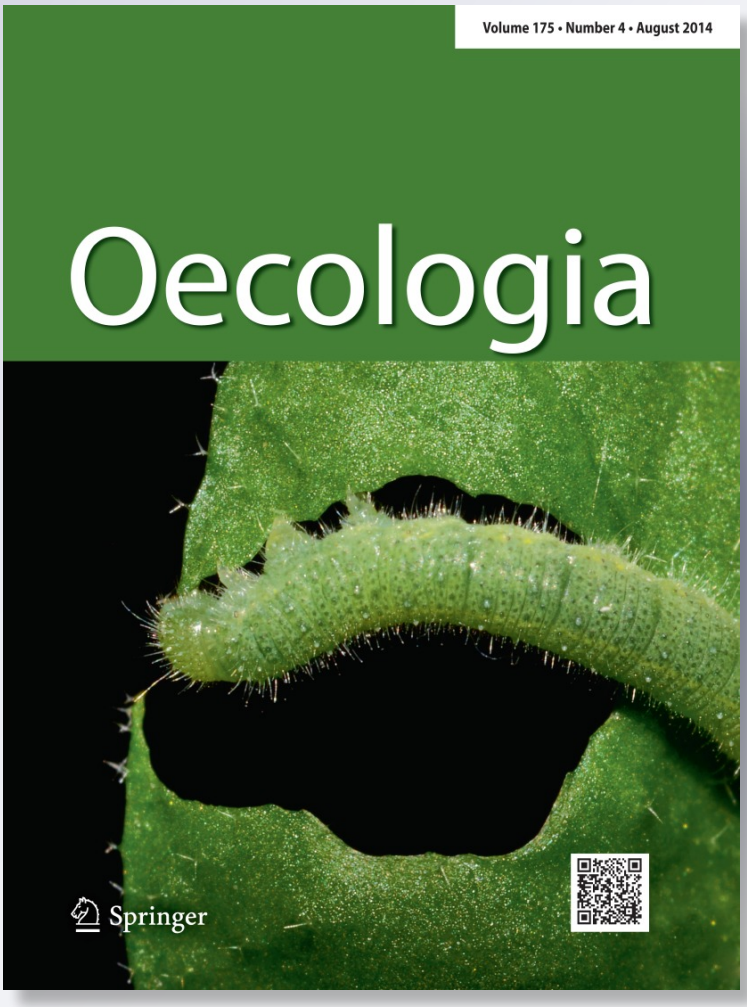

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PHYSIOLOGICAL ECOLOGY - ORIGINAL RESEARCH

Relaxed selection causes microevolution of seawater osmoregulation and gene expression in landlocked Alewives

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Abstract Ecological transitions from marine to freshwater environments have been important in the creation of diversity among fishes. Evolutionary changes associated with these transitions likely involve modifications of osmoregulatory function. In particular, relaxed selection on hypo-osmoregulation should strongly affect animals that transition into novel freshwater environments. We used populations of the Alewife (*Alosa pseudoharengus*) to study evolutionary shifts in hypo-osmoregulatory capacity and ion regulation associated with freshwater transitions. Alewives are ancestrally anadromous, but multiple populations in Connecticut have been independently restricted to freshwater lakes; these landlocked populations complete their entire life cycle in freshwater. Juvenile landlocked and anadromous Alewives were exposed to three salinities (1, 20 and 30 ppt) in small enclosures within the lake. We detected strong differentiation between life history forms: landlocked Alewives exhibited reduced seawater tolerance and hypo-osmoregulatory performance compared to anadromous Alewives. Furthermore, gill Na^+/K^+-ATPase activity and transcription of genes for seawater osmoregulation (*NKCC*—$Na^+/K^+/2Cl^-$ cotransporter and *CFTR*—cystic fibrosis transmembrane conductance regulator) exhibited reduced responsiveness to seawater challenge. Our study demonstrates that adaptations of marine-derived species to completely freshwater life cycles involve partial loss of seawater osmoregulatory performance mediated through changes to ion regulation in the gill.

Keywords *Alosa pseudoharengus* · Anadromy · Na^+/K^+-ATPase activity · Physiological ecology · Salinity challenge

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J. P. Velotta (✉) · E. T. Schultz
Department of Ecology and Evolutionary Biology, University of Connecticut, 75 North Eagleville Road, Unit 3043, Storrs, CT 06269, USA
e-mail: jonathan.velotta@uconn.edu

S. D. McCormick
USGS, Conte Anadromous Fish Research Center, Turners Falls, MA 01376, USA

R. J. O'Neill
Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269, USA

Introduction

Among fishes, ecological transitions from marine to freshwater environments have often involved episodes of diversification and adaptive radiation (Schultz and McCormick 2013). Freshwater environments contain a substantial amount of earth's fish diversity (approximately 40 %; Nelson 2006) in only a fraction (0.01 %) of the available water. The boundary between seawater and freshwater, however, may be a formidable one to cross, since these habitats differ strongly in osmotic pressure and ion concentration (Lee and Bell 1999). Euryhaline species may be uncommonly suited for ecological movement into freshwater due to their ability to tolerate a wide range of salinities (Schultz and McCormick 2013). Adaptive changes that facilitate freshwater transitions have been studied in a number of taxa (e.g., Lee et al. 2011; Whitehead et al. 2011; DeFaveri et al. 2011), but we lack a full understanding of how osmoregulatory mechanisms evolve in response to such movements. Modern ecological transitions in which populations

of extant euryhaline species are restricted to freshwater through natural or anthropogenic land-locking events offer an opportunity to study evolutionary changes to osmoregulatory function. Here, we report on micro-evolutionary shifts in osmoregulatory function in an ancestrally anadromous species in which multiple landlocked, entirely freshwater populations exist.

Osmoregulation in teleost fishes involves integrated molecular and biochemical processes that take place within a variety of organs, including the gills (Evans et al. 2005; McCormick and Saunders 1987). These processes differ considerably between salinity environments. In freshwater, fishes passively gain external water and lose ions across all exposed surfaces, especially the gills. Passive ion loss is actively opposed by taking in environmental Na^+ and Cl^- at the gills, which maintains internal ion concentrations above that of the environment (hyper-osmoregulation). Fish in seawater passively lose water and gain ions from the environment. To maintain internal concentrations below ambient, marine fish drink large quantities of seawater and actively secrete the excess Na^+ and Cl^- at the gills (hypo-osmoregulation). Several well-studied ion transporters are responsible for ion secretion by gill ionocytes of seawater fishes (Evans et al. 2005): Na^+/K^+-ATPase (NKA), $Na^+/K^+/2Cl^-$ cotransporter (NKCC), and cystic fibrosis transmembrane conductance regulator homolog (CFTR).

Transitions from euryhaline to freshwater-restricted life cycles are likely to be followed by strong evolutionary adjustments favoring mechanisms for hyper-osmoregulation. The consequences of such transitions to existing hypo-osmoregulatory mechanisms have not been well characterized for fishes (but see Lee et al. 2003, 2007, 2011 for such work in copepods). Theory predicts that traits with constitutive energetic costs and/or traits subject to neutral evolutionary processes will decay over time following the elimination of a source of selection, a process known as relaxed selection (Lahti et al. 2009). Therefore, when fish become restricted to freshwater, relaxed selection is likely to reduce or eliminate hypo-osmoregulatory function (e.g., in *Salmo salar*; Nilsen et al. 2007). The rate at which trait decay occurs will depend on whether or not the trait bears an energetic cost. Hypo-osmoregulatory mechanisms should decay rapidly upon transition to freshwater if they bear constitutive energetic costs, since traits that reduce fitness should be selected against. If, however, hypo-osmoregulatory mechanisms bear no underlying cost in freshwater, neutral processes alone may still eliminate trait function, albeit more slowly, via genetic drift. Despite its importance to fish diversity and evolution, we do not yet have a full understanding of the details and rapidity of evolutionary changes in osmoregulation after transitions into permanent freshwater habitats, and how function in seawater is affected by such changes.

Research characterizing the evolutionary consequences of freshwater restriction on hypo-osmoregulation has primarily involved comparisons of landlocked and anadromous species in the family Salmonidae, which evolved and diversified in freshwater judging from the life history of basal species (Crespi and Fulton 2004; Stearley 1992; Wilson and Li 1999). For example, reduced seawater tolerance in landlocked populations of anadromous salmonids has been shown for Arctic Char (*Salvelinus alpinus*; Staurnes et al. 1992), Sockeye Salmon (*Oncorhynchus nerka*: Foote et al. 1992) and Atlantic Salmon (*Salmo salar*: Barbour and Garside 1983; Birt and Green 1986; Burton and Idler 1984; Nilsen et al. 2007). These studies indicate that greater seawater sensitivity of landlocked salmonids is also associated with a reduced ability to regulate blood Na^+ and Cl^- in seawater (Staurnes et al. 1992; Foote et al. 1992; Nilsen et al. 2007). Research conducted on species with marine ancestry, in which hypo-osmoregulation is a basal condition, and for which a freshwater life history is secondarily derived, may shed more light on the evolutionary processes associated with ecological transitions to freshwater.

Studies of freshwater forms of Threespine Stickleback (*Gasterosteus aculeatus*; Bell and Foster 1994) and killifishes of the genus *Fundulus* (Whitehead 2010), both of which are ancestrally marine, has revealed intraspecific changes in osmoregulatory physiology associated with transitions to a completely freshwater life history. In stickleback, survival in seawater is reduced among freshwater or lake populations (DeFaveri and Merila 2014; McCairns and Bernatchez 2010; E. Schultz, unpublished data). Loci under positive selection in the transition from marine to freshwater environments have been identified in stickleback, and include Na^+/K^+-ATPase and other genes involved in osmoregulation (DeFaveri et al. 2011; Jones et al. 2012; Shimada et al. 2011). Studies in the killifish *Fundulus heteroclitus* have revealed intraspecific differences in the molecular mechanisms that drive seawater osmoregulation between northern and southern populations (Scott and Schulte 2005); fish with northern genotypes appear better adapted to freshwater (Able and Palmer 1988; Scott et al. 2004) and occur more frequently in freshwater habitats compared to individuals with southern genotypes (Powers et al. 1986). Changes in the molecular response of northern killifish to seawater are not associated with hypo-osmoregulatory costs; killifish from northern and southern populations maintain plasma Na^+ and Cl^- balance after seawater transfer (Scott and Schulte 2005). Few studies have tied evolutionary changes in seawater tolerance and osmoregulatory capacity to associated physiological and molecular mechanisms in a marine-derived species.

Alewife (*Alosa pseudoharengus*) populations in Connecticut provide a distinctive opportunity to study the consequences of freshwater transitions and relaxed selection

on seawater osmoregulation. In Alewives, two life history forms exist: an ancestral anadromous form that migrates from seawater to freshwater to spawn, and a landlocked form, in which seawater migration has been eliminated from the life cycle. Population genetic analyses using mitochondrial and microsatellite loci indicate that multiple landlocked Alewife populations in Connecticut are independently derived from a genetically homogeneous anadromous stock (Palkovacs et al. 2008). Divergence estimates using microsatellite loci suggest that landlocked Alewives diverged from the anadromous ancestor no more than 5,000 years ago, and as recently as 300 years ago, depending on the microsatellite mutation rate assumed (Palkovacs et al. 2008). The most likely explanation is that Alewives became restricted to their natal lakes as a result of dam construction during European settlement approximately 300–500 years ago (Palkovacs et al. 2008).

Alewives belong to a predominately marine family of fishes, the Clupeidae, which apparently diversified in seawater (Li and Orti 2007) and in which hypo-osmoregulation is an ancestral capability. The development of seawater tolerance differs considerably between species in the Salmonidae and Clupeidae, evidence of their distinctive evolutionary histories. Juvenile anadromous salmonids prepare for entry into seawater through a series of preparatory physiological changes that lead to an increase in seawater tolerance just prior to migration, an ontogenetic phase known as smolting (McCormick 2013). Limited data available suggests that clupeids can tolerate seawater well before downstream migration; American Shad (*Alosa sapidissima*) can survive direct transfer to seawater at the larval-juvenile transition when gills develop (Zydlewski and McCormick 1997b), and Alewife tolerance to seawater appears to develop even earlier (Yako 1998). Since hypo-osmoregulation is deeply rooted in Alewife ancestry, the tempo and mode by which they adapt to freshwater restriction may be different than that experienced by ancestrally-freshwater salmonid fishes.

By comparing landlocked and anadromous Alewives, we investigated if relaxed selection on seawater function results in evolutionary changes to seawater tolerance (measured as survival), hypo-osmoregulatory capacity (measured as plasma osmolality after seawater exposure), the expression of two key seawater osmoregulation genes (NKCC and CFTR) and the enzymatic activity of Na^+/K^+-ATPase. We exposed wild-caught juvenile Alewives from one anadromous population and two landlocked populations in Connecticut to a series of 60 h in situ salinity challenge experiments, in which fish were transferred directly from their natal lake to 1 ppt (freshwater), 20 ppt (brackish water) and 30 ppt (seawater). We collected juvenile (age 0) Alewives from their natal lakes, since at this life history stage anadromous and landlocked Alewives live in identical salinity environments (approximately 0 ppt) and are naïve to seawater. We hypothesized that land-locking in Alewives would result in significant loss of osmoregulatory function in seawater due to relaxed selection. We predicted that seawater-challenged landlocked Alewives would experience reductions in tolerance, hypo-osmoregulatory capacity, Na^+/K^+-ATPase activity and expression of two seawater genes, *NKCC* and *CFTR,* compared to anadromous Alewives.

Materials and methods

Animals and experimental procedures

Anadromous and landlocked young-of-the-year (YOY) Alewives (*Alosa pseudoharengus*) were collected from their natal lakes in coastal Connecticut on six dates in 2009 (Table 1). All animals were handled in accordance with the University of Connecticut's Institutional Animal Care and Use Committee (protocol A09-24). We captured Alewives from three locations: anadromous population from Bride Lake (East Lyme, Connecticut), and landlocked populations from Pattagansett (East Lyme, Connecticut) and Rogers Lakes (Old Lyme, Connecticut, Table 1). The salinity of all three lakes was approximately 0 ppt. Three separate experimental trials were run in separate months (trial 1: September; trial 2: October; trial 3: November; Table 1) towards the end of juvenile anadromous Alewife out-migration (Gahagan et al. 2010). Within each trial, we subjected 10–15 Alewives from one anadromous and one landlocked population to three salinity treatments (1, 20, and 30 ppt) for 60 h (Table 2). We chose this time frame based on the results of salinity challenge experiments in other species (Scott and Schulte 2005; Staurnes et al. 1992;

Table 1 Details of salinity challenge experiments

Trial	Life history form	Location	Date (2009)	Length (mm)
Sep	Landlocked	Rogers Lake	09-Sep–11-Sep	48.5 ± 0.9
Sep	Anadromous	Bride Lake	15-Sep–18-Sep	56.8 ± 0.8
Oct	Landlocked	Pattagansett Lake	29-Sep–02-Oct	61.6 ± 0.7
Oct	Anadromous	Bride Lake	06-Oct–09-Oct	70.2 ± 0.5
Nov	Landlocked	Pattagansett Lake	17-Nov–20-Nov	78.2 ± 0.6
Nov	Anadromous	Bride Lake	10-Nov–12-Nov	73.2 ± 0.6

Trials consisted of one comparison between landlocked and anadromous Alewives, each consisting of 60-h salinity challenges at 1, 20, and 30 ppt. Lakes are located in East Lyme, Connecticut (Bride Lake and Pattagansett Lake) and Old Lyme, Connecticut (Rogers Lake). Mean standard length ± standard error of the mean of fish from each salinity challenge is also reported

Sep September, *Oct* October, *Nov* November

Table 2 The number of Alewives stocked from each population (A: Anadromous; L: Landlocked) in each salinity treatment (1, 20, 30 ppt) during each trial (Sep: September; Oct: October; Nov: November)

Trial	1 ppt		20 ppt		30 ppt	
	A	L	A	L	A	L
Sep	15	12	15	11	14	12
Oct	10	14	15	14	13	15
Nov	14	11	12	11	13	11

Zydlewski and McCormick 1997a) indicating that most mortality occurred over the first three days of exposure, and that perturbations in plasma osmolality were greatest between one day and three days. In addition, a 60-h time frame allows for measurement of critical changes in transcription (mRNA levels) in response to seawater (Scott and Schulte 2005). Salinity treatment of each population occurred in consecutive weeks, such that only one population was treated during a given week, followed by the other population the next week (Table 1), allowing for direct comparison of an anadromous and landlocked population at each trial.

Salinity challenges were conducted immediately after capture, when approximately fifteen Alewives were directly transferred to each of the three salinity treatments. Experiments were conducted in situ at Bride Lake in 150-l oval tanks filled with Bride Lake water. Tanks were immersed in the lake to maintain temperature. Landlocked Alewives were transported from their home environment to Bride Lake. To do this, we placed captive Alewives in covered oval tanks (150-l) filled with lake water and drove them immediately to the Bride Lake site (8 km on average). For consistency, anadromous Alewives were similarly transported after capture, but returned to Bride Lake. Treatment salinities were achieved by dissolving artificial sea salt (Instant Ocean, Spectrum Brands, Madison, WI) in water from Bride Lake. Tanks were aerated with battery-powered units for the duration of the experiment. Experimental tanks were checked for mortalities within the first 6–8 h after the start of salinity treatment, and then approximately every 12 h thereafter. Any dead fish found were immediately removed and measured for standard length (hereafter, length).

At the end of the 60-h treatment period, we euthanized remaining fish in 250 mg l^{-1} tricaine methanesulfonate (Argent, Redmond, WA, USA) and measured length. Immediately after euthanasia, blood was drawn from the caudal vessel with 1 ml heparinized hematocrit tubes and centrifuged at 3200*g* for 5 min. Plasma was removed and transferred to 0.5 mL tubes and stored at −80 °C. Plasma osmolality (i.e., total plasma ion concentration measured in mosmol kg^{-1}) was subsequently measured on a vapor pressure osmometer (Wescor Inc., Logan, Utah) using 8 µL of plasma and following the manufacturer's instructions. For fish in which <8 µL of plasma was collected, samples within a life history form and salinity treatment group were pooled (a total of 27 individuals were pooled). The mRNA expression by quantitative real-time polymerase chain reaction (qPCR, $n = 5$ per treatment per life history form) was performed on tissue from four gills arches from each side of the fish, placed directly in liquid nitrogen, and stored at −80 °C. The first right gill arch was excised from additional fish ($n = 8$ per treatment per life history form), placed immediately in 100 µl 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 measurement of Na^+/K^+-ATPase activity.

Na^+/K^+-ATPase activity and mRNA expression assays

Gill Na^+/K^+-ATPase activity (hereafter NKA activity) was determined by the microplate method outlined by McCormick (1993). Following this method, ouabain-sensitive ATPase activity was measured by coupling the production of ADP to NADH using lactic dehydrogenase and pyruvate kinase, in the presence and absence of 0.5 mmol l^{-1} ouabain. Homogenized gill samples were run in duplicate in 96-well microplates at 25 °C and read at a wavelength of 340 nm for 10 min on a THERMOmax microplate reader using SOFTmax software (Molecular Devices, Menlo Park, CA, USA). The total protein content of the homogenate was determined using a BCA (bicinchoninic acid) Protein Assay (Pierce, Rockford, IL, USA) in order to normalize NKA activity to the total amount of protein. Activity was calculated as the difference in ATP hydrolysis in the absence and presence of ouabain, expressed as µmol ADP mg $protein^{-1}$ h^{-1}.

Expression of candidate genes was measured by quantitative real-time PCR (qPCR). Total RNA for gene expression analysis was extracted from approximately 30 mg of gill tissue per sample using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Tissue was homogenized using a Kinematica Polytron PT 2100 bench top homogenizer (Kinematica, Inc, Bohemia, NY). We quantified RNA spectrophotometrically, assessed purity (260/280 > 1.8) and checked integrity on a 1 % agarose gel. Purified RNA was DNase treated using the TURBO DNA-free kit (Life Technologies, Grand Island, NY) as described by the manufacturer. First strand synthesis of cDNA for use in qPCR was achieved using

Table 3 Primer sequences (F: forward; R: reverse) for each candidate gene (*CFTR* and *NKCC*) and a reference gene (*EF1α*)

Gene	Primer sequence	Product size (bp)
CFTR	F: TTCCCTGACAAGCTGGACT R: GTGCAGGTGGAGAAGGAGTC	197
EF1α	F: GCTGGAAAATCGAGCGTAAG R: CACGGGTACGGTTCCAATAC	155
NKCC	F: ACCACCATTACTGGCGTCTC R: TACATGGCTACTGCCACAGC	158

Product size indicates the size of the pcr amplicon expressed in number of base pairs (bp)

Primers were designed from Alewife gill transcriptome sequence (J. Velotta, unpublished)

500 ng RNA and qScript reverse transcriptase (Quanta Biosciences, Gaithersburg, MD). Quantitative real-time PCR primers for *NKCC*, *CFTR* and elongation factor 1α (*EF1α*, reference gene) were designed using reads generated from the gill-specific transcriptome sequence of wild-caught juvenile Alewives (J. Velotta, unpublished). Primer sequences are reported in Table 3.

Target cDNAs were amplified in triplicate by qPCR using a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA) and PerfeCTa SYBR Green Fastmix (Quanta Biosciences). All qPCR reactions were performed using the following cycle conditions: 10 min at 95 °C, 45 cycles of 95 °C for 20 s and 59.5 °C for 50 s. Melt curve analysis was performed following each reaction to confirm that only a single product was produced. We arbitrarily selected a sample of gill tissue from a landlocked Alewife that was not subjected to a salinity challenge experiment to serve as a standard material, referred to as a calibrator sample. Standard curves derived from triplicate dilutions of calibrator samples yielded estimates of amplification efficiency (E), the ability of a primer set to double the target amplicon after each PCR cycle. E values for each primer set were close to the ideal value of 2 (EF1α: 1.94, NKCC: 1.95, CFTR: 1.98). Samples of the calibrator were included on each PCR plate in triplicate. Relative expression was calculated using the $\Delta\Delta C_T$ method (Pfaffl 2001),

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

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, 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. Note that the purpose of the calibrator C_T value is to account for variance arising from random differences in run conditions from plate to plate. The cDNA samples were loaded onto plates in sequential order by time and date of collection. A total of five plates were needed for each gene of interest.

Statistical analyses

We used survival analysis to determine differences in survivorship between life history forms (whether anadromous or landlocked) and salinity treatments. Length was included as a continuous covariate since it differed among life history forms and between trials (Table 1). Data were analyzed by fitting a non-parametric survival model (the Cox proportional hazards model) in R version 2.12.1. The Cox method models death rate as a log-linear function of predictors, where regression coefficients give the relative effect of covariates on survivorship (i.e., the proportion of individuals alive at a given time). The model computes a baseline hazard function (i.e., the instantaneous risk of death at baseline levels of covariates) that is modified multiplicatively by the covariates (Venables and Ripley 1999). Trial was used as a stratification variable, which permitted the calculation of separate baseline hazard functions for each case. We computed the hazard ratios (HR) for each parameter of the Cox model using maximum likelihood estimates in order to compare the hazard rates among treatments and life history forms. For discrete factors in the model (i.e., life history form and salinity) the HR represents the ratio of the hazard rates between a given category and the reference category (selected as anadromous for life history form and 30 ppt for salinity). For continuous covariates (i.e., length), the HR is the ratio of hazard rates for an increase of one unit of the variable.

We used linear mixed effects models to determine differences in mean NKA activity ($n = 70$ for anadromous; $n = 49$ for landlocked), log-transformed plasma osmolality ($n = 54$ for anadromous; $n = 36$ for landlocked), and log-transformed relative gene expression ($n = 27$ for anadromous; $n = 22$ for landlocked). Models included life history form and salinity treatment as fixed effects, with length as a covariate (log-transformed length was used for plasma osmolality and gene expression data). We included two random effects in our model: (1) trial (i.e., September, October or November trial month), and (2) tank (experimental tanks at each level of salinity treatment, life history form and trial were given a unique identifier). This experiment was intended to be a full factorial block design, where trial represents a random blocking variable, and tank represents a plot within a block. As random effects, both trial and tank were expected to influence the variance of the dependent variables; in a mixed effects model these random effects are accounted for in order to properly infer the impact of the fixed effects. We chose this approach over an alternative

approach treating trial dates as fixed treatments of interest. Although the latter approach would provide an opportunity to explore seasonal variation in osmoregulatory physiology, more dispersion in sampling dates would be required for an adequate test of seasonal influence. Models were run using the lmer function (*lme4* package) in R version 2.15.2 (R Core Team 2012). Significance testing of linear mixed effects models was conducted using the function pvals.fnc in R (*languageR* package). This function calculates p-values from a linear mixed model fit with the lmer function by generating confidence intervals from the posterior distribution of 10,000 parameter estimates obtained by Markov chain Monte Carlo simulations (MCMC randomization test). Full models included three-way interactions of life history form, salinity treatment and length, but were reduced where non-significant interactions ($p > 0.05$) were found. A table summarizing results of final linear mixed effects models (including MCMC upper and lower confidence limits and p values) is available in electronic supplemental material (Online Resource 1). In models where length was a significant ($p < 0.05$) factor, we estimated least-squares means (LSmeans; Searle et al. 1980) using the PROC GLM procedure in SAS version 9.3. This procedure calculates the mean of each factor at a mean common length. Differences between LSmeans for each factor were determined using a Student's t test. We analyzed two separate datasets for plasma osmolality values. The first dataset consisted of all samples including those pooled during measurement, but excluding length as a covariate. In the second dataset, we removed pooled samples in order to use length as a covariate in a linear mixed model. Blood samples for plasma osmolality and gill samples for real-time PCR were not obtained during the September trial and as such, analyses for these measures include data from October to November trials only. Eliminating the September trial data from the NKA activity dataset does not affect the findings.

Results

The average length of Alewives differed between life history forms and trials (Table 1), which was expected given that landlocked Alewives tend to be smaller overall at age (Scott and Crossman 1973), and that trials were run one month apart. An analysis of variance revealed a significant effect of life history form ($F_{1,228} = 135$; $p < 0.001$) and trial ($F_{2,228} = 483$; $p < 0.001$) on standard length. Survivorship of Alewives differed between salinity treatments and life history forms (Fig. 1). A Cox proportional hazards model revealed a significant effect of life history form ($z = 2.44$, $p = 0.01$), salinity ($z = 3.85$, $p < 0.001$) and length ($z = -3.83$, $p < 0.001$) on survivorship among all trials. Anadromous Alewives survived nearly all salinity treatments in each trial (the one exception being the 30 ppt treatment during the September trial in which there was 14 % mortality). In contrast, 10–40 % of landlocked Alewives died at each salinity treatment, and mortality rate was higher in seawater than in freshwater (Fig. 1). A Cox proportional hazards model run within landlocked Alewives revealed a significant effect of treatment at 30 ppt ($z = 3.34$, $p < 0.001$) as compared to 1 ppt. The effect of 20 ppt on survival compared to 1 ppt was non-significant ($z = 0.67$, $p = 0.5$). With respect to the main effect of life history form, the hazard ratio for anadromous Alewives was approximately one-fifth that of landlocked Alewives (HR = 0.17). For the main effect of salinity, separate hazard ratios were computed for 1 ppt (HR = 0.15) and 20 ppt (HR = 0.29). These ratios indicate that, compared to 30 ppt, hazard was reduced by 85 % and 71 % at 1 and 20 ppt, respectively. The estimated hazard ratio for standard length was HR = 0.87, i.e., for every 10 mm increase in length, the risk of death decreased by about 75 %.

We detected strong differences in plasma osmolality between life history forms and salinity treatments. Landlocked Alewives had higher plasma osmolality than anadromous Alewives in seawater treatments, and there was a positive effect of salinity on plasma osmolality (Fig. 2). Overall, seawater treatment resulted in higher osmolality among landlocked and anadromous Alewives (MCMC randomization test; $p = 0.002$) in a reduced linear mixed effects model where length was included as a covariate. The strength of the increase in plasma osmolality with salinity differed between landlocked and anadromous Alewives; we found a significant interaction between life history form and salinity treatment (MCMC randomization test; $p = 0.008$). The strongest between-life history form difference in plasma osmolality occurred in

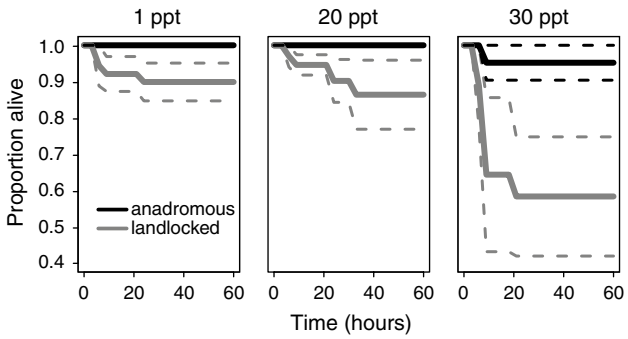

Fig. 1 Survival of Alewives for each of three salinity treatments: 1, 20, 30 ppt. *Solid lines* represent the mean proportion of anadromous (*black lines*) and landlocked (*gray lines*) Alewives alive over time. Treatments were checked every 12 h for mortality. *Dashed lines*are mean proportion alive ± standard error of the mean. There is a significant main effect of population ($p = 0.01$)

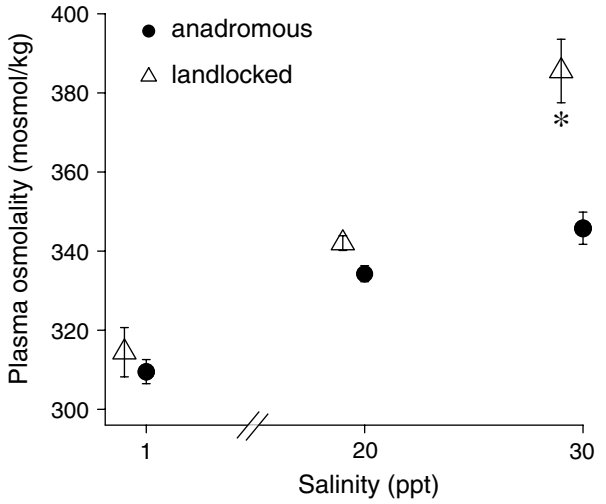

Fig. 2 Plasma osmolality of anadromous Alewives (*black circles*) at 1 ppt ($n = 15$), 20 ppt ($n = 25$), and 30 ppt ($n = 23$); and landlocked Alewives (*open triangles*) at 1 ppt ($n = 16$), 20 ppt ($n = 18$), and 30 ppt ($n = 12$). Values are mean plasma osmolality (mosmol kg^{-1}) ± standard error of the mean. *$p = 0.05$ for population effect at 30 ppt. Values represent plasma osmolality from pooled-sample dataset. Values were offset by 0.5 ppt for landlocked Alewives to eliminate overlap of data points

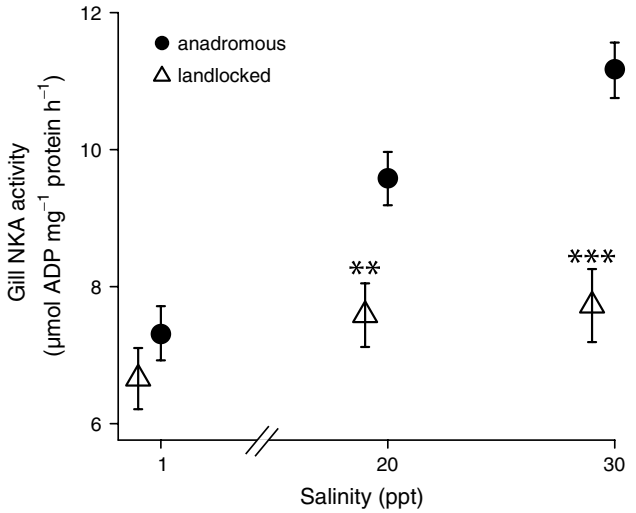

Fig. 3 Gill Na^+/K^+-ATPase activity of anadromous Alewives (*black circles*) at 1 ppt ($n = 23$), 20 ppt ($n = 24$), and 30 ppt ($n = 23$); and landlocked Alewives (*open triangles*) at 1 ppt ($n = 19$), 20 ppt ($n = 17$), and 30 ppt ($n = 13$). Values are LSmeans of NKA activity (µmol ADP mg protein^{-1} hour^{-1}) ± standard error of the mean. **$p = 0.002$, ***$p < 0.0001$ for population effect at 20 and 30 ppt, respectively. Values were offset by 0.5 ppt for landlocked Alewives to eliminate overlap of data points

Alewives treated at 30 ppt; on average, plasma osmolality of landlocked Alewives at 30 ppt was approximately 30 mosmol kg^{-1} (8 %) higher than anadromous Alewives. A within-treatment linear mixed effects model revealed a significant effect of life history form at 30 ppt (MCMC randomization test; $p = 0.05$). At 20 ppt, plasma osmolality among landlocked Alewives was 10 mosmol kg^{-1} higher (3 %) than anadromous Alewives (MCMC randomization test; $p = 0.06$), and only 2 mosmol kg^{-1} (0.6 %) higher at 1 ppt (MCMC randomization test; $p = 0.70$). Analyses that included pooled plasma osmolality samples yielded similar results regarding salinity and life history form effects; we detected a significant life history form by salinity interaction (MCMC randomization test; $p = 0.02$). The pooled osmolality data are plotted in Fig. 2 for completeness.

Gill Na^+/K^+-ATPase (NKA) activity increased with salinity treatment for landlocked and anadromous Alewives, but upregulation was weaker among landlocked individuals (Fig. 3). A reduced linear mixed effects model, including two-way interactions among salinity, life history form and length, revealed a significant interaction of life history form and salinity (MCMC randomization test; $p = 0.02$) as well as an interaction of length with salinity (MCMC randomization test; $p = 0.01$). We subsequently evaluated life history form differences in NKA activity separately in each of the three salinity treatments by running separate linear mixed effects models. NKA activity was significantly lower in landlocked Alewives compared to anadromous Alewives at 20 ppt (MCMC randomization test; $p = 0.04$) and 30 ppt (MCMC randomization test; $p = 0.002$). We found no significant differences in NKA activity between life history forms at 1 ppt (MCMC randomization test; $p = 0.30$). At 30 ppt, we also detected a significant main effect of length on NKA activity (MCMC randomization test; $p = 0.02$); NKA activity is negatively correlated with length ($r = -0.39$; $p = 0.02$). To account for the effect of length, we calculated values for NKA activity at a mean common length (LSmeans) for each level of life history form and salinity treatment (Fig. 3). The LSmeans NKA activity was lower among landlocked Alewives as compared to anadromous Alewives by approximately 20 % at 20 ppt (*t* test; $p = 0.002$) and 30 % at 30 ppt (*t* test; $p < 0.0001$). We detected no difference between life history forms at 1 ppt (*t* test; $p = 0.30$). These results are consistent with those from the linear mixed effects models.

Landlocked and anadromous Alewives differed in expression of seawater osmoregulation genes *NKCC* and *CFTR* at the gill. Expression of *CFTR* varied with life history form and salinity (Fig. 4). A reduced linear mixed effects model, including only main effects of salinity, life history form and length, revealed that *CFTR* mRNA expression increased significantly with salinity exposure (MCMC randomization test; $p = 0.03$) and that mRNA expression was significantly reduced in landlocked Alewives compared to anadromous Alewives (MCMC randomization test; $p = 0.008$).

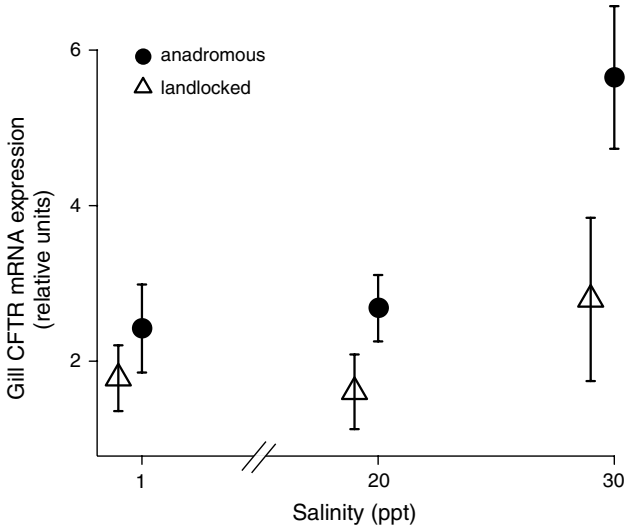

Fig. 4 Relative gill mRNA expression of *CFTR* of anadromous Alewives (*black circles*) at 1 ppt ($n = 8$), 20 ppt ($n = 9$), and 30 ppt ($n = 10$); and landlocked Alewives (*open triangles*) at 1 ppt ($n = 9$), 20 ppt ($n = 8$), and 30 ppt ($n = 5$). Values are mean relative units (expression value relative to *EF1α*) ± standard error of the mean. There is a significant main effect of population ($p = 0.008$). Values were offset by 0.5 ppt for landlocked Alewives to eliminate overlap of data points

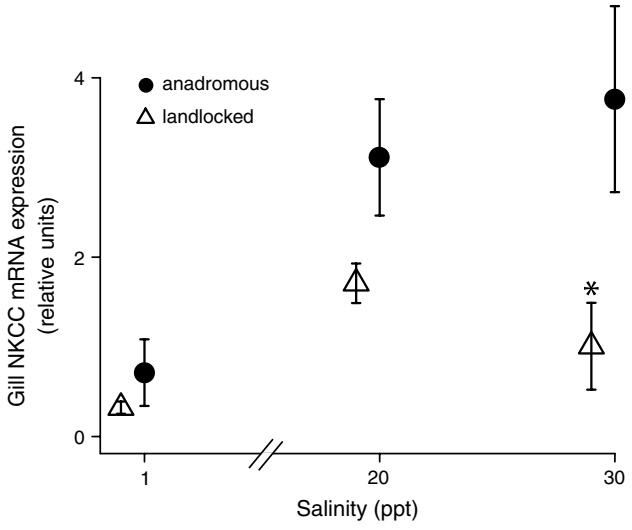

Fig. 5 Relative gill mRNA expression of *NKCC* of anadromous Alewives (*black circles*) at 1 ppt ($n = 7$), 20 ppt ($n = 9$), and 30 ppt ($n = 10$); and landlocked Alewives (*open triangles*) at 1 ppt ($n = 9$), 20 ppt ($n = 8$), and 30 ppt ($n = 5$). Values are mean relative units (expression value relative to *EF1α*) ± standard error of the mean. *$p = 0.04$ for population effect at 30 ppt. Values were offset by 0.5 ppt for landlocked Alewives to eliminate overlap of data points

Although anadromous Alewives had higher *CFTR* expression across all salinity treatments, between-life history form differences in *CFTR* expression were highest at 30 ppt (twofold difference between life history forms; Fig. 4). Expression of *NKCC* also varied with life history form and salinity (Fig. 5). A linear mixed effects model revealed a significant three-way interaction of life history form, salinity and length (MCMC randomization test; $p = 0.04$). Because this three-way interaction is difficult to interpret, we ran linear mixed effects models separately for each salinity treatment. We found that *NKCC* expression was fourfold greater among anadromous Alewives at 30 ppt (MCMC randomization test; $p = 0.04$), and over twofold greater at 20 ppt (though this effect was non-significant: MCMC randomization test; $p = 0.11$). There were no significant differences in *NKCC* expression between life history forms at 1 ppt (MCMC randomization test; $p = 0.12$).

Discussion

Ecological transitions from marine to freshwater environments involve the elimination of seawater as a source of selection (relaxed selection; Lahti et al. 2009). Since relaxed selection is thought to weaken or eliminate trait expression, we predicted that hypo-osmoregulatory function, and the molecular machinery that underlies it, would be reduced in freshwater-restricted, landlocked Alewives, compared to their seawater-migrating anadromous ancestor. We found that permanent freshwater residency in Alewives results in significant reductions in seawater survival and hypo-osmoregulatory capacity, and a weaker response of multiple molecular pathways that drive seawater osmoregulation.

As a model for studying the evolution of osmoregulatory function and associated mechanisms, Alewives are distinct from other species studied previously in several important respects. Alewives are in a predominantly marine family (Nelson 2006). In contrast to freshwater populations of *Fundulus spp.*, landlocked Alewife populations clearly arose as a result of multiple independent isolating events, and there is little or no gene flow of anadromous genotypes into landlocked populations (Palkovacs et al. 2008); this system is, therefore, ideal for testing parallel evolutionary change. Landlocked populations were founded recently (300–500 year ago; Palkovacs et al. 2008) compared to most landlocked populations of Threespine Stickleback (circa 10,000 years ago; Bell and Foster 1994), allowing us to test whether the osmoregulatory system can evolve on shorter time scales than has been previously established. Comparison of Alewives to sticklebacks, killifishes, and salmonids, provide an opportunity to examine whether freshwater restriction results in convergent changes to osmoregulatory function. Given their ancestry and unique life history, landlocked Alewives are ideal for examining the outcome of relaxed selection on hypo-osmoregulation.

Juvenile anadromous Alewives were more tolerant of all salinities than landlocked Alewife juveniles, and in

particular, there was a pronounced difference in survivorship between life history forms challenged at 30 ppt seawater (Fig. 1). Survival of anadromous Alewives at all salinity treatments was high (no more than 14 % mortality at 30 ppt, and no deaths at 1 or 20 ppt). The broad tolerance of anadromous Alewives was expected given their life history. Survival differences between life history forms were the least pronounced in 1 ppt freshwater (an 11 % difference on average), slightly greater in 20 ppt seawater (a 17 % difference on average), and dramatically different in 30 ppt seawater (a 40 % difference on average; Fig. 1). We did not expect to find life history form differences in tolerance at 1 ppt since both landlocked and anadromous individuals inhabit freshwater as juveniles. Lowered survival in 1 ppt among landlocked Alewives may be caused by a higher sensitivity to the stress of handling (J. Velotta, personal observation), rather than a true reduction in tolerance of 1 ppt. Regardless, the strong effect of 30 ppt treatment on landlocked Alewife survival, as well as prominent life history form differences at this salinity, indicate that landlocked Alewives have a reduced ability to tolerate seawater compare to anadromous Alewives. This evolutionary shift may be caused in part by changes to the physiological and molecular mechanisms that regulate ion secretion in seawater.

Reduced seawater survival in landlocked Alewives is consistent with findings in landlocked life history forms of salmonids (e.g., Arctic Char: Staurnes et al. 1992) and of species with marine ancestry (e.g., Threespine Stickleback: McCairns and Bernatchez 2010; killifish: Scott and Schulte 2005). Interspecific differences in seawater tolerance have also been observed, particularly among closely related species of killifishes. In the genus *Lucania*, the stenohaline freshwater species *L. goodei* survives considerably less well in seawater than its euryhaline congener *L. parva* (Fuller 2008). Whitehead (2010) examined 23 species of *Fundulus* and demonstrated that each of five independent transitions into freshwater has resulted in a significant loss of salinity tolerance. Taken together, evolution of reduced seawater tolerance accompanying adaptation to an entirely freshwater environment appears to be common among highly divergent groups of fishes, both intra-specifically and inter-specifically, and may, therefore, represent a ubiquitous evolutionary consequence of ecological transitions to freshwater.

There are several physiological explanations for the observed differences in hypo-osmoregulatory capacity between landlocked and anadromous Alewives. One possibility is that landlocked Alewives experience reductions in osmosensing, a process involving the detection of osmotic changes, which activates ion transport processes that restore homeostasis (Evans 2010). Another possible (though not mutually exclusive) explanation is that land-locking has resulted in reduced ion secretory capacity or control over ion permeability. Reductions in ion secretory capacity are likely to be the result of changes in the function of ion transporters in gill ionocytes, which are equipped with a suite of well-characterized ion transport proteins (Evans et al. 2005). We chose to analyze life history form differences in several ion transporters involved in seawater osmoregulation as a way of assessing changes in the mechanisms that promote ion secretion at the gills. Although we did not attempt to assess differences in osmosensing between life history forms, future work should be aimed at establishing whether landlocked Alewives show reduced osmosensing capabilities in seawater.

We found that Na^+/K^+-ATPase activity increased with seawater exposure among anadromous Alewives (Fig. 3). This result corresponds with previous studies of anadromous Alewives that demonstrated that NKA activity increased by 75 % after long-term seawater acclimation (Christensen et al. 2012; McCormick et al. 1997). Upregulation of NKA activity in seawater has also been reported in American Shad (a congener of the alewife; Zydlewski and McCormick 1997b). In our study, upregulation of NKA activity with salinity was dramatically reduced in landlocked Alewives (Fig. 3), which may, at least in part, account for their reduced hypo-osmoregulatory capacity. Such reductions in upregulation of NKA activity among freshwater-adapted populations is consistent with that found in the copepod *Eurytemora affinis* by Lee et al. (2011), and the killifish by Scott and Schulte (2005).

We found a negative correlation between gill NKA activity and fish length, which was unexpected. Ion flux rates may be greater for smaller fish and it is possible that higher gill NKA activity is due to higher demand for active ion uptake in smaller individuals. Alternatively, there may be size-dependent developmental differences in gill NKA activity related to the acquisition of salinity tolerance and subsequent migration. However, since migrating anadromous individuals are generally larger than non-migrants (Gahagan et al. 2010), we would have expected a positive (rather than negative) relationship with size and NKA activity, which is upregulated prior to seawater migration in other alosines (Zydlewski and McCormick 1997a). Regardless, when we calculate NKA activity at a mean common length (LSmeans), we find consistent patterns as with linear mixed effects models, indicating that differences in fish length is not the likely driver of reduced NKA activity among landlocked Alewives.

Among anadromous and landlocked Alewives, seawater exposure resulted in upregulation of *NKCC* and *CFTR* mRNA (Figs. 4, 5), which is consistent with these transporters' roles in ion secretion (Evans et al. 2005). *NKCC* and *CFTR* expression among landlocked Alewives, however, showed a weaker response to seawater relative to expression

by anadromous Alewives (Figs. 4, 5). Land-locking, therefore, appears to have resulted in reduced responsiveness of hypo-osmoregulatory pathways to seawater, which is consistent with findings in freshwater adapted populations of the killifish *Fundulus heteroclitus* (Scott and Schulte 2005). Recent work has identified interspecific changes in seawater function that mirror the intraspecific changes found here. *NKA* and *NKCC* mRNA is expressed at lower levels in the stenohaline freshwater *L. goodei* compared to its euryhaline congener, *L. parva,* when fish are transferred to seawater (Berdan and Fuller 2012). Currently, we have no evidence to suggest that landlocked and anadromous life history forms differ constitutively in hypo-osmoregulatory function. Our results strongly suggest that the physiological plasticity associated with the response to seawater challenge (i.e., the upregulation of NKA activity, *NKCC* and *CFTR* expression in seawater) has been reduced in landlocked forms. Constitutive expression differences between landlocked an anadromous forms of fish have been explored previously (in Atlantic salmon; Nilsen et al. 2007), and future studies will address this issue in Alewives.

A weaker response of the physiological pathways involved in hypo-osmoregulation among landlocked Alewives likely accounts for reduced ion secretory capabilities in seawater, and may have contributed to their higher mortality relative to anadromous counterparts. NKA, NKCC and CFTR allow for the secretion of excess Na^+ and Cl^- out of gill ionocytes in hypo-osmoregulating fishes. Na^+/K^+-ATPase is the primary driving force for ion secretion at the gill; it establishes a strong electrochemical gradient by maintaining low intracellular Na^+ levels and keeping ionocytes negatively charged. NKCC co-transports Na^+, K^+ and Cl^- into the cell, and Cl^- is then secreted through CFTR, an apical ion channel. Na^+ ions are subsequently secreted paracellularly through shallow tight junctions between ionocytes and accessory cells (Evans et al. 2005; Hwang and Lee 2007; Marshall and Grosell 2006). Lowered activity of NKA and expression of *NKCC* and *CFTR* in landlocked Alewives likely reduces Na^+ and Cl^- secretion at gill ionocytes, which may account for the observed reductions in hypo-osmoregulatory capacity. In particular, NKCC and CFTR are the primary ion transporters by which Cl^- is secreted at the gill, and the same upstream transcription factors and/or hormones may control their expression.

Evolutionary changes in seawater survival, hypo-osmoregulatory capacity and the expression and activity of pathways involved in ion secretion among landlocked Alewives may be the result of relaxed selection, since the presumed source of selection for hypo-osmoregulatory function (i.e., seawater) has been eliminated from their life cycle. Traits that regulate hypo-osmoregulatory function may bear constitutive maintenance costs even in freshwater environments where they are not being expressed. Trait loss is predicted to occur rapidly where constitutive costs are high since they would reduce fitness (Lahti et al. 2009). Trait decay would proceed more slowly (or not at all) when positively correlated with other functional traits or if there were no energetic costs to maintaining hypo-osmoregulatory function in the freshwater environment. Given that the time since divergence from the anadromous ancestor is short (circa 300–500 years), and that we have demonstrated significant differences in survival and osmoregulatory function in seawater, it is likely that hypo-osmoregulatory function bears a high maintenance cost in Alewives, and that natural selection is acting to reduce or eliminate it in landlocked forms. Rapid evolutionary reductions in seawater survival and NKA activity have been observed previously in the euryhaline copepod *Eurytemora affinis* following invasion into freshwater (Lee et al. 2003, 2007, 2011). To our knowledge, the decay of hypo-osmoregulatory function in landlocked Alewives presented here is the most rapid of such declines in a marine derived fish documented to date (e.g., several hundred years compared to approximately 10,000 years in threespine stickleback; Bell and Foster 1994). Convergent patterns of reduced hypo-osmoregulatory function and changes to salt-secreting pathways among freshwater forms of salmonids (e.g., Nilsen et al. 2007), killifish (e.g., Scott and Schulte 2005), Threespine Stickleback (e.g., McCairns and Bernatchez 2010), and now Alewives, suggests that such changes represent important adaptations to ecological movement into freshwater and are ubiquitous consequences of relaxed selection on seawater function.

The tempo and mode of evolutionary change in hypo-osmoregulation may differ among independently derived landlocked Alewife populations, particularly if they differ in time since divergence from the anadromous ancestor. The possibility of among-landlocked population differences is interesting and will be the subject of future experimentation, but could not be adequately addressed with the current study design. It is also possible that the life history form divergence in hypo-osmoregulatory function we observe is the result of environmental effects on osmoregulatory phenotypes acting on the young Alewives or through maternal effects. One way to eliminate such effects is to breed and rear animals from different life history forms in a common laboratory environment (a common-environment experiment sensu Conover and Schultz 1995). Since the lakes in this study do not differ in salinity regime, and since no individuals from either life history form experienced seawater prior to testing, environmental effects that would have caused divergence in salinity tolerance or osmoregulatory capacity are likely to be minimal or non-existent.

An alternative, but not mutually exclusive, explanation is that reductions in seawater function among landlocked Alewives may be caused by selection favoring enhanced

freshwater performance. Selection for improved ion uptake in freshwater may increase fitness in landlocked forms, especially by enhancing survival in winter months. In anadromous Alewives, cold freshwater induces mortality and reduces plasma and muscle sodium levels relative to fish at warmer temperatures (McCormick et al. 1997; Stanley and Colby 1971). Some freshwater populations of euryhaline species have higher survival in freshwater (Lee et al. 2003, 2007) and better regulation of plasma ions (Whitehead et al. 2011) during freshwater challenge relative to seawater or brackish populations of the same species. These studies have also identified several ion transporters under selection in freshwater (e.g., V-type H + -ATPase: Lee et al. 2011). Freshwater challenges in killifish indicate that enhanced freshwater tolerance among freshwater-associated populations corresponds with improved plasma Cl^- regulation (Scott et al. 2004; Whitehead et al. 2012). If there is a physiological tradeoff associated with osmoregulatory function in different salinity environments, enhanced osmoregulatory function in freshwater could lead to reduced function in seawater. In particular, selection for improved plasma Cl^- regulation in freshwater may trade-off against Cl^- secretion in seawater, which may explain the strong reductions in transcription of *NKCC* and *CFTR* (the main Cl^- transporters in seawater ionocytes of the gill). It will be of value to determine ion uptake capacity in landlocked and anadromous Alewives in order to elucidate the effects of evolutionary changes in freshwater capacity as a result of land-locking.

In summary, ecological transitions into freshwater have led to substantial reductions in seawater tolerance and hypo-osmoregulatory capacity in landlocked Alewives, which are likely driven by changes in the molecular machinery that regulate ion secretion, namely the reduced response of NKA, CFTR and NKCC to seawater. Because fish in landlocked lakes no longer need to function in seawater, we interpret these changes as the result of relaxed selection on hypo-osmoregulatory function. The present study represents a novel combination of findings and a greater integration of the molecular, physiological and whole-organism level responses to seawater than has been conducted previously. This work also confirms that Connecticut Alewives can serve as a model system to explore the evolution of osmoregulatory function after adaptation to a fully freshwater life cycle. Although our targeted approach has identified specific ion transporters that may be subject to evolutionary change associated with freshwater transitions, more research is needed on other important osmoregulatory effectors in seawater (e.g., aquaporins) and freshwater (e.g., Na^+/H^+ exchanger, Na^+/Cl^- cotransporter, V-type H^+-ATPase) in order to provide insight into the suite of evolutionary changes associated with movement in freshwater.

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