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Rapid evolution of body fluid regulation following independent invasions into freshwater habitats

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Abstract

Colonizations from marine to freshwater environments constitute among the most dramatic evolutionary transitions in the history of life. Colonizing dilute environments poses great challenges for acquiring essential ions against steep concentration gradients. This study explored the evolution of body fluid regulation following freshwater invasions by the copepod *Eurytemora affinis*. The goals of this study were to determine (1) whether invasions from saline to freshwater habitats were accompanied by evolutionary shifts in body fluid regulation (hemolymph osmolality) and (2) whether parallel shifts occurred during independent invasions. We measured hemolymph osmolality for ancestral saline and freshwater invading populations reared across a range of common-garden salinities (0.2–25 PSU). Our results revealed the evolution of increased hemolymph osmolality (by 16–31%) at lower salinities in freshwater populations of *E. affinis* relative to their saline ancestors. Moreover, we observed the same evolutionary shifts across two independent freshwater invasions. Such increases in hemolymph osmolality are consistent with evidence of increased ion uptake in freshwater populations at low salinity, found in a previous study, and are likely to entail increased energetic costs upon invading freshwater habitats. Our findings are consistent with the evolution of increased physiological regulation accompanying transitions into stressful environments.

Introduction

The transition from marine to freshwater environments constitute among the most dramatic evolutionary transitions in the history of life that relatively few taxa have been able to penetrate (Hutchinson, 1957; Little, 1983, 1990; Miller & Labandeira, 2002). Most animals evolved in the sea, and marine animals (other than most chordates) tend to possess body fluids that resemble the surrounding seawater in ionic composition. Colonizing dilute environments poses great challenges for acquiring essential ions against steep concentration gradients (Beyenbach, 2001; Morris, 2001; Tsai & Lin, 2007; Lee *et al.*, 2011). As organisms in fresh water must maintain elevated body fluid concentrations (osmolalities) relative to the very dilute environment (i.e. they must be hyperosmotic), life in fresh water tends to be energetically costly (Péqueux, 1995; Morgan & Iwama, 1999; Łapucki & Normant, 2008). Thus, the evolution of body fluid regulation constitutes a critical step that limits colonizations into freshwater habitats. Moreover, freshwater adaptations have broader implications for terrestrial invasions, as many key physiological adaptations resulting from saline to freshwater transitions are thought to have provided stepping-stones for the colonization of land (Wolcott, 1992; Anger, 2001; Morris, 2001; Glenner *et al.*, 2006).

In recent years, saline to freshwater invasions have become increasingly common with increases in shipping, dumping of ballast water, and the stocking of coastal fish and invertebrates into inland waters (De Beaufort, 1954; Miller, 1958; Mordukhai-Boltovskoi, 1979; Jazdzewski, 1980; Taylor & Harris, 1986; Lee, 1999; Lee & Bell, 1999; Ricciardi & MacIsaac, 2000; May *et al.*, 2006). These

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saline to freshwater invasions are noteworthy, as saline and freshwater invertebrates are typically separated by a biogeographic boundary (salinity of ~5 PSU = osmolality of ~150 mOsm kg^{-1}) that is impenetrable for most species (Khlebovich & Abramova, 2000). What physiological mechanisms might enable some species to cross this biogeographic boundary?

The common estuarine and salt marsh copepod *Eurytemora affinis* is notable in its ability to successfully invade freshwater habitats (Lee, 1999). Within the past century, this small crustacean invaded freshwater habitats multiple times independently from coastal waters in North America, Europe, and Asia (Lee, 1999). Previous research has revealed evolutionary shifts associated with freshwater invasions by *E. affinis*, toward increases in freshwater tolerance and reductions in high salinity tolerance (Lee *et al.*, 2003, 2007). Negative genetic correlations between survival at low and high salinities suggested tradeoffs between low and high salinity tolerance (Lee *et al.*, 2003, 2007). Most notably, the freshwater populations exhibited rapid evolution of ion transport capacity, showing increases in activity and expression of the ion transport enzyme V-type H^+ ATPase in fresh water (0.2 PSU salinity, 300 µS cm^{-1} conductivity) and declines at higher salinity (15 PSU) relative to saline populations (Fig. 1; Lee *et al.*, 2011). In contrast, the ion transport enzyme Na^+/K^+-ATPase, previously considered to be the principal driving force for ion uptake from the environment (Lucu & Towle, 2003; Kirschner, 2004), showed declines in activity and expression across all salinities in the freshwater populations relative to their saline ancestors. Given this result, this enzyme (Na^+/K^+-ATPase) is unlikely to be implicated in freshwater adaptation in *E. affinis* (Lee *et al.*, 2011). On the other hand, the evolutionary increase in V-type H^+ ATPase activity in fresh water suggests an evolutionary increase in ion uptake capacity following freshwater invasions. These evolutionary shifts were repeatable, with parallel evolutionary shifts across independent invasions in the wild (Fig. 1a), as well as in selection experiments in the laboratory (Fig. 1b) (Lee *et al.*, 2011).

Given the evolutionary shifts in activity and expression of a key ion transport enzyme (Fig. 1; Lee *et al.*, 2011), what consequences would we observe in physiological function? Would we find evolutionary changes in body fluid concentration following freshwater invasions? And what pattern of evolution would the freshwater populations exhibit relative to their saline ancestors? The concentration gradient between the extracellular body fluids (hemolymph) and the environment affects the amount of work required to maintain hemolymph concentrations (Willmer *et al.*, 2004). At very low salinities, organisms must retain a hemolymph osmolality that is elevated relative to the environment (i.e. hyperosmotic) in order to maintain physiological function. The question is whether the freshwater populations would show greater or lower hemolymph osmolalities relative to saline populations under freshwater conditions.

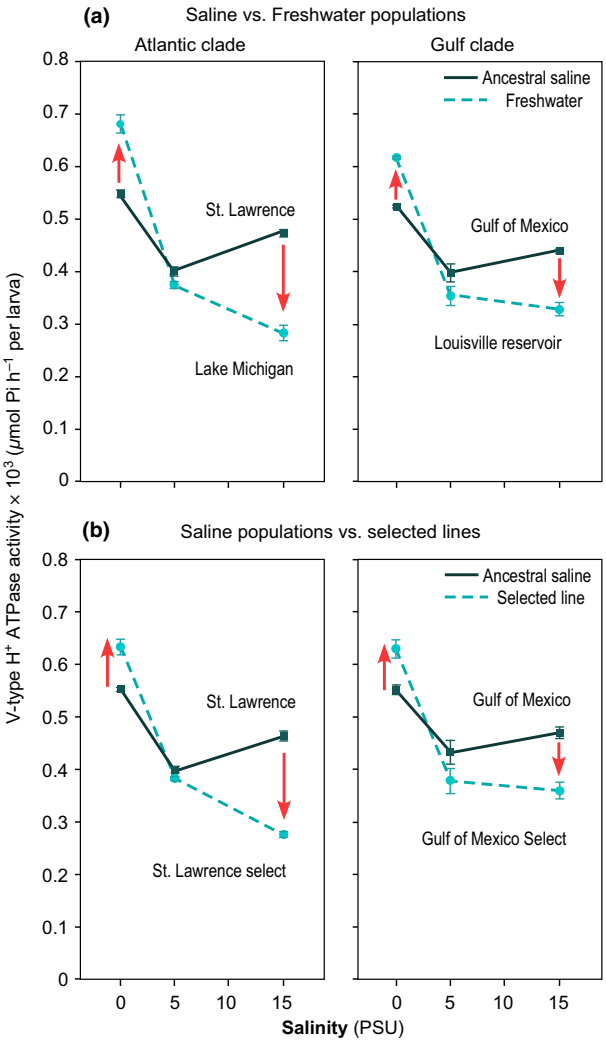

Fig. 1 Evolutionary shifts in ion transport enzyme V-type H^+ ATPase activity between saline and freshwater populations from the Atlantic (left graphs) and Gulf clades (right graphs). (a) Enzyme activity of ancestral saline populations relative to natural freshwater populations. (b) Enzyme activity of saline populations relative to freshwater-selected populations, where saline populations were selected for freshwater tolerance over 12 generations. Arrows depict evolutionary shifts in the freshwater populations (dashed lines), toward increased enzyme activity in fresh water (0.2 PSU) and reduced activity at high salinity (15 PSU) relative to the saline populations (solid lines). Data points are mean ± SE for six replicate treatments. Data taken from Lee *et al.* (2011).

Relative to saline populations, freshwater populations might evolve the capacity to maintain higher hemolymph osmolality at lower salinities (Fig. 2, Hypothesis A). Maintaining elevated hemolymph osmolality might be adaptive for optimal physiological functioning under freshwater conditions. The elevated activity of the ion transport enzyme V-type H^+ ATPase in the freshwater populations (Fig. 1; Lee *et al.*, 2011) might result in

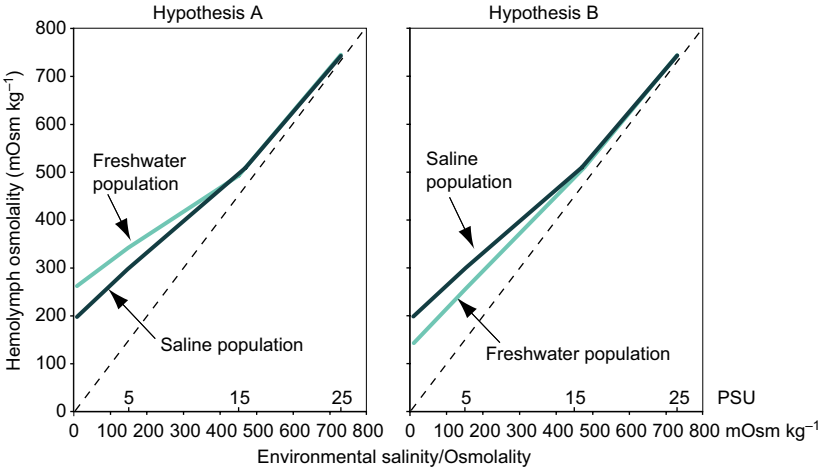

Fig. 2 Hypotheses regarding the evolution of hemolymph osmolality following freshwater invasions. Hypothesis A, increased hyperregulation: freshwater populations show evolutionary increases in hemolymph osmolality at lower salinities, relative to saline populations. Hypothesis B, reduced hyperregulation: freshwater populations show evolutionary declines in hemolymph osmolality at lower salinities, relative to saline populations. The dashed lines represent the isosmotic line, where hemolymph osmolality would equal the environmental osmolality. Higher salinity ranges are not shown on the graphs.

increased uptake of essential ions and the maintenance of enhanced hemolymph osmolality (Ahearn *et al.*, 1999; Donini *et al.*, 2007). On the other hand, freshwater populations might have evolved lowered hemolymph osmolality relative to saline populations under freshwater conditions (Fig. 2, Hypothesis B) in order to reduce the ionic concentration gradient with the environment. Such a reduction in hemolymph osmolality could reduce the energetic cost at lower salinities, especially for smaller species with relatively large surface area (Brand & Bayly, 1971; Péqueux, 1995). Under such conditions, freshwater species would need to evolve mechanisms to tolerate low hemolymph concentrations. In some comparative studies of arthropod species from different salinities, freshwater species showed lower hemolymph osmolalities relative to saline species (Brand & Bayly, 1971; Schubart & Diesel, 1999; Augusto *et al.*, 2009; Thurman *et al.*, 2010). However, these comparisons were typically not performed under common-garden conditions, and consequently did not distinguish whether differences in hemolymph osmolality among the species resulted from acclimation to native conditions (salinity) or evolutionary differences (see 'Discussion' section).

Given the hypotheses above, the goals of this study were to determine (1) whether evolutionary shifts in the regulation of hemolymph concentration occurred during invasions from saline to freshwater habitats and (2) whether parallel shifts took place during independent invasions. To determine evolutionary shifts in hemolymph concentration following saline to freshwater invasions, we measured hemolymph osmolality for ancestral saline and freshwater invading populations that were reared across a range of common-garden salinities (0.2, 5, 15 and 25 PSU) from metamorphosis until adulthood. To detect parallel evolutionary shifts across independent invasions, we examined pairs of saline and freshwater populations from two genetically distinct clades, representing two independent invasions (Fig. 3).

This study probed rapid evolution of a key functional trait following saline to freshwater transitions. Measuring hemolymph osmolality provides direct information on the magnitude of the osmotic gradient between body fluids and the environment, and consequently provides insights into the degree of regulation that would be required across different environments. An evolutionary increase in hemolymph osmolality in freshwater populations under freshwater conditions would represent evolutionary shifts toward greater regulation following invasions into freshwater habitats.

Materials and methods

Population sampling

Saline ancestral and freshwater invading populations were collected from two genetically distinct clades (Fig. 3; Atlantic, Gulf) within 2006–2008. A saline

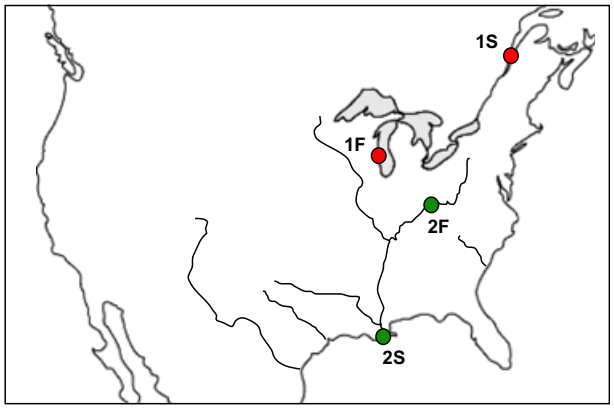

Fig. 3 Population sampling for this study, including saline and freshwater populations of *E. affinis* from the Atlantic (red, 1F and 1S) and Gulf (green, 2F and 2S) clades. 1S = St. Lawrence salt marsh (saline), 1F = Lake Michigan (fresh), 2S = Gulf of Mexico (saline) and 2F = Louisville Reservoir (fresh).

population from the Atlantic clade was collected from a salt marsh pond in Baie de L'Isle Verte, Quebec, Canada (Fig. 3, population 1S; 48°00′14″N, 69°25′31″W) adjacent to the St. Lawrence estuary at a salinity of 15 PSU (practical salinity unit, SI unit for salinity ≈ parts per thousand). Populations here are typically found at salinities ranging between 5 and 40 PSU (Lee, 1999). A saline population from the Gulf clade was collected from Blue Hammock Bayou, Fourleague Bay, LA, USA (Fig. 3, population 2S; 29°17′18″N, 91°6′59″W) at a salinity of 5 PSU. *E. affinis* is found here at salinities ranging between 1 and 15 PSU.

A freshwater population from the Atlantic clade was collected from Lake Michigan at Racine Harbor, WI, USA (Fig. 3, population 1F; 42°43′46″N, 87°46′44″W). The copepod *E. affinis* first invaded the Great Lakes in 1958 (Anderson & Clayton, 1959), approximately 200–300 generations ago (~4–5 generations year^{-1}; seasonality June–October). Conductivity, which is a finer measure of ionic concentration at lower salinities, is in the range of 300 μS cm^{-1} in Lake Michigan (Lee, unpublished data). A freshwater population from the Gulf clade was collected from McAlpine pool (reservoir) in the Ohio River at Louisville, KY, USA (Fig. 3, population 2F; 38°15′36″N, 85°45′00″W). *E. affinis* was first reported from this location in 1985 (Bowman & Lewis, 1989), approximately 70–100 generations ago (~3 generations year^{-1}; seasonality July–September). Conductivity of 338 μS cm^{-1} has been measured at this location (Westerhoff *et al.*, 2005). The usage of 'fresh water' as a noun and 'freshwater' as an adjective is adopted throughout this paper.

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, unpublished data). Therefore, shared genetic mechanisms underlying freshwater adaptation in the two clades would represent labile mechanisms evolving independently and in parallel in each clade.

Common garden experiment

To remove effects of environmental acclimation to native salinities, hemolymph osmolality was measured for saline and freshwater populations (Fig. 3) that were reared under common-garden conditions. 'Common-garden' conditions are used in evolutionary laboratory or field experiments, where different populations (or species) are placed under the same conditions to remove effects of acclimation and determine whether traits differences are due to evolutionary differences. Attempts were made to reduce effects of acclimation in order to determine the evolutionary (heritable) differences in hemolymph osmolality between the saline and freshwater populations, measured across a range of salinities (0.2, 5, 15 and 25 PSU). The 0.2 PSU treatment consisted of Lake Michigan water with conductivity of ~300 μS cm^{-1} (registering as 0.2 PSU on the salinity scale). While 'salinity' measurements of Lake Michigan water is approximately 0.2 PSU, much of the ionic load is due to high concentrations of calcium (and also sulphate) rather than sodium. At Racine Harbor, Lake Michigan, measurements of Na^+ concentration was 15 mg L^{-1}, whereas Ca^{2+} concentration was 39 mg L^{-1} in June 2007 (University of Wisconsin Soil and Plant Analysis Lab; Lee, unpublished data). Considerable effort was also made to minimize effects of selection during the experiment by minimizing mortality in response to low salinities (see below).

Prior to the common-garden experiment, saline and freshwater populations (Fig. 3) were reared at their native salinities for at least two generations in the laboratory (0.2 PSU Lake Michigan water for the freshwater populations, 5 PSU for the Gulf population, and 15 PSU for the St. Lawrence population). From each laboratory population, 30 egg clutches (embryos) were taken from females and gradually (over a couple of days) transferred to a common salinity of 5 PSU. The freshwater and saline populations were reared to metamorphosis at this common salinity to remove effects of environmental acclimation to the different native salinities. In addition, a common salinity of 5 PSU was chosen for rearing all populations from hatching to metamorphosis (the larval stage) to avoid imposing strong selection on the populations due to the more extreme treatment salinities (Lee *et al.*, 2003, 2007). We did not rear the four populations at the common salinity of 5 PSU for more than a generation in the laboratory because rearing at this salinity during an entire life cycle (including the formation of eggs) would impose selection on the freshwater populations (Lee *et al.*, 2003, 2007). While being maintained at 5 PSU from hatching to metamorphosis, all four populations were fed the saline alga *Rhodomonas salina* on a daily basis. Most of the larvae from all four populations metamorphosed to the juvenile stage after 10–11 days.

Upon reaching metamorphosis (at ~12 days), post-metamorphic juveniles from the four populations were transferred from 5 PSU to the four treatment salinities (0.2, 5, 15 and 25 PSU) and reared at these salinities until adulthood. The copepods could not be moved to these treatment salinities at an earlier larval (naupliar) stage, as they would suffer very high mortalities due to osmotic shock and undergo selection in response to salinity (Lee *et al.*, 2003, 2007). Approximately 150–200 juveniles were randomly chosen and transferred gradually to each treatment salinity. In particular, the transfer of saline populations (Gulf and St. Lawrence) from 5 to 0.2 PSU was performed over 4 days, with salinity declining by about half for each day. Survival from metamorphosis to adulthood was high (96–98%) with no observable differences among populations or treatments (salinities). Following transfers to treatment salinities, cultures at

0.2 PSU were fed the freshwater alga *Rhodomonas minuta,* while all other cultures (5, 15 and 25 PSU) were fed the nutritionally similar saline alga *R. salina*. Feeding freshwater algae to the low salinity treatment (0.2 PSU) was necessary because the saline algae would experience osmotic shock at the low salinity. Upon reaching adulthood, hemolymph osmolality was measured for adult females from each population (see next section).

Experiments were performed at 13 °C for the Atlantic clade populations. For the Gulf clade populations, experiments were performed at 15 °C, as 13 °C tends to cause high mortality in the Louisville Reservoir (Gulf clade) population. All populations were exposed to a 15L:9D photoperiod. To avoid bacterial infections all populations were treated with 20 mg L^{-1} of the antibiotic Primaxin® every fourth day.

Measurement of hemolymph osmolality

In order to determine evolutionary shifts in hemolymph osmolality between saline and freshwater populations, hemolymph osmolality (defined as the concentration of solutes in a solution) was measured from individual copepods (10–17 per population) for the four populations described above (Fig. 3). Hemolymph osmolality measurements were made for female copepods only, because the small size of males (prosome length for Lake Michigan population: males = 0.657 mm ± 0.027 SE, females = 0.790 mm ± 0.018 SE) made it difficult to extract a sufficient volume of fluid from single individuals. Hemolymph osmolality was determined in adult females after 7–18 days of exposure to the treatment salinities (0.2, 5, 15 and 25 PSU). Exposure at 0.2 PSU was shorter for the saline populations (7 days) because transitions from high to low salinity were performed over several days to avoid osmotic shock and selection (see previous section). The exposure time period was long enough to allow for osmotic stabilization in each medium which, in small-sized individuals, requires only a few hours (reviewed in Charmantier *et al.*, 2009). The copepods were superficially dried on filter paper, and then quickly immersed in mineral oil to avoid evaporation and desiccation. Remaining adherent water was removed using a glass micropipette. Another hand-made micropipette was then inserted into the dorsal sinus to sample the hemolymph. Hemolymph osmolality was measured with the medium osmolality on a Kalber–Clifton nanoliter osmometer (Clifton Technical Physics, Hartford, CT, USA) requiring about 30 nL.

Statistical analyses

For each clade (Atlantic and Gulf) effects of population, salinity, and population × salinity interaction on hemolymph osmolality were analysed in a linear model framework using the PROC GLM Procedure in SAS 9.1 (SAS, 2003). Assumptions of normality of distribution of the data and constancy of variance were not violated (Sokal & Rohlf, 1995). A *post hoc* Tukey–Kramer test was used to determine differences in hemolymph osmolality between saline and freshwater populations at each salinity (0.2, 5, 15 and 25 PSU).

Results

Evolutionary shifts in hemolymph osmolality

Freshwater populations of *E. affinis* exhibited the evolution of increased hemolymph osmolality (by 16–31%) at low salinities relative to their saline (brackishwater) ancestors (Fig. 4; Table 1). This pattern conformed to Hypothesis A (Fig. 2) of increased hyperregulation following freshwater invasions. Moreover, the two genetically distinct clades (Fig. 3, Atlantic, Gulf) showed parallel evolutionary shifts in hemolymph osmolality across independent freshwater invasions (Fig. 4a and b; Table 1).

Overall, saline and freshwater populations differed significantly in hemolymph osmolality for both the Atlantic and Gulf clades, indicating evolutionary shifts in body fluid regulation between saline and freshwater populations (Fig. 4) (ANOVA, main effect of population; Atlantic: $F_{1,80} = 16.92$, $P < 0.0001$; Gulf: $F_{1,94} = 63.00$, $P < 0.0001$). Salinity had significant effects on hemolymph osmolality for both clades (ANOVA; Atlantic: $F_{3,80} = 1896.36$, $P < 0.0001$; Gulf: $F_{3,94} = 5451.47$, $P < 0.0001$). Population by salinity interaction was significant for both clades, indicating evolutionary shifts in osmoregulatory response across salinities (slope of response) between the saline and freshwater populations (ANOVA; Atlantic: $F_{3,80} = 11.87$, $P < 0.0001$; Gulf: $F_{3,94} = 17.52$, $P < 0.0001$).

All populations appeared to hyperregulate more extensively below 15 PSU, showing greater hemolymph concentration relative to the environment, while remaining relatively isosmotic in the 15–25 PSU range (Fig. 4). However, the freshwater populations showed a greater extent of hyperregulation at lower salinities than their saline ancestors, indicating an evolutionary shift toward greater ionic and osmotic regulation (Fig. 4; Table 1). Freshwater populations (Lake Michigan and Louisville Reservoir) showed evolutionary increases in hemolymph osmolality, relative to their saline ancestral populations (St. Lawrence and Gulf), at the lower salinities of 0.2 and 5 PSU (Table 1, Tukey–Kramer $P < 0.001$; Fig. 4). Under freshwater conditions, the freshwater populations exhibited minimum hemolymph osmolalities of ~240–260 mOsm kg^{-1}, whereas minimum hemolymph osmolality was about 200 mOsm kg^{-1} in the saline populations (Fig. 4; Table 1). In contrast, at higher salinities of 15 and 25 PSU hemolymph osmolalities of the saline and freshwater populations were very similar to one another (Table 1, Tukey–Kramer $P > 0.50$; Fig. 4), indicating no evolutionary change at these salinities.

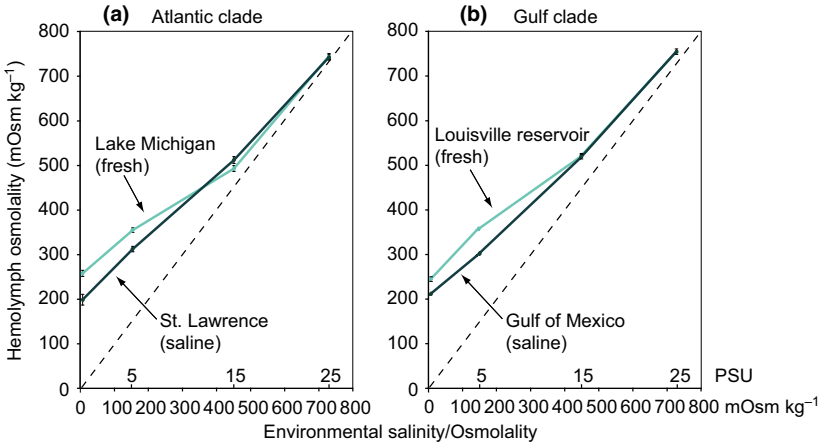

Fig. 4 Evolutionary shifts in hemolymph osmolality from saline to freshwater habitats. Parallel evolutionary shifts were apparent for independent invasions in the two genetically distinct (a) Atlantic and (b) Gulf clades. The freshwater populations in Lake Michigan and Louisville reservoir (light lines) showed significantly elevated hemolymph osmolalities at lower salinities (0.2 and 5 PSU) relative to their saline ancestors in the St. Lawrence salt marsh and Gulf of Mexico (dark lines) (Tukey-Kramer, $P < 0.001$). The dashed lines represent the isosmotic line, where the environmental and hemolymph osmolalities would be equivalent. The independent axis shows environmental osmolality in mOsm kg^{-1} and salinity in PSU (practical salinity units ≈ parts per thousand). The data points are mean osmolality ± standard error for 10–17 adult females.

Table 1 Hemolymph osmolality (mOsm kg^{-1}) of saline (brackish) and freshwater populations of the copepod *Eurytemora affinis* across a range of treatment salinities or osmolalities.

Environmental salinity (PSU)	0.2	5	15	25
Osmolality (mOsm kg^{-1})	7	150	455	737
(a) Atlantic clade populations				
St. Lawrence (saline)	198.82 ± 12.05 (11)	312.33 ± 6.13 (12)	512.00 ± 7.67 (10)	743.00 ± 6.67 (10)
Lake Michigan (fresh)	257.83 ± 6.90 (12)	354.83 ± 4.99 (12)	493.00 ± 6.46 (10)	745.45 ± 5.29 (11)
P-value (St. Lawrence vs. L. Michigan)	< 0.0001	0.0011	0.645	1.000
(b) Gulf clade populations				
Gulf of Mexico (saline)	207.18 ± 3.82 (17)	294.50 ± 4.46 (12)	514.36 ± 4.72 (11)	753.00 ± 5.12 (10)
Louisville Reservoir (fresh)	239.53 ± 4.69 (17)	352.00 ± 3.99 (12)	517.17 ± 3.79 (12)	752.27 ± 4.28 (11)
P-value (Gulf vs. Louisville)	< 0.0001	< 0.0001	0.999	1.000

Values are mean ± SE. Sample sizes are in parentheses. *P*-values are for Tukey–Kramer comparisons between saline and freshwater populations at each salinity. 0.2 PSU salinity here refers to Lake Michigan water with a conductivity of 300 μS cm^{-1}

Discussion

Evolutionary shifts in body fluid regulation

The saline to freshwater transition represents a formidable barrier that most invertebrate species have been unable to penetrate (Hutchinson, 1957). Yet, the copepod *E. affinis* has been able to breach this boundary multiple times independently, within decades in the wild (Lee, 1999; Lee *et al.*, 2003, 2011) and a few generations in laboratory selection experiments (Lee *et al.*, 2011). Such invasions impose serious challenges for ionic and osmotic regulation, in terms of acquiring ions from dilute solutions against steep concentration gradients. Previous studies have found evolutionary increases in freshwater tolerance and in ion uptake activity following freshwater invasions by *E. affinis* (Lee *et al.*, 2003, 2007, 2011). In this study, contrasts between ancestral saline and derived freshwater populations suggest that increases in body fluid regulation could evolve very rapidly following contemporary habitat shifts (Fig. 4; Table 1).

This study revealed the evolution of increased body fluid regulation at low salinities, in terms of increased hemolymph osmolality in freshwater populations of *E. affinis* relative to their saline progenitors (Fig. 4; Table 1). This evolutionary increase conformed to Hypothesis A, of greater hyperregulation associated with freshwater adaptation (Fig. 2). This pattern was consistent with the increased activity of ion uptake enzyme V-type H^+ ATPase observed in freshwater populations under freshwater conditions (Fig. 1) (Lee *et al.*, 2011). Moreover, the parallel evolutionary shifts in hemolymph osmolality that we observed in the two independently derived freshwater populations (Fig. 4a and b) were

concordant with the parallel evolutionary increases in V-type H^+ ATPase activity and expression found in those same freshwater populations (Lee *et al.*, 2011).

The key question is why the freshwater populations would undergo an evolutionary increase in ionic regulation (in fresh water) relative to the saline populations. In freshwater environments, the need for ion absorption increases due to elevated diffusional loss of ions from animals in fresh water. Additionally, ion uptake becomes more energetically costly as the fluid becomes more dilute. Moreover, the evolutionary increase in hemolymph osmolality in the freshwater-adapted populations (Fig. 4) would require an even greater energetic cost associated with the increase in ionic regulation in fresh water. Elevated hemolymph osmolality might be critical for survival under freshwater conditions, given that saline populations of *E. affinis* cannot be sustained in fresh water across generations without imposing strong selection. Maintaining elevated hemolymph osmolality in fresh water might be important for preserving enzymatic function and easing the burden of cell volume regulation.

The transition from saline to freshwater habitats requires a switch in physiological strategy for maintaining hemolymph concentration, from one of relative conformation (of osmotic pressure and ionic concentration) in saline environments to that of increased osmo- and iono-regulation in freshwater habitats. Hemolymph is the extracellular body fluid that forms the buffer between the variable external environment and the cell. The cell maintains a relatively constant ionic composition across taxa and environments, whereas osmotic concentration (osmolality) of the cell varies to a greater degree and is kept close to that of the hemolymph in order to maintain near-constant cell volume (Péqueux, 1995; Willmer *et al.*, 2004; Charmantier *et al.*, 2009). On the other hand, both the ionic and osmotic concentrations of hemolymph may vary greatly according to the environment, especially for osmoconformers (Willmer *et al.*, 2004). Most taxa in the sea are osmoconformers (e.g. most marine invertebrates) and tend to have hemolymph osmolalities that match that of the environment (i.e. they are isosmotic). In contrast, osmoregulators (e.g. most vertebrates) tend to show much more constant hemolymph or blood osmolalities across a broad range of salinities, maintaining gradients with the environment (Willmer *et al.*, 2004). Invertebrate species that live in fluctuating salinities tend to be hyper–hypo-osmoregulators (e.g. many estuarine crustaceans); that is, they are isosmotic across intermediate salinities (ca. 15–30 PSU), but then hyperregulate at lower salinities (hemolymph osmolality > ambient water) and hyporegulate at higher salinities (hemolymph osmolality < ambient water). In general, freshwater species face serious challenges in very dilute environments of maintaining hyperosmotic body fluid concentrations against steep concentration gradients with the environment. Such gradients are maintained by mechanisms such as increased ion uptake, reduced ion efflux, or excretion of dilute urine (Péqueux, 1995; Patrick *et al.*, 2001; Willmer *et al.*, 2004; Augusto *et al.*, 2007; Charmantier *et al.*, 2009; Lee *et al.*, 2011).

Based on our results and those of Roddie *et al.* (1984), saline populations of *E. affinis* displayed patterns of hemolymph osmolality typical of hyper–hypo-osmoregulating estuarine species (Fig. 4; Table 1) (Roddie *et al.*, 1984). The saline (brackish) populations in this study were close to isosmotic in the 15–25 PSU salinity range and became increasingly hyperosmotic below 15 PSU, with a minimum hemolymph osmolality of about 200 mOsm kg^{-1} (Fig. 4; Table 1), similar to results of Roddie *et al.* (1984). While we did not measure hemolymph osmolalities above 25 PSU, we would expect regulation to be hyposmotic above this salinity, as found by Roddie *et al.* (1984).

Few studies have examined evolutionary shifts in osmoregulation across a habitat cline. One study did find evolutionary differences in hemolymph osmolality between anadromous and freshwater landlocked populations of the South American shrimp *Macrobrachium amazonicum* (Charmantier & Anger, 2011). While the two populations showed similar hemolymph osmolalities at low salinities across most life history stages, the freshwater population showed the loss of ability to hyporegulate at higher salinities (Charmantier & Anger, 2011). Most previous comparative studies did not uncover evolutionary differences in hemolymph regulation, as they tended to assay animals directly from the field or acclimate adults to common treatment salinities for a few days to a few weeks prior to measurement assays (Bayly, 1969; Brand & Bayly, 1971; Schubart & Diesel, 1999; Freire *et al.*, 2003; Augusto *et al.*, 2007, 2009; Thurman *et al.*, 2010). As such, these studies would not have removed effects of developmental acclimation to native salinities. Rearing conditions during development could have profound effects on adult physiology (Huey & Berrigan, 1996; Huey *et al.*, 1999; Lee & Petersen, 2003). In addition, measurements of hemolymph of animals collected from the wild, or acclimated for short periods, would likely be confounded by pre-existing ion stores, which might dissipate very slowly over time (Beadle & Shaw, 1950; Lockwood, 1959; Scheide & Dietz, 1982; Wilcox & Dietz, 1995; Lin *et al.*, 2002).

While this study focused on osmoregulation in adults, it would be worth investigating the evolutionary shifts in body fluid regulation of the larval (naupliar) stages (although this would be difficult due to small size). Previous results revealed that the naupliar stages of *E. affinis* are much more susceptible to mortality under low ionic conditions than adults (Lee *et al.*, 2003, 2007, 2011). In addition, hemolymph osmolality has been found to vary among life history stages in some crustaceans (Guerin & Stickle, 1997; Charmantier, 1998; Charmantier & Anger, 2011). Given the small size of copepod larval stages (~300 μm diameter), and larger surface area relative to volume, osmotic and ionic regulation of hemolymph might be much more difficult

and energetically costly to maintain in fresh water at the early life history stages.

The evolution of increased body fluid regulation would entail increases in energetic costs in freshwater habitats. A higher ionic or osmotic gradient with the environment at lower salinities would require greater rates of ion uptake and/or reduced ion efflux (e.g. lower integument permeability). Higher energetic costs of osmoregulation are apparent from the increase in oxygen consumption of copepods at salinities above and below the isosmotic range, where they are hyper- or hypo-osmoregulating (Lance, 1965; Gyllenberg & Lundquist, 1978, 1979). Adequate food consumption might be critical to fuel the energetic costs associated with increased ionic and osmotic regulation. The increased energetic requirements might explain why invaders from brackishwater environments tend to invade freshwater habitats with elevated food availability or harbouring specific types of algae (van den Brink *et al.*, 1993; Vanderploeg *et al.*, 1996; Lauringson *et al.*, 2007).

Increases in body fluid regulation, and associated increases in energetic costs, are problems that face organisms as they become farther removed from the sea (Withers, 1992; Willmer *et al.*, 2004). This study is the first to reveal evolutionary shifts in body fluid regulation across a habitat cline, following rapid transitions from saline into freshwater habitats. Overall, our results are consistent with the evolution of increased physiological regulation accompanying transitions into stressful habitats.

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