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PUMPING IONS: RAPID PARALLEL EVOLUTION OF IONIC REGULATION FOLLOWING HABITAT INVASIONS

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Marine to freshwater colonizations constitute among the most dramatic evolutionary transitions in the history of life. This study examined evolution of ionic regulation following saline-to-freshwater transitions in an invasive species. In recent years, the copepod *Eurytemora affinis* has invaded freshwater habitats multiple times independently. We found parallel evolutionary shifts in ion-motive enzyme activity (V-type H^+ ATPase, Na^+/K^+-ATPase) across independent invasions and in replicate laboratory selection experiments. Freshwater populations exhibited increased V-type H^+ ATPase activity in fresh water (0 PSU) and declines at higher salinity (15 PSU) relative to saline populations. This shift represented marked evolutionary increases in plasticity. In contrast, freshwater populations displayed reduced Na^+/K^+-ATPase activity across all salinities. Most notably, modifying salinity alone during laboratory selection experiments recapitulated the evolutionary shifts in V-type H^+ ATPase activity observed in nature. Maternal and embryonic acclimation could not account for the observed shifts in enzyme activity. V-type H^+ ATPase function has been hypothesized to be critical for freshwater and terrestrial adaptations, but evolution of this enzyme function had not been previously demonstrated in the context of habitat transitions. Moreover, the speed of these evolutionary shifts was remarkable, within a few generations in the laboratory and a few decades in the wild.

KEY WORDS: **Adaptation, colonization, gene expression, invasive species, ion transport, Na^+/K^+-ATPase, osmoregulation, phenotypic plasticity, V-type H^+ ATPase.**

Colonization from marine to freshwater habitats constitutes among the most dramatic evolutionary transitions in the history of life. Relatively few animal taxa have breached these habitat boundaries, as they impose serious challenges for ionic regulation (Hutchinson 1957; Little 1983, 1990; Lee and Bell 1999; Morris 2001; Miller and Labandeira 2002). Life in fresh water requires the ability to acquire essential ions from dilute environments. The evolution of ion regulatory mechanisms in fresh water has been thought to often provide stepping stones for the colonization of land, where conservation of water and ions become important (e.g., through the regulation of urine concentration) (Wolcott 1992; Morris 2001; Glenner et al. 2006). Such regulatory functions are energetically driven by ion-motive ATPase enzymes, which are ubiquitous across all three domains of life (bacteria, archaea, and eukarya) (Gogarten and Starke 1992; Palmgren and Axelsen 1998). Of these enzymes, evolution of function of V-type H^+ ATPase and Na^+/K^+-ATPase has been hypothesized to be critical for the colonization of fresh water and land for many taxa (Beyenbach 2001; Morris 2001; Tsai and Lin 2007).

In recent years, movement across biogeographic boundaries has increased in prevalence with the rapid spread of invasive species. For example, many species invading freshwater habitats have originated from more saline environments,

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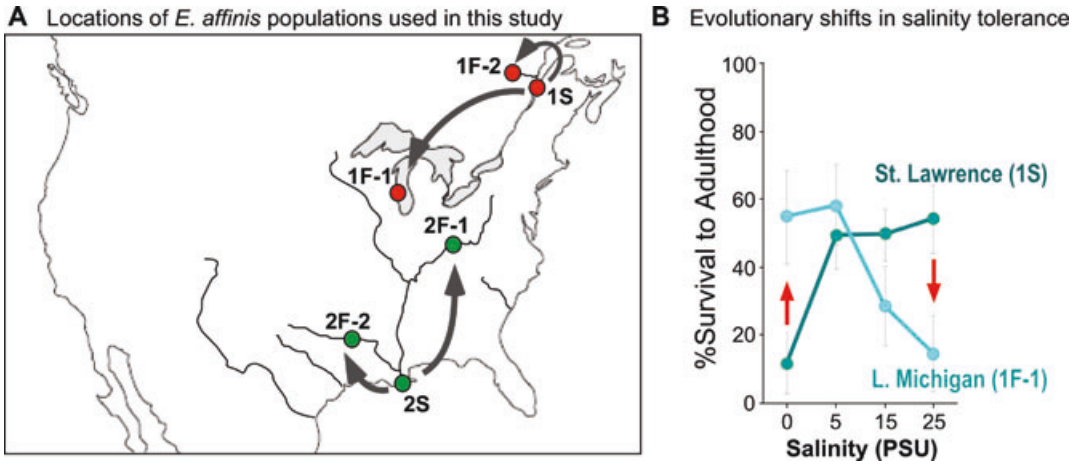

Figure 1. **(A) Population sampling for this study. Arrows show independent invasions from saline to freshwater habitats for two genetically distinct clades of *E. affinis* (Atlantic, Gulf). The invasions occurred within the past ~70 years (Lee 1999). Populations 1F-2 and 2F-2 were used for gene expression analysis only (see Supporting Information).**

Clade	Saline	Freshwater
Atlantic (red)	(1S) St. Lawrence	(1F-1) Lake Michigan, WI, USA
		(1F-2) Lac Saint Jean, PQ, Canada
Gulf (green)	(2S) Gulf of Mexico	(2F-1) Louisville, KY, USA
		(2F-2) Lake Texoma, OK, USA

(B) Background information: Evolutionary shifts in salinity tolerance following an invasion from saline (St. Lawrence) to freshwater (Lake Michigan) habitats, based on data from Lee et al. (2003). Arrows represent shifts in physiological tolerance between the populations. Data shown are mean %survival to adulthood ± SE for eight clutches.

disproportionate to their rate of transport (De Beaufort 1954; Miller 1958; Mordukhai-Boltovskoi 1979; Jazdzewski 1980; Taylor and Harris 1986; Lee 1999; Lee and Bell 1999; Ricciardi and MacIsaac 2000; May et al. 2006). Such invasions are remarkable, as saline and freshwater invertebrates are typically separated by a biogeographic boundary (of ~5 PSU, practical salinity units ≈ parts per thousand, 150 mOsm/kg), across which most species are physiologically unable to cross (Khlebovich and Abramova 2000). The species that have been able to invade freshwater habitats appear to face serious physiological challenges and high energetic costs (Taylor and Harris 1986; Dietz et al. 1996; Lee et al. 2003). These recent invasions provide valuable opportunities to study physiological evolution between populations separated by minimal evolutionary distance. How are these invaders able to survive radical habitat shifts? Might common mechanisms govern the ability to invade fresh water from more saline sources?

In a notable example, the coastal copepod *Eurytemora affinis* has made remarkably rapid and frequent saline to freshwater transitions within the past century. This copepod has invaded freshwater habitats multiple times independently with the advent of ballast water shipping and discharge into freshwater lakes (Lee 1999) (examples shown in Fig. 1A). During these invasions, *E. affinis* experienced heritable physiological shifts, with increases in freshwater tolerance and reduced saltwater tolerance (Fig. 1B; Lee et al. 2003, 2007). Which particular mechanisms might underlie the evolutionary changes in freshwater tolerance?

Given the requirements for ion uptake in dilute environments, saline to freshwater invasions are likely to involve evolution of ion transport function. In all cells, ion transport enzymes are responsible for regulating ionic concentrations across the cell membrane (Fig. 2). Until relatively recently, Na^+/K^+-ATPase (Na/K-ATPase) was thought to be the principal driving force for ion uptake from the environment (Lucu and Towle 2003; Kirschner 2004). Na/K-ATPase is often located on the basolateral side of epithelial cells, transporting Na^+ from the epithelial cell to the blood (hemolymph) (Fig. 2A; Towle and Kays 1986). However, Na/K-ATPase alone cannot provide the driving force for Na^+ uptake in dilute media. An environmental concentration below ~1.0 mM NaCl creates a gradient with body fluids that exceeds the thermodynamic limit for ion uptake by Na/K-ATPase alone (Larsen et al. 1996). Although Na/K-ATPase function would be insufficient for ion uptake from dilute environments, this enzyme might still constitute a critical step for ion uptake in saline habitats (Fig. 2A).

In freshwater habitats, the function of vacuolar-type H^+ ATPase (V-ATPase) might be critical for ion uptake from dilute media (Fig. 2B). Increasingly, localization and activity of V-ATPase has been considered to be crucial for the transport of ions against

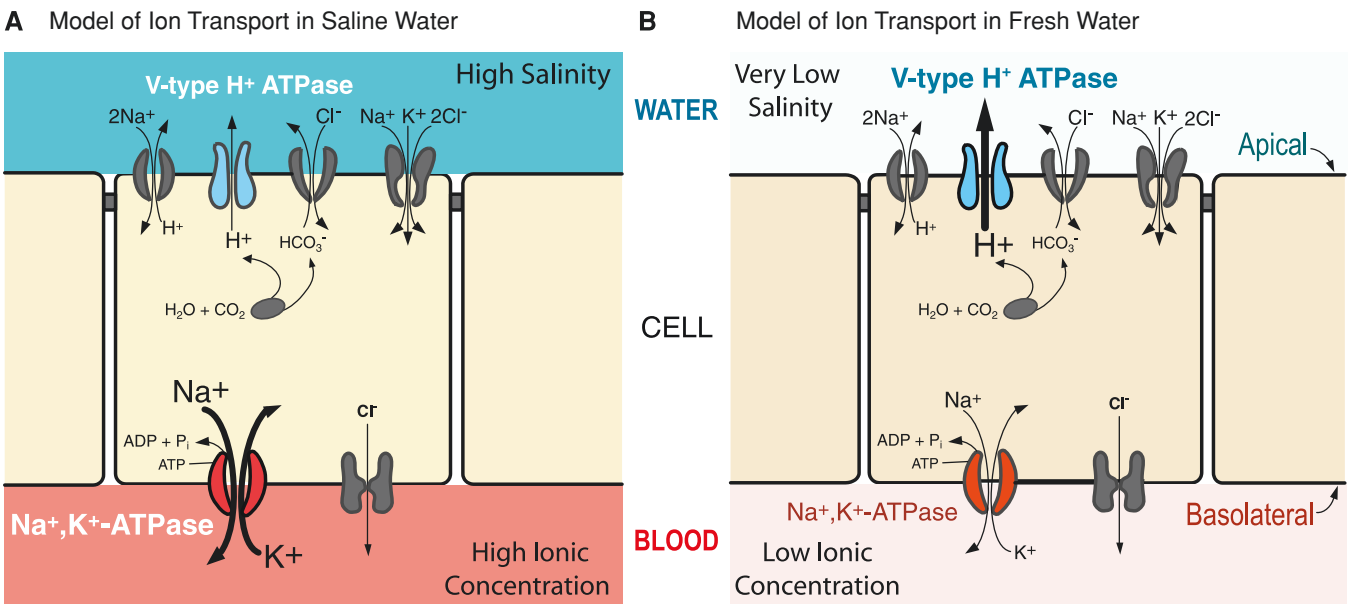

Figure 2. **Hypothesized model of ion uptake across the epithelial tissue showing a subset of putative ion transporters, based on a model for decapod crustaceans (Towle and Weihrauch 2001). Several enzymes are responsible for ionic regulation in crustaceans, including Na^+/K^+-ATPase, V-type H^+ ATPase, carbonic anhydrase, Cl^-/HCO_3^- exchanger, $Na^+/K^+/2Cl^-$ cotransporter, and Na^+/H^+ exchanger. (A) In saline habitats, ionic concentration of the ambient fluid is high relative to that of the epithelial cell (~300 mOsm/kg), so that Na^+ could simply diffuse into the cell. Thus, the limiting step for ion uptake is across the basolateral membrane, where Na^+/K^+-ATPase (red) transports Na^+ from the cell into the hemolymph (blood). Ion transport from the cell to the hemolymph is energetically more costly for invertebrates in saline than in freshwater habitats because ionic concentration of the hemolymph tends to be elevated in high salinity environments. (B) In freshwater habitats, ionic concentration of the ambient fluid is extremely low, such that ions must be transported into the cell against a steep concentration gradient. The limiting step for ion uptake is from the water into the cell, across the apical membrane, where V-type H^+ ATPase (blue) energizes the transmembrane electrochemical potential by pumping H^+ out of the cell. This proton gradient then drives the transport of Na^+ into the cell via other transporters. Ion transport from the cell to the hemolymph is less energetically costly than in saline habitats because ionic concentration of the hemolymph is relatively low.**

steep concentration gradients for many species (Ehrenfeld and Klein 1997; Weihrauch et al. 2004; Patrick et al. 2006; Tsai and Lin 2007). V-ATPase generates a H^+ gradient across the apical membrane, which enables cations (such as Na^+) to be transported into the cell via other transporters (Fig. 2B; Ehrenfeld and Klein 1997; Wieczorek et al. 1999; Beyenbach and Wieczorek 2006). Despite its hypothesized importance for ion uptake from dilute environments, no study has demonstrated the evolution of V-ATPase function in the context of habitat shifts.

In this study, we examined V-ATPase and Na/K-ATPase activity in the context of rapid evolutionary change during invasions from saline into freshwater habitats by the copepod *E. affinis*. Our goals were to (1) determine whether ion-motive ATPase activity could evolve in response to salinity change, during natural invasions and in laboratory selection experiments, (2) confirm whether shifts in ATPase activity during freshwater invasions result from selection rather than merely from acclimation, and (3) observe whether parallel evolutionary changes take place during independent invasions in the wild and in selection experiments in the laboratory. We predicted that under freshwater conditions V-ATPase activity would be elevated in the freshwater populations relative to their saline ancestors, to absorb ions from very dilute environments (Fig. 2B). We also predicted higher Na/K-ATPase activity in saline than in freshwater populations, as Na/K-ATPase might play a more important role for ion uptake in saline environments (Fig. 2A).

In addition, according to Lande's model (2009) and Waddington's argument (1953), the evolution of plasticity could accelerate adaptation during extraordinary environmental change. The evolution of plasticity could arise via selection favoring the more extreme phenotype in the novel environment (resulting in steeper reaction norm slope). Such a process would enable invasive populations to survive radical habitat shifts and avoid extinction (Lande 2009). Given this model, our final goal was to (4) examine whether the evolution of increased plasticity was associated with freshwater invasions. We predicted that freshwater populations would exhibit greater plasticity in V-ATPase activity (greater reaction norm slope) relative to their saline ancestors.

As noted above, the evolution of V-ATPase function in the context of habitat shifts has not been demonstrated previously.

In the case of Na/K-ATPase, some comparative studies have examined its function across more ancient habitat transitions (Scott et al. 2004; Brooks and Mills 2006; Bystriansky et al. 2006; Hiroi and McCormick 2007; Nilsen et al. 2007; Tsai and Lin 2007; Kang et al. 2008; McCairns and Bernatchez 2010). But, this study examined evolution of function for both enzymes across very recent invasions, avoiding the potential confounding effects of additional extraneous evolutionary changes that would separate more distantly related taxa or populations. Most notably, this study represents the most explicit test of evolution of ion-motive ATPase activity in response to a single variable (salinity) through laboratory selection experiments. Few studies have used laboratory selection to confirm the evolutionary lability of a critical trait limiting habitat invasions. Parallel evolution in the laboratory and field would suggest labile evolutionary mechanisms that could enable rapid adaptation during these extraordinary habitat shifts.

Materials and Methods

POPULATION SAMPLING

Saline and freshwater populations from two genetically distinct clades (Fig. 1A; Atlantic, Gulf) were collected within 2006–2008. A saline population from the Atlantic clade was collected from Baie de L'Isle Verte salt marsh pond, Quebec, Canada (Fig. 1A, site 1S; 48°00′14″N, 69°25′31″W) adjacent to the St. Lawrence estuary at a salinity of 15 PSU. Populations here are typically found at a salinity range of 5–40 PSU (Lee 1999). A saline population from the Gulf clade was collected from Blue Hammock Bayou, Fourleague Bay, LA, USA (Fig. 1A, site 2S; 29°17′18″N, 91°6′59″W) at a salinity of 5 PSU. *Eurytemora affinis* is found at this location at salinities ranging 2–15 PSU.

A freshwater invading population from the Atlantic clade was collected from Lake Michigan at Racine Harbor, WI, USA (Fig. 1A, site 1F-1; 42°43′46″N, 87°46′44″W). Conductivity, which is a more sensitive measure at lower salinities, was in the range of 300 μS/cm (0 PSU). The copepod *E. affinis* first invaded the Great Lakes in 1958 (Anderson and Clayton 1959), approximately 200–300 generations ago (∼4–5 generations/year; seasonality June-October). A freshwater population from the Gulf clade was collected from McAlpine pool (reservoir) on the Ohio River in Louisville, KY, USA (Fig. 1A, site 2F-1; 38°15′36″N, 85°45′00″W). The occurrence of *E. affinis* in this location was first reported in 1985 (Bowman and Lewis 1989), approximately 70–100 generations ago (∼3 generations/year; seasonality July-September). Conductivity of the Ohio River at Louisville was measured at 338 μS/cm (0 PSU) (Westerhoff et al. 2005).

The Atlantic and Gulf clades constitute separate sibling species on independent evolutionary trajectories, as they are genetically divergent (∼13% at COI) and show evidence of reproductive isolation (Lee 2000; Lee et al. unpubl. ms.). Therefore, shared genetic mechanisms underlying freshwater adaptation in the two clades would represent labile mechanisms evolving independently and in parallel in each clade. The usage of "fresh water" as a noun and "freshwater" as an adjective is adopted throughout this article.

POPULATION COMPARISONS OF ION-MOTIVE ATPASE ACTIVITY

All populations were reared in the laboratory for at least two generations prior to experimentation. Freshwater populations were maintained in fresh water from Lake Michigan (0 PSU, ∼7 mOsm/kg; conductivity of 300 μS/cm) collected at Racine Harbor, WI, USA and filtered with a 0.2-μm filter to remove bacteria. Saline populations were maintained at a salinity of 15 PSU (450 mOsm/kg) made from mixtures of filtered Lake Michigan water and Instant Ocean®. All populations were maintained at 13°C except for the population from McAlpine pool (Louisville) reservoir (Fig. 1, site 2F-1), which could not tolerate 13°C and was maintained at 15°C. Saline populations were fed the saline cryptophyte alga *Rhodomonas salina,* whereas freshwater populations were fed the freshwater congener *R. minuta.* Cultures were treated approximately weekly with 20 mg/L of the antibiotic Primaxin® to prevent infection.

Saline versus freshwater populations

To determine evolutionary shifts in ion-uptake function following freshwater invasions, we compared V-ATPase and Na/K-ATPase activity in ancestral saline populations (St. Lawrence, Gulf) relative to freshwater derived populations (Lake Michigan, Louisville reservoir) (Fig. 1A, 1S vs. 1F-1 and 2S vs. 2F-1). We examined shifts in ATPase activity across independent invasions in two genetically distinct clades (Fig. 1A; Atlantic, Gulf clades).

We found in previous experiments that rearing the populations at a common salinity for multiple generations prior to the enzyme assays imposes selection on both saline and freshwater populations and alters their physiological tolerances (Lee et al. 2007). Thus, we reared the populations close to their native salinities (saline population at 15 PSU, freshwater population at 0 PSU) to avoid effects of selection. We then placed approximately 200 gravid females from each population for 2 days at a common salinity of 5 PSU to reduce effects of acclimation to native salinities. We then removed the egg sacs from the females and placed them at the treatment salinities (0, 5, 15 PSU). Under these conditions, we were concerned that maternal environmental effects might have remained (due to the salinity at which the mother was reared and produced the eggs). Thus, we performed a separate experiment (see below) to determine whether maternal and embryonic developmental acclimation could affect enzyme activity.

Selection for freshwater tolerance

To examine whether the evolutionary shifts found in nature could be replicated in the laboratory, we compared V-ATPase activity of saline populations versus saline populations selected for freshwater tolerance. We created freshwater-selected lines by transferring the saline populations (Fig. 1A, St. Lawrence [1S] and Gulf [2S]) to declining salinities over a period of seven generations (~five months), and then maintaining the populations at 0 PSU for an additional four to five generations (for a total of 11–12 generations of selection).

The saline populations were maintained at 15 PSU in the laboratory for at least four generations prior to selection, and then subjected to declining salinities for a period of five months (seven to eight generations). For each saline population, a starting population of 400 adult copepods (200 male and 200 females) were initially transferred from 15 PSU to 5 PSU, and then to declining salinities of 1 PSU, 0.1 PSU, 0.05 PSU, and finally 0 PSU (Lake Michigan water). Populations were held at each salinity for one month (>1 copepod generation). The populations suffered highest mortalities (>>50%) during the final salinity transitions (0.1 → 0.05 → 0 PSU). The populations were subsequently reared at 0 PSU for four (block 1) or five generations (block 2) prior to the enzyme assays. Cultures were treated approximately weekly with 20 mg/L Primaxin® and 150 mg/L Timentin to prevent infection.

Effects of maternal and embryonic developmental acclimation on V-ATPase activity

We wished to verify that the observed differences in enzyme activity between saline and freshwater populations (or freshwater-selected lines) (Figs. 3 and 4) were not influenced by maternal and embryonic developmental acclimation to different salinities, but by selection alone. Thus, we compared V-ATPase activity of the saline St. Lawrence population (Atlantic clade) reared at 0 versus 15 PSU (~7 or 450 mOsm/kg), from metamorphosis of the parental population (the prior generation) to embryonic development at the following generation. Because selection for freshwater tolerance appears to act primarily on the larval stages right before metamorphosis (Lee et al. 2003), we avoided exposing the saline population to fresh water (0 PSU) until after metamorphosis. We first reared the parental generation of the saline St. Lawrence population to metamorphosis at 15 PSU. We then transferred the postmetamorphic juveniles either to fresh water (gradually to 0 PSU) or 15 PSU (controls) and reared them to adulthood. We allowed the adult females to mate and produce egg clutches at 0 or 15 PSU. We then split the clutches across salinities (0, 5, 15 PSU), and measured enzyme activity of the larvae that hatched from these clutches (next section). Using this procedure, we were able to achieve high survival (> 98%) during maternal and embryonic development of the saline population at 0 PSU, and avoided imposing selection.

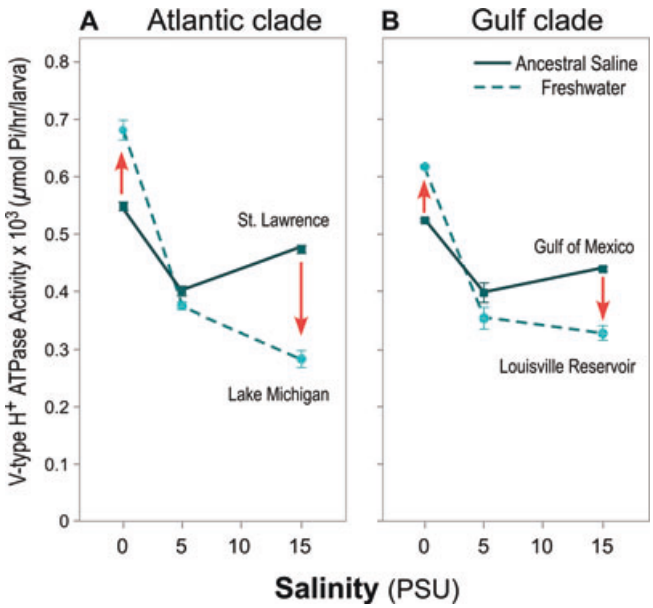

Figure 3. Evolutionary shifts in V-type H^+ ATPase activity between saline and freshwater populations from the (A) Atlantic and (B) Gulf clades. Red arrows depict evolutionary shifts in the freshwater populations (dashed lines), toward increased enzyme activity in fresh water (0 PSU) and reduced activity at high salinity (15 PSU) relative to the saline populations (solid lines). Freshwater populations also showed increased plasticity (i.e., greater slope) relative to the saline populations. Datapoints are mean ± SE. Data from both blocks are combined (six biological replicates, 120 larvae per biological replicate). Block effects were accounted for when calculating standard error bars.

We compared enzyme activity of the two acclimated treatments (0 or 15 PSU) to the same saline population selected for freshwater tolerance for ~12 generations in the laboratory (same population as in the previous section, but assayed again here for V-ATPase activity). This contrast allowed us to clearly observe the differences between effects of selection versus maternal + embryonic developmental acclimation on enzyme activity.

MEASUREMENT OF ION-MOTIVE ATPASE ACTIVITY

Experimental design of treatments

For each comparison described above (i.e., saline vs. freshwater populations; saline vs. freshwater-selected lines; saline population acclimated to 15 PSU vs. acclimated to 0 PSU), egg clutches from adult females from each population were harvested and placed across three salinities (0, 5, 15 PSU; ~7, 150, 450 mOsm/kg). For each population, ~20 egg clutches (> 400 eggs) were placed into each of six replicate containers per salinity per population. The treatments were held at the three salinities for 7–11 days, depending on development rate (as the freshwater populations develop more slowly). Enzyme kinetic assays were performed when the

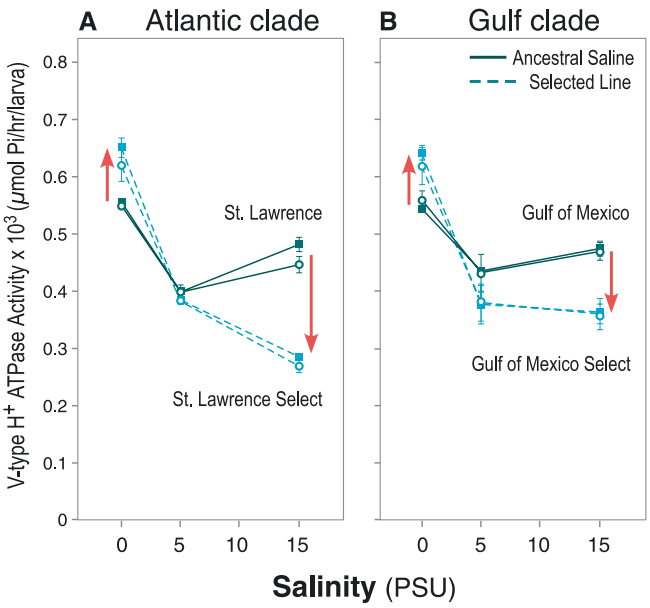

Figure 4. Evolutionary shifts in V-type H^+ ATPase activity in laboratory selected lines from the (A) Atlantic and (B) Gulf clades. Red arrows depict the evolutionary shifts in the selected lines (dashed lines; saline populations selected for freshwater tolerance over ~12 generations). Selected lines show increased enzyme activity in fresh water (0 PSU) and reduced activity at high salinity (15 PSU), relative to the saline ancestral populations (solid lines). The selected lines also show increased plasticity (i.e., greater slope) relative to the saline populations. Results are shown for two experimental blocks, where solid squares (■) represent results from the first block, whereas the open circles (o) represent results for the second block. Datapoints are mean ± SE for three biological replicates per block, 120 larvae per biological replicate.

larvae were at an identical developmental stage (and size) across populations and treatments. Enzyme assays were performed on larvae (nauplii) because it is the life-history stage that experiences mortality in response to low or high salinity stress (Lee et al. 2003, 2007).

Each of the six biological replicates (containing 400 larvae) was split into 10 parts containing the equivalent of 40 larvae, so that three technical replicates could be measured for enzyme activity, three for enzyme activity with an inhibitor (bafilomycin or ouabain, see next section), and three for protein concentration (see below). The technical replicates were used to reduce measurement error for each biological replicate. Enzyme assays were performed in two independent blocks on different dates for each population comparison, with each block containing three biological replicates.

Ion-motive ATPase activity assay

Larvae were prepared and homogenized using a standard EIS buffer of pH 7.2 (Lee and Watts 1994). The homogenate was centrifuged in a microfuge at 4°C for 5 min at 500 g (~2400 rpm in our case). Enzyme activity was measured using a coupled pyruvate kinase/lactate dehydrogenase assay, where hydrolysis of ATP is coupled to the oxidation of NADH (Scharschmidt et al. 1979). Enzyme activity was assayed using a standard reaction buffer (Lee and Watts 1994). Enzyme activity was measured in the absence and presence of specific inhibitors, using bafilomycin A_1 as an inhibitor of V-ATPase (Bowman et al. 1988) and ouabain as an inhibitor of Na/K-ATPase (Lee and Watts 1994). Difference in the rates of NADH oxidation between the assays, with and without the inhibitor, was used to determine either V-ATPase or Na/K-ATPase activity.

The reaction was initiated by adding copepod homogenate containing the equivalent of 40 larvae into each cuvette containing the reaction buffer (for each technical replicate). Total reaction volume was 500 μl. Reactions were incubated at room temperature (23°C) in disposable polycarbonate cuvettes (1-cm path length) for 10 min prior to enzyme activity measurements. Enzyme activity was measured by following the disappearance of NADH at 340 nm for 5 min using a Molecular Devices SpectraMax-Plus spectrophotometer (Molecular Devices, Inc., 1311 Orleans Drive Sunnyvale, CA). The rate of oxidation of NADH at 340 nm (OD/min) was converted to ATPase activity by dividing by an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ (Dawson et al. 1986). Enzyme activity was estimated per larva and also per milligram protein (see below).

Ion-motive ATPase activity normalized per milligram of protein

In addition to estimating ion-motive ATPase activity per larva, we also determined enzyme activity per milligram of protein of copepod larvae. Enzyme activity and protein concentration was measured for each biological replicate. Protein concentration was determined using the Bradford method with bovine serum albumin as a standard (Bradford 1976). Copepod homogenate equivalent to 40 larvae per reaction was placed in disposable polycarbonate cuvettes (1-cm path length). Deionized water was added to equal 800 μl, after which 200 μl of Bio-Rad Protein Assay dye concentrate was added to achieve a 1 mL total volume. After thorough mixing, samples were incubated for 10 min at 23°C. Protein concentration was measured at 595 nm using a Molecular Devices SpectraMax-Plus spectrophotometer.

STATISTICAL ANALYSES

For each enzyme and clade, effects of population, block, salinity, and population × salinity on enzyme activity were analyzed in a linear model framework using the PROC GLM procedure in SAS 9.1 (SAS 2003). All effects were treated as fixed effects. A post-hoc Tukey test was used to determine differences in ATPase activity between populations at each salinity.

Table 1. Statistical analysis comparing V-ATPase activity of saline versus freshwater populations (Fig. 3), using an ANOVA and post-hoc Tukey test. Results show effects of population (saline vs. freshwater populations), salinity (0, 5, 15 PSU), population × salinity, and block (date of assay) on V-ATPase activity. *F* = test statistic from the ANOVA; df = degrees of freedom.

Comparison	*F*	df	*P*-value
Atlantic clade			
Main effect of population (St. Lawrence vs. Lake Michigan)	6.94	1	0.0134
Main effect of salinity (0, 5, 15 PSU)	230.26	2	<0.0001
Population by salinity interaction	83.29	2	<0.0001
Tukey:			
0 PSU, fresh > saline population			<0.0001
5 PSU, no significant difference			0.719
15 PSU, saline > fresh population			<0.0001
Effect of block (two different dates of assay)	0.03	1	0.857
Gulf clade			
Main effect of population (Gulf vs. Louisville Reservoir)	3.75	1	0.0626
Main effect of salinity (0, 5, 15 PSU)	136.30	2	<0.0001
Population by salinity interaction	29.62	2	<0.0001
Tukey:			
0 PSU, fresh > saline population			0.0006
5 PSU, no significant difference			0.230
15 PSU, saline > fresh population			<0.0001
Effect of block (two different dates of assay)	12.29	1	0.0015

Results

EVOLUTIONARY SHIFTS IN V-TYPE H^+ ATPASE ACTIVITY

When we measured V-type H^+ ATPase activity for two pairs of saline and freshwater populations, representing independent salt-to-freshwater invasions from two genetically distinct clades (Fig. 1A, 1S vs. 1F-1 and 2S vs. 2F-1), we found rapid evolutionary shifts in enzyme function associated with invasions from saline into freshwater habitats (Fig. 3, Table 1). Most notably, saline and freshwater populations differed significantly in their response to salinity (population by salinity interaction) in both clades ($P < 0.0001$, Table 1), indicating significant shifts in response to salinity (V-ATPase activity) between the saline and freshwater populations. Pairwise Tukey comparisons indicated evolutionary shifts in response (V-ATPase activity) between the populations at both low (0 PSU) and high (15 PSU) salinities (Table 1). The freshwater populations (Lake Michigan [1F-1], Louisville [2F-1]) exhibited significant increases in V-ATPase activity in fresh water (0 PSU, ~7 mOsm/kg) and strikingly large declines at higher salinity (15 PSU, 450 mOsm/kg) relative to their saline ancestors (Fig. 3; see Table 1). At the intermediate salinity (5 PSU, 150 mOsm/kg), ATPase activity did not differ significantly between the fresh and saline populations (Fig. 3; see Table 1). The same pattern of evolutionary change was evident in the two genetically distinct Atlantic and Gulf clades (Fig. 3), indicating parallel evolution across the independent invasions from distinct ancestors.

The significant population by salinity interaction also indicated evolutionary shifts in plasticity between the saline and freshwater populations in both clades (i.e., Fig. 3, shift in reaction norms; Table 1). The plastic response to salinity was greater in the derived freshwater populations than in the ancestral saline populations, evident from the increase in slope in the freshwater populations (Fig. 3).

Saline and freshwater populations differed significantly in overall V-ATPase activity for the Atlantic clade (ANOVA, main effect of population, $F = 6.94$, DF = 1, $P = 0.0134$), but not for the Gulf clade ($F = 3.75$, DF = 1, $P = 0.063$). This latter result was not surprising, given that there was a rank order shift in enzyme activity between saline and freshwater populations across salinities, without much change in mean values between the populations (Fig. 3). Salinity had significant effects on V-ATPase activity for both the Atlantic and Gulf clades (Table 1), indicating a significant plastic response to salinity. For all assays, enzyme activity per larva (Fig. 5A) and per milligram protein (Fig. 5B) yielded the same pattern.

EVOLUTIONARY SHIFTS IN V-TYPE H^+ ATPASE ACTIVITY IN LABORATORY SELECTED LINES

When we compared V-ATPase activity of the ancestral saline populations (Fig. 1A, 1S and 2S) relative to these same saline populations selected for freshwater tolerance for ~12 generations, the selected lines exhibited evolutionary shifts in enzyme activity that were remarkably close to patterns found in the natural freshwater populations (compare Figs. 3 and 4). In addition, the independent selection experiments from each saline population showed the same evolutionary shifts (Fig. 4).

Population by salinity interaction was significant for both clades (Fig. 4 and Table 2A, $P < 0.0001$), indicating significant evolutionary shifts in plastic response between the saline and freshwater-selected populations. In concordance with the natural freshwater populations, the freshwater selected lines showed a significant increase in plastic response to salinity relative to the ancestral saline populations. Similar to the natural freshwater populations (see Fig. 3), the freshwater-selected populations showed significantly higher V-ATPase activity at 0 PSU, and lower activity at 15 PSU than the saline populations for both clades (Fig. 4; see Table 2A). These results indicated that the patterns of evolution

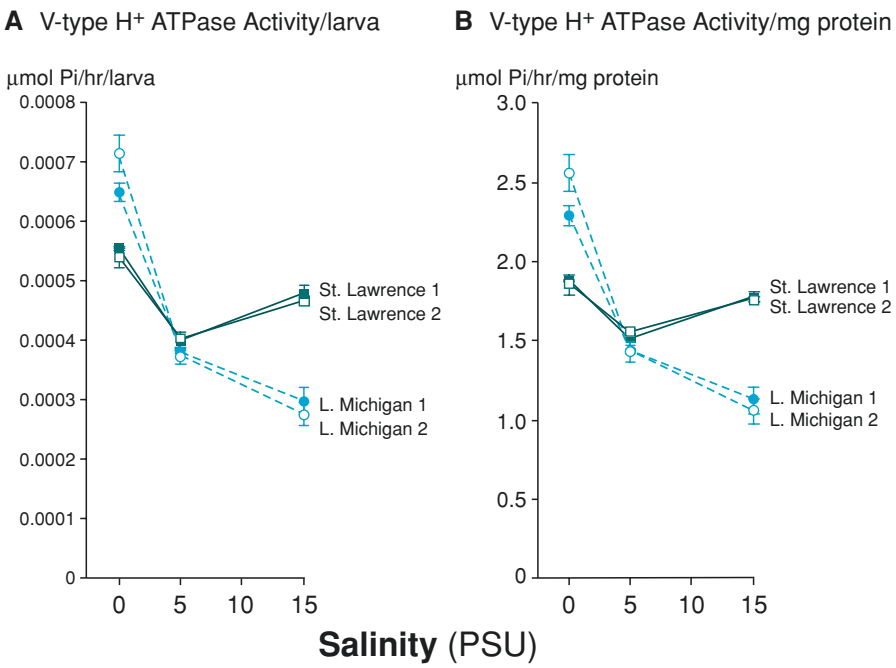

Figure 5. Evolutionary shifts in V-type H^+ ATPase activity between saline and freshwater populations from the Atlantic clade, showing results for two blocks (1 and 2; where the reaction norm experiments were performed and enzyme activity was measured on different dates). (A) V-type H^+ ATPase activity per larva. (B) V-type H^+ ATPase activity per milligram protein of copepod larvae. Datapoints shown are mean ± standard error. Each block contains three biological replicates (120 larvae per biological replicate).

observed in the wild populations could be reproduced in the laboratory in only a few generations. Thus, we observed parallel evolution of V-ATPase function arising both repeatedly from the same ancestral saline populations, as well as from different ancestral populations in genetically divergent clades (Figs. 3 and 4). Salinity had significant effects on V-ATPase activity for both clades (Table 2A), indicating a significant plastic response to salinity.

EFFECTS OF MATERNAL AND EMBRYONIC DEVELOPMENTAL ACCLIMATION ON V-TYPE H^+ ATPASE ACTIVITY

Maternal + embryonic developmental acclimation (developmental plasticity) could not account for the shifts in V-ATPase activity between saline and freshwater/freshwater-selected populations (Figs. 3 and 4). Rearing the saline population (Fig. 1, St. Lawrence [1S]) during maternal and embryonic development in fresh water (0 PSU) and higher salinity (15 PSU) yielded the same pattern of enzyme activity (Fig. 6, solid lines; Table 2B). Meanwhile, V-ATPase activity of the freshwater-acclimated population (Fig. 6, light solid line) did differ significantly from the freshwater-selected population (Fig. 6, dashed line) at 0 and 15 PSU (Table 2B, $P = 0.0054$ and $P < 0.0001$, respectively), in a manner that resembled the contrast between natural saline and freshwater populations (compare Figs. 3 and 6). Thus, maternal + embryonic developmental acclimation (at 0 PSU) could not replicate the pattern of V-ATPase activity found in the natural freshwater or laboratory freshwater-selected populations, and could not account for differences in enzyme activity observed between saline and freshwater populations (Figs. 3 and 4).

EVOLUTIONARY SHIFTS IN NA^+/K^+-ATPASE ACTIVITY

When we compared Na/K-ATPase activity across salinities (0, 5, 15 PSU; equivalent to ~7, 150, 450 mOsm/kg) for two saline-freshwater population pairs, representing independent salt-to-freshwater invasions (Fig. 2, 1S vs. 1F-1 and 2S vs. 2F-1, Atlantic and Gulf clades), we found evolution of Na/K-ATPase activity in the freshwater populations relative to their saline ancestors (Fig. 7; Table 3). Population had a significant effect on Na/K-ATPase activity, with saline populations showing higher enzyme activity across all salinities relative to the freshwater populations (Fig. 7; Table 3, $P < 0.0001$ for Atlantic clade, $P = 0.0026$ for Gulf clade). This result suggested a greater role for this enzyme in the saline populations.

In contrast to the case for V-ATPase (Tables 1, 2), population by salinity interaction was not significant for Na/K-ATPase activity in both the Atlantic and Gulf clades (Table 3). There was no change in rank order in V-ATPase activity between populations across salinities and no evolution of plasticity following invasions. Salinity had significant effects on Na/K-ATPase activity for both the Atlantic and Gulf clades (Table 3), indicating a significant plastic response to salinity.

Table 2. **Statistical analysis examining effects of selection (Fig. 4) and maternal + embryonic developmental acclimation (Fig. 6) on V-ATPase activity, using an ANOVA and post-hoc Tukey test. Results show effects of treatment (natural, selected, acclimated), salinity (0, 5, 15 PSU), treatment × salinity, and block (date of assay) on V-ATPase activity. *F* = test statistic from an ANOVA; df = degrees of freedom.**

Comparison	*F*	df	*P*-value
(A) Natural saline populations versus selected lines (saline populations selected for freshwater tolerance)			
Atlantic clade			
Main effect of population (St. Lawrence vs. St. Lawrence selected)	29.01	1	<0.0001
Main effect of salinity (0, 5, 15 PSU)	368.66	2	<0.0001
Population by salinity interaction	111.30	2	<0.0001
Tukey:			
0 PSU, fresh > saline population			<0.0001
5 PSU, no significant difference			0.875
15 PSU, saline > fresh population			<0.0001
Effect of block (two different dates of assay)	4.01	1	0.055
Gulf clade			
Main effect of population (Gulf vs. Gulf selected)	4.71	1	0.038
Main effect of salinity (0, 5, 15 PSU)	81.90	2	<0.0001
Population by salinity interaction	17.39	2	<0.0001
Tukey:			
0 PSU, fresh > saline population			0.024
5 PSU, no significant difference			0.201
15 PSU, saline > fresh population			0.0007
Effect of block (two different dates of assay)	0.03	1	0.867
(B) Effects of maternal and embryonic developmental acclimation			
(Saline St. Lawrence population acclimated at 0 PSU vs. acclimated at 15 PSU vs. selected at 0 PSU)			
Atlantic clade			
Main effect of treatment (population)	5.69	2	0.0063
Main effect of salinity (0, 5, 15 PSU)	145.46	2	<0.0001
Population by salinity interaction	20.68	4	<0.0001
Tukey (maternal + embryonic acclimation at 0 PSU vs. acclimation at 15 PSU):			
For larvae hatched and reared at:			
0 PSU			0.879
5 PSU			1.00
15 PSU			0.994
Tukey (maternal + embryonic acclimation at 0 PSU vs. selected at 0 PSU):			
For larvae hatched and reared at:			
0 PSU			0.0054
5 PSU			1.00
15 PSU			<0.0001
Effect of block (two different dates of assay)	1.51	1	0.225

BLOCK EFFECTS ON ENZYME ACTIVITY

Block (different dates of assay) typically had no significant effects on enzyme activity, except in two cases (Tables 1–3; Fig. 5). Block had a significant effect on V-ATPase activity for the comparison of saline and freshwater populations from the Gulf clade (Table 1). This significant block effect was due to a 9% upward shift in enzyme activity for block 2, while shape of the response curves remained very similar between the blocks. Block also had a significant effect on Na/K-ATPase activity for the comparison of saline and freshwater populations from the Atlantic clade (Table 3). The significant block effect here was due to an 8% upward shift in enzyme activity values for the second block, with no difference in rank order and near identical shape of response curves for the two blocks.

Discussion

Life originated in the sea, and most marine taxa have little need to regulate their internal osmotic and ionic concentrations relative to the environment. As osmoconformers, the extracellular ionic

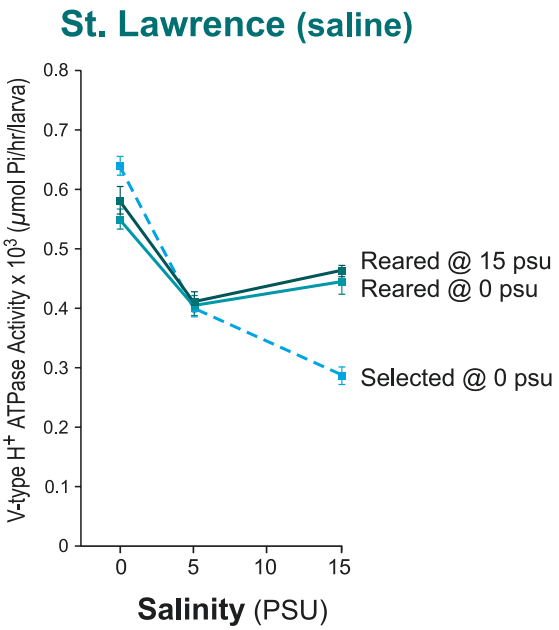

Figure 6. Effects of maternal + embryonic developmental acclimation on V-type H^+ ATPase activity. Graph shows V-type H^+ ATPase activity of the saline St. Lawrence population (Atlantic clade) reared from the parental generation (postmetamorphosis) through embryonic development in the following generation in either (1) fresh water (0 PSU; solid light line) or (2) saline conditions (15 PSU; solid dark line). The acclimated lines were compared to (3) the saline population selected for fresh water tolerance for ~12 generations (dashed line). Rearing salinity (0 or 15 PSU) did not significantly affect enzyme activity, in contrast to the significant shifts due to selection at 0 PSU. Datapoints shown are mean ± SE for six biological replicates, split into two blocks, 120 larvae per biological replicate. Block effects were accounted for when calculating standard error bars.

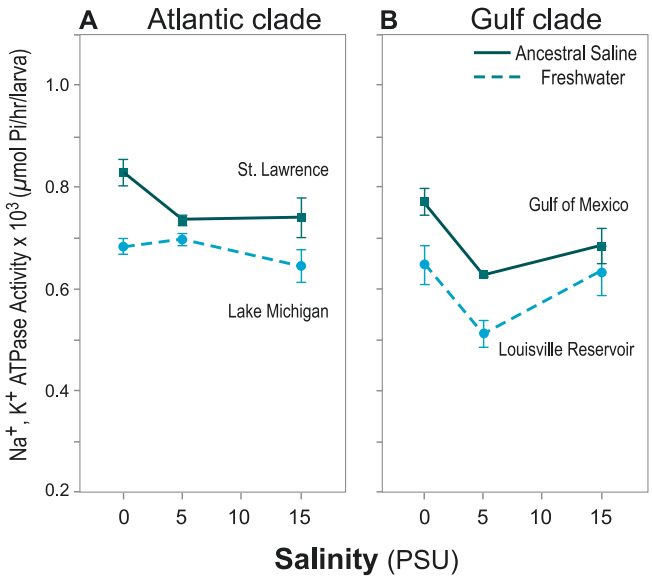

Figure 7. Evolutionary shifts in Na^+/K^+-ATPase activity between saline and freshwater populations from the (A) Atlantic and (B) Gulf clades. Freshwater populations (dashed lines) showed decreased enzyme activity across salinities relative to the saline populations (solid lines). Datapoints shown are mean ± SE for six biological replicates, split into two blocks, 120 larvae per biological replicate. Block effects were accounted for when calculating standard error bars.

concentrations of most marine invertebrates are similar to that of the surrounding water. However, major habitat transitions into estuarine, freshwater, and terrestrial habitats required the evolution of internal body fluid regulation to maintain ionic gradients with the external environment. Over geological time scales, throughout the Phanerozoic, marine taxa were extremely slow to evolve the capacity to colonize freshwater niches (Miller and Labandeira 2002). The targets of selection and the nature of selection during such transitions have remained poorly understood.

Recent human-mediated transport has been introducing saline species into freshwater habitats at unprecedented levels (Jazdzewski 1980; Lee and Bell 1999; Ricciardi and MacIsaac 2000). This study revealed rapid and repeated evolution of ion regulatory function following extraordinary environmental change during rapid habitat shifts, on time scales as short as decades in the wild and a few generations in the laboratory. Comparisons between ancestral saline populations and recent freshwater invaders (Fig. 1) uncovered striking patterns of evolution for both V-ATPase and Na/K-ATPase activity (Figs. 3, 7). Most notably, we found that manipulating a single variable in the laboratory (i.e., salinity) could reproduce the same evolutionary shifts found in nature (Fig. 4), on very short time scales and starting with small population size. Few studies have used laboratory selection to confirm the evolutionary lability of a critical trait limiting habitat transitions.

Here, we found parallel evolution at the level of enzyme function, arising both repeatedly from the same ancestral saline populations (in replicated selection experiments and natural invasions) as well as from genetically divergent ancestral populations from different clades (from St. Lawrence and Gulf populations) (Figs. 3, 4, and 7). Parallel evolution involved the same ion-motive enzymes to alter ion-uptake capacity in a similar manner.

The term "parallel evolution" has classically referred to the manifestation of the same phenotype during repeated evolutionary events from the same ancestral population (i.e., "replaying the evolutionary tape of life"; Gould 1989). On the other hand, a more recent definition accounts for the prevalence of shared genetic mechanisms underlying parallel phenotypic evolution, even across genetically divergent taxa, as many traits that were once considered a result of convergent evolution are now known to be governed by the same genetic mechanisms (Majerus and Mundy 2003; ffrench-Constant et al. 2004; Mundy et al. 2004; Colosimo

Table 3. Statistical analysis comparing Na/K-ATPase activity of saline versus freshwater populations (Fig. 7), using an ANOVA and post-hoc Tukey test. Results show effects of population (saline vs. freshwater populations), salinity (0, 5, 15 PSU), population × salinity, and block (date of assay) on Na/K-ATPase activity. *F* = test statistic from the ANOVA; df = degrees of freedom.

Comparison	*F*	DF	*P*-value
Atlantic clade			
Main effect of population (St. Lawrence vs. Lake Michigan)	22.65	1	<0.0001
Tukey:			
0 PSU, fresh < saline population			0.0021
5 PSU, no significant difference			0.871
15 PSU, saline > fresh population			0.083
Main effect of salinity (0, 5, 15 PSU)	3.75	2	0.035
Population by salinity interaction	2.56	2	0.095
Effect of block (two different dates of assay)	8.92	1	0.0057
Gulf clade			
Main effect of population (Gulf vs. Louisville Reservoir)	10.85	1	0.0026
Tukey:			
0 PSU, fresh > saline population			0.171
5 PSU, no significant difference			0.233
15 PSU, saline > fresh population			0.925
Main effect of salinity (0, 5, 15 PSU)	8.13	2	0.0016
Population by salinity interaction	0.66	2	0.523
Effect of block (two different dates of assay)	0.89	1	0.353

et al. 2005; Wood et al. 2005). The parallelisms that we found arising both within and between clades suggest labile evolutionary mechanisms that could respond rapidly and readily to environmental change.

EVOLUTIONARY SHIFTS IN V-TYPE ATPASE ACTIVITY

This study is the first to demonstrate rapid evolution of V-ATPase function, and the evolutionary response of this enzyme to environmental change. V-ATPase has been hypothesized to be crucial for ion uptake from a dilute environment for many species. The transmembrane potential generated by V-ATPase (Fig. 2B) could energize the absorption of ions against a steep concentration gradient (Ehrenfeld and Klein 1997; Ehrenfeld 1998; Wieczorek et al. 1999; Beyenbach 2001; Beyenbach and Wieczorek 2006). Specifically, an apical localization of V-ATPase pumps H^+ from the epithelial cell into the external fluid, creating a H^+ gradient that then enables ions, such as Na^+, to flow into the cell via other transport proteins, such as Na^+ channels or the Na^+/H^+ exchanger (Fig. 2B). Thus, we predicted that V-ATPase would exhibit an evolutionary shift toward elevated activity in the freshwater populations at low salinities.

Concordant with our expectations, V-ATPase activity was significantly higher in the freshwater populations relative to the saline populations under freshwater conditions (0 PSU) (Fig. 3 and Table 1). Simultaneously, the freshwater populations showed a drastic decline in V-ATPase activity at higher salinity (15 PSU) that was both striking and unexpected (Fig. 3 and Table 1). Artificial freshwater populations, namely, saline populations selected for freshwater tolerance over ~12 generations, showed the same evolutionary shifts as the natural freshwater populations (Fig. 4 and Table 2A). These shifts could not be accounted for by maternal + embryonic developmental acclimation, as rearing of the saline population at 0 PSU (from postmetamorphoric parents to hatching at the next generation) could not generate the pattern of activity found in the freshwater populations or freshwater-selected lines (Fig. 6 and Table 2B).

The evolutionary shifts in V-ATPase activity we observed in the natural and laboratory-selected freshwater populations (Figs. 3 and 4) were reminiscent of the pattern seen for survival, where freshwater populations showed increased survival at low salinity coupled with reduced survival at high salinity relative to saline populations (Fig. 1B; Lee et al. 2003, 2007). This pattern, along with negative genetic correlations between survival at low versus high salinities (Lee et al. 2003, Lee et al. 2007), suggested trade-offs between low- and high-salinity tolerance. The increase in V-ATPase activity at low salinity concomitant with the decline at high salinity (Figs. 3 and 4) might similarly reflect negative correlations in V-ATPase function at low versus high salinity conditions, congruent with (and potentially contributing to) the pattern seen for survival.

EVOLUTIONARY SHIFTS IN Na/K-ATPASE ACTIVITY

Na/K-ATPase activity was significantly lower across salinities in the freshwater populations relative to the saline populations for both clades (Fig. 7 and Table 3). Na/K-ATPase activity might have evolved to be constitutively higher (across all salinities) in the saline *E. affinis* populations because of its rate-limiting role in ionic regulation in saline habitats (Fig. 2A). Ionic concentration of the cell tends to be constant across environments, whereas hemolymph concentration tends to be higher in saline populations (Fig. 2). Thus, ion transport across the basolateral membrane (from the cell to hemolymph) would be more energetically costly in saline populations, perhaps requiring enhanced Na/K-ATPase activity.

The overall elevated activity of Na/K-ATPase in the saline populations was concordant with some previous studies that compared saline and freshwater species. For instance, marine and brackish amphipod (gammarid) species tended to show higher Na/K-ATPase activity (1.5–2 fold) than freshwater species, particularly at lower salinities (Brooks and Mills 2006). In studies of fish, the more saline species showed higher Na/K-ATPase

activity under freshwater conditions (Bystriansky et al. 2006; Nilsen et al. 2007; Kang et al. 2008), but not necessarily under saline conditions (Kang et al. 2008).

ACCLIMATION IN RESPONSE TO SALINITY

All populations showed significant plastic responses in V-ATPase and Na/K-ATPase activity in response to salinity (0, 5, 15 PSU), as indicated by the significant effect of salinity on ATPase activity (Tables 1–3). The saline populations displayed a U-shaped pattern of activity for both V-ATPase and Na/K-ATPase, with the lowest activity at 5 PSU (Figs. 3, 4, and 7). Several studies have likewise found a characteristic U-shaped pattern for Na/K-ATPase activity in brackish water species. In many organisms, Na/K-ATPase activity is often lowest at the salinities to which the population is adapted, and elevated at lower (for ion uptake) and higher (for ion excretion) salinities (Jensen et al. 1998; Lin et al. 2004; Kang et al. 2008). In contrast, the freshwater populations did not show a U-shaped pattern for V-ATPase activity (Figs. 3 vs. 7), perhaps as a result of selection for increased plasticity following freshwater invasions, in accordance to predictions of Lande's (2009) model (see next section).

EVOLUTION OF PLASTICITY FOLLOWING HABITAT CHANGE

Our results revealed a dramatic increase in plasticity following adaptation to freshwater conditions (Figs. 3 and 4). Natural and laboratory-selected freshwater populations exhibited much greater slope in V-ATPase activity in response to salinity, relative to saline populations (Figs. 3 and 4), revealing the evolution of plasticity during freshwater invasions. Our results were consistent with Waddington's argument (1953) and Lande's theoretical model (2009) indicating that under sudden extraordinary environmental change, substantially exceeding typical background environmental fluctuations, evolution of increased plasticity could accelerate adaptation to novel conditions (Waddington 1953; Lande 2009). In the native ancestral saline habitat, when environmental conditions are not fully predictable, an intermediate level of plasticity would be favored (as an extreme phenotype produced by extreme developmental plasticity would be more disadvantageous upon environmental change; Lande 2009). According to Lande's model (2009), genetic variation in plasticity in the native range could greatly facilitate adaptation during invasions. In the case of *E. affinis*, ancestral saline populations do exhibit high levels of genetic variation in survival reaction norms, consistent with genetic variation in plasticity for ionoregulatory mechanisms upon which selection could act (Lee et al. 2003, 2007). Adaptation via selection on plasticity might be a common mechanism permitting habitat shifts into radically novel environments.

Lande's model (2009) also predicts that following the initial increase in plasticity in the novel habitat, stabilizing selection for the optimum phenotype would lead to slow genetic assimilation and a reduction in plasticity (canalization) over time (on time scales of $>10^3$ generations in Lande's simulations). Thus, one might expect more ancient freshwater invading populations to exhibit flatter reaction norms (lower slope, reduced plasticity) in response to salinity than more recently invading populations. In our study, the selected lines and natural freshwater populations exhibited virtually no difference in plasticity (i.e., slope) (compare Figs. 3 and 4). However, the natural freshwater populations, which invaded ~25–50 years (~150–300 generations) ago, are too recently derived to display signs of genetic assimilation as described in Lande's model.

Lande's model (2009) assumes a time lag between (1) sensitivity to the environment during development (which, via developmental plasticity, determines an organism's phenotypic value) and (2) the time at which selection acts on the resultant phenotype. A consequence of this time lag is the reduction of the optimum reaction norm slope in the native range (because the future environment is not perfectly predicted by the early environment, given stochastic environmental fluctuations). Subsequent invasion of an extreme novel habitat, favoring an extreme phenotype, would then result in evolution of increased reaction norm slope (i.e., the evolution of plasticity; Lande 2009). Such a time lag arising from developmental plasticity pertains to *E. affinis*, as osmoregulatory function is influenced by a developmental response to salinity at the early life stages, leading to relatively irreversible tolerances at later stages (although, a fully saline population cannot achieve freshwater tolerance through developmental plasticity alone; Lee and Petersen 2003). In addition, in decapod crustaceans, as well as in other taxa, the abundance and localization of ATPase-rich ionocytes (and ion-motive ATPase expression within ionocytes) have been found to show a developmental response to salinity (Piermarini and Evans 2000; Lignot and Charmantier 2001; Cieluch et al. 2004; Charmantier et al. 2009).

Although we observed a dramatic increase in plasticity for V-ATPase activity following freshwater invasions (Figs. 3 and 4), we did not see a corresponding increase in plasticity for Na/K ATPase activity (Fig. 7). V-ATPase activity is a critical trait that would be under strong selection during freshwater adaptation, whereas evolution of Na/K-ATPase activity unlikely to be as critical for freshwater survival. In fact, the evolutionary shift toward reduced Na/K-ATPase activity in freshwater populations might be driven by either conservation of resources or tolerance of mutations that reduce expression of the enzyme.

POTENTIAL TARGETS OF EVOLUTIONARY CHANGE

Adaptation could occur either by selection on new mutations or on standing genetic variation in ancestral populations. Given the rapid time scale of invasions (as well as reduced effective population size during colonization), selection during invasions

is likely to occur from standing genetic variation (Innan and Kim 2004; Hermisson and Pennings 2005; Lee and Gelembiuk 2008; Lande 2009). It would be worthwhile to investigate whether the native range harbors a high degree of genetic variation in ion-motive ATPase function, upon which selection could act.

Evolutionary shifts in ion-motive ATPase activity between ancestral saline and freshwater invading populations could arise from either changes in specific activity of the enzyme or changes in amount of protein expressed in the tissue (e.g., due to changes in gene expression). Results from custom cDNA microarrays (Supporting Information) indicate that evolutionary shifts in expression of genes underlying the enzymes (*V-type H^+ ATPase B-subunit, Na^+/K^+ ATPase ⟨-subunit*) show the same trends as that for enzyme activity. Expression of *V-type H^+ ATPase B-subunit* was consistently elevated in freshwater populations relative to saline populations at 0 PSU ($P = 0.0019$ for a one-tailed test of upregulation in freshwater vs. saline populations; see Supporting Information) whereas expression of *Na^+/K^+ ATPase ⟨-subunit* was generally reduced in the freshwater populations relative to saline populations at both 0 PSU and 5 PSU (Supporting Information, Table S1). This concordance between enzyme activity and expression suggests that evolutionary shifts in gene expression might contribute to changes in enzyme activity.

Changes in ion-motive ATPase expression might reflect either increased gene expression within ionocytes (epithelial cells responsible for ion transport) or increases in the number of ionocytes expressing V-ATPase or Na/K-ATPase. Differential patterns of localization of cell types within organs could also affect osmoregulatory functioning (Smith et al. 2008). In situ approaches (e.g., immunohistochemical, RNA expression) would help identify where ion-motive ATPase activity is localized in the organism, and help determine whether ATPase activity evolves via changes in expression within cells, cell type, number of cells, or localization of cells. Several regions in the copepod might be involved in ionic regulation (where ion-motive ATPase activity would be localized), such as integumental windows (Hosfeld and Schminke 1997b; Hosfeld 1999), maxillary glands (Le Borgne 1986), antennal glands of larvae (Mercadé 1982), and segmental extranephridial podocytes (Hosfeld and Schminke 1997a).

IMPLICATIONS FOR RANGE SHIFTS IN OTHER TAXA

Ion-motive ATPase enzymes are highly conserved, ubiquitous across all domains of life (bacteria, archaea, eukaryotes), and essential for ion transport in all cells (Gogarten and Starke 1992; Palmgren and Axelsen 1998). V-ATPase and NA/K-ATPase tend to be evolutionarily quite conserved, with the catalytic subunits showing high degrees of homology across highly divergent taxa (>70% amino acid sequence identity across vertebrate and invertebrate species) (Vasilets and Schwarz 1993; Weihrauch et al. 2001; Lucu and Towle 2003). This high degree of conservation suggests the preservation of functions that are fundamentally important across eukaryotic organisms. This conservation in amino acid sequence increases the likelihood that evolutionary shifts in ion-motive ATPase function are due to changes in gene regulation (but see Jorgensen and Amat 2008).

The small numbers of taxa that are able to transition from saline to freshwater environments tend to comprise a relatively large portion of the freshwater invaders (De Beaufort 1954; Miller 1958; Mordukhai-Boltovskoi 1979; Jazdzewski 1980; Taylor and Harris 1986; Lee 1999; Lee and Bell 1999; May et al. 2006). Thus, understanding mechanisms of such physiological transitions has key relevance for numerous aquatic invasions. In particular, the evolutionary parallelism observed here might have general relevance for the numerous brackish water species invading the Great Lakes and inland waters. Ionic regulation is a common challenge for many of these invaders, such as zebra mussels and many microcrustaceans, as they are inefficient osmoregulators under freshwater conditions (Taylor and Harris 1986; Dietz et al. 1996; Lee and Bell 1999). A question remains regarding the degree to which the evolutionary mechanisms here are shared across a wide variety of taxa invading diverse landscapes. V-ATPase and Na/K-ATPase are universal primary energizing pumps for ionoregulation in eukaryotes. Thus, these enzymes are very likely to provide evolutionary targets in any habitat shift that involves the need for changes in ion regulatory function (e.g., freshwater, terrestrial, arid, dietary environments).

An intriguing question remains as to why some groups show the evolutionary capacity to invade fresh water, whereas most do not. The taxa that do possess the ability to invade fresh water from more saline habitats appear to be phylogenetically diverse (Jazdzewski 1980; Lee and Bell 1999; Ricciardi and MacIsaac 2000), but appear to have in common an evolutionary history in fluctuating environments (Lee and Gelembiuk 2008). Evolution under such conditions might lead to the evolutionary lability that we observe in this study, as evinced by the repeatable and parallel evolution in both natural and laboratory populations. The common-garden and selection experiments demonstrate evolutionary shifts in ion-motive ATPase activity in response to salinity. However, additional work that links genotype with phenotype would be required to directly establish a link between enzyme activity and fitness under freshwater conditions (e.g., transgenic or association approaches). In particular, it would be useful to determine the precise causal mutations that distinguish freshwater-adapted populations relative to their saline ancestors. Identifying the specific genetic mechanisms that underlie physiological evolution during freshwater invasions might bring us closer to determining why particular populations have the capacity to cross this formidable boundary, and also provide general insights into why certain species have the ability to invade.

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Supporting Information

The following supporting information is available for this article:

Table S1. Evolutionary shifts in gene expression based on cDNA microarrays.

Supporting Information may be found in the online version of this article.

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