Genotype-by-Environment Interaction for Salinity Tolerance in the Freshwater-Invading Copepod *Eurytemora affinis*

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ABSTRACT

This study examined the extent of phenotypic plasticity for salinity tolerance and genetic variation in plasticity in the invasive copepod *Eurytemora affinis*. *Euryemora affinis* is a species complex inhabiting brackish to hypersaline environments but has invaded freshwater lakes and reservoirs within the past century. Reaction norm experiments were performed on a relatively euryhaline population collected from a brackish lake with fluctuating salinity. Life history traits (hatching rate, survival, and development time) were measured for 20 full-sib clutches that were split and reared at four salinities (fresh, 5, 10, and 27 practical salinity units [PSU]). On average, higher salinities (10 and 27 PSU) were more favorable for larval growth, yielding greater survival and faster development rate. Clutches differed significantly in their response to salinity, with a significant genotype-by-environment interaction for development time. In addition, genetic (clutch) effects were evident in response to low salinity, given that survival in fresh (lake) water was significantly positively correlated with survival at 5 PSU for individual clutches. Clutches raised in fresh water could not survive beyond metamorphosis, suggesting that acclimation to fresh water could not occur in a single generation. Results suggest the importance of natural selection during freshwater invasion events, given the inability of plasticity to generate a freshwater phenotype, and the presence of genetic variation for plasticity upon which natural selection could act.

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Introduction

Phenotypic plasticity is a change in the average phenotype expressed by a genotype in different environments (Bradshaw 1965) and is a key trait affecting fitness of organisms in changing environments. How phenotypic plasticity evolves is controversial (Via et al. 1995), but all hypothesized mechanisms require genetic variation among individuals. The set of phenotypes expressed in different environments by a genotype is called the "reaction norm" of that genotype. Varying effects of environment on phenotype among genotypes result in variation in reaction norms and a significant genotype-by-environment (G × E) interaction. G × E interaction is the type of genetic variation required for the evolution of phenotypic plasticity (Via and Lande 1985).

Few studies have distinguished rigorously between phenotypic plasticity and selection operating on physiological polymorphisms within populations. For invasive species, a few studies have examined the role of phenotypic plasticity during range expansions (Weinig 2000*a*, 2000*b*), but this topic has been inadequately explored. Some investigators have argued that "eurytolerance" (broad physiological tolerance) could explain the ability to invade (Baker 1965; Strayer 1999; Wolff 2000). Their argument becomes circular because eurytolerance of natural populations is often inferred from geographic distributions rather than from rigorous common garden experiments. In fact, what these investigators call eurytolerance is, in many cases, the result of natural selection (see critique by Davis and Shaw [2001] of Baker's [1965] concept of "general purpose" genotypes). Broad tolerances and plasticity might play critical roles under certain circumstances during invasions but often are unable to account fully for the ability to invade (Lee 2002).

This study examined the extent of phenotypic plasticity in salinity tolerance and genetic variation for phenotypic plasticity in the invasive copepod *Eurytemora affinis*. *Eurytemora affinis* is a sibling species complex (Lee 2000) that has invaded fresh water independently within and among genetically distinct clades throughout its range—North America, Asia, and Europe. This species complex occurs in saline and hypersaline salt marshes (25–40 practical salinity units [PSU] = parts per thousand in mass) and brackish estuaries and lakes (0.5–25 PSU) but has invaded freshwater lakes and reservoirs (≤0.5 PSU) within the past 100 yr (Lee 1999), probably as a result of human activity. Each genetically divergent clade (or sibling species) contains populations in diverse habitats that vary in salinity, except for the clade in the Pacific Northwest of North America,

which has no known freshwater descendants (Lee 2000). The pattern of freshwater invasions raises several questions, such as the following: What is the relative importance of phenotypic plasticity versus natural selection during freshwater invasion events? Is there genetic variation in phenotypic plasticity upon which natural selection can act? Can freshwater tolerance be induced through developmental acclimation in a single generation?

Thus, specific goals of this study were to determine (1) the presence of genetic variation for optimal developmental salinity; (2) the extent of plasticity, that is, whether plasticity could induce freshwater tolerance in a single generation; and (3) the presence of genetic variation for phenotypic plasticity. To achieve these goals, reaction norms were determined for a range of salinities (fresh, 5, 10, and 27 PSU) using a population of *E. affinis* from a brackish lake with fluctuating salinity (Edgartown Great Pond, Martha's Vineyard, Mass.). This population was chosen because it was more tolerant of fresh water than other saline populations tested (Lee 1999). Genetic variation for phenotypic plasticity was measured using two statistical approaches. First, pairwise genetic correlations of survival and development time were determined among split broods reared in four salinities to determine whether these traits were distinct at different environments (Falconer 1952; Bradley 1986; Via 1994). Second, the significance of the genotype (clutch)-by-environment (salinity) interaction component of variance was determined for development time using a mixed-model analysis of variance (ANOVA; Fry 1992) followed by a likelihood-ratio test.

Material and Methods

Study Population

Eurytemora affinis (Poppe 1880) was collected in May 1996 from Edgartown Great Pond, Martha's Vineyard, Massachusetts. This population occurs in a large brackish lake separated from the ocean by a sandbar. A few hundred copepods were collected by towing a 100-μm plankton net. The sample used for this study was collected when the lake was at a salinity of 11 PSU. This population was chosen for this study because it was more tolerant of fresh water than other saline populations tested and achieved a 1.1% survival to metamorphosis over two generations in fresh water (Lee 1999).

Hatching, Survival, and Development Time

Populations were maintained at 10 PSU in a 13°C environmental chamber for at least two generations before the experiments. Juvenile females were paired individually with adult males to produce full-sib clutches. Twenty egg sacs were excised from adult females with a pin and quartered into four salinity treatments (about four to 11 eggs each) of lake water and 5, 10, and 27 PSU. The treatment salinity of 10 PSU was chosen as the control because it was proximate to the salinity at which the population was found. Treatment water was made from mixtures of water from Lake Union or Lake Washington in Seattle and Puget Sound, Washington (27 PSU). Lakes Union and Washington range from 100 to 400 μS/cm in conductivity, which is approximately equivalent to 0.05–0.2 PSU.

Each clutch of eggs was placed in a 20-mL scintillation vial and maintained one-third-full of fluid with caps left ajar to allow for oxygen exchange. Vials were kept in a 13°C environmental chamber maintained on an 8D : 16L light cycle. To avoid altering salinity of treatments and to provide algal cells that would not burst because of osmotic shock, copepods in saline water were fed a mixture of estuarine algae (*Rhodomonas* sp., *Isochrysis galbana*, *Thalassiosira pseudonana*), while those in fresh water were fed a mixture of freshwater algae (*Scenedesmus* sp., *Ankistodesmus* sp., *Chlamydomonas* sp.). Because different food sources were used, two separate controls (at 10 PSU) were maintained on either marine or freshwater algae. Developing copepods were fed in excess every 2 or 3 d. Every 10 d, 50% of the water was replaced. All water and vials were autoclaved before use, and algal cultures were axenic.

Visual inspection of vials was performed daily. Development time and survivorship were measured at three life history stages: on hatching, at metamorphosis (to copepodid 1 stage), and on reaching adulthood. Individuals were classified as adults when males developed geniculate right antennules and females developed large winglike processes on the posterior end of their prosome (body). The number of clutches measured for development time depended on number that survived. Effects of salinity on survival and development time were determined using ANOVA. Hatching and survival data were arcsine transformed to approach normal distributions (Zar 1999).

Extent of Developmental Plasticity

If invasions of fresh water by *E. affinis* were the result of euryhalinity, or broad tolerance (Wolff 2000), one would expect individuals from a brackish habitat to survive and reproduce under freshwater conditions, without suffering high mortalities due to natural selection. Hatching rate and number of days to survival were observed for the treatment placed in fresh water and were compared with 10 PSU controls reared on freshwater algae.

Clutch Effect on Salinity Tolerance and Performance

ANOVA was performed within and among clutches to determine whether clutches differed significantly in development time and survival time in fresh water (~0.2 PSU). In addition, correlations were determined between survival time in fresh water and development time and survival at 5 PSU to ascertain whether clutch effects persisted across both low-salinity treatments. Survival time was the only meaningful measure of per-

formance in fresh water because hatching rate did not predict survival, and survival to metamorphosis was nearly zero. For 5 PSU, survival and development time to metamorphosis were used because survival to adulthood at 5 PSU was very low, leading to small sample size. Survival data were arcsine transformed to approach a normal distribution.

Genetic Variation in Plasticity

Genetic Correlations across Salinity Treatments. Genetic correlations in response (survival, development time) across different salinities were used to determine genetic variance in response to the environment (Via and Lande 1985; Falconer and Mackey 1996). These correlations indicate whether tolerance and performance at different salinities are related traits, with a positive correlation suggesting that acclimation to high and low salinities involves the same genes or alleles (Falconer and Mackey 1996). If genetic correlations across environments are high and positive, then individuals can be considered generalists. In contrast, negative genetic correlations in performance are indicative of variation among individuals and potential trade-offs in different environments (Via 1984). Pairwise correlations between three salinities (5, 10, and 27 PSU) were determined for percentage survival to metamorphosis and adult stages for 20 clutches. Pairwise correlations were also determined between salinities for mean development time to metamorphosis and adult stages, with the number of clutches dependent on survivorship. The lowest salinity treatment (~0.2 PSU) was not used because of lack of survivors to metamorphosis.

Genotype (*Clutch*)*-by-Environment* (*Salinity*) *Interaction.* The G × E interaction term was computed to determine variation in response to salinity according to genotype upon which natural selection could operate (Via and Lande 1985; Via 1994). Interaction terms were computed for development time but not for survival data because survival was determined as proportions of survivors for each clutch. A mixed-model two-way ANOVA (Fry 1992) was performed to determine effects of genotype (clutch) and environment (salinity) on development time (to metamorphosis and adult) using the Proc Mixed option in the statistical software package SAS (version 4.0; SAS Institute, Cary, N.C.). Independent variables were "clutch," designated as a random factor (assumed to be randomly selected from an infinite population of possible levels), and "salinity," designated as the fixed factor (levels are predetermined). The G × E term was also designated as random. To determine significance of the G × E term, likelihood-ratio tests were performed between models with and without the random interaction effect. Replication was not equal because survivorship of hatchlings varied among clutches. Full-sib clutches were assumed to represent distinct genotypes. This was a reasonable assumption given that juvenile virgin females were paired with different males from a genetically diverse population to yield the clutches.

Results

Hatching and Survival in Response to Salinity

Patterns of hatching and survival indicated that (1) salinity had no significant effect on hatching success but did have significant effects on (2) survival to metamorphosis and (3) survival to adulthood (Fig. 1). Fresh water had profoundly adverse effects on survival, with only one clutch with survivors that reached metamorphosis and none that reached adulthood.

Overall, hatching success (percentage hatching within clutches) did not vary significantly among salinities (Fig. 1*A*; ANOVA, $N = 19$ clutches, $F = 1.672$, $P = 0.181$). In addition, a multiple comparison among means (Tukey's test, $\alpha = 0.05$) found no significant difference in hatching rate between salinities. Salinity did have an overall significant effect on survival to metamorphosis (ANOVA, $N = 19$ clutches, $F = 11.27$, $P < 0.0001$) but only because survival in fresh water was nearly zero. Salinities of 5, 10, and 27 PSU had no significant effect on survival to metamorphosis (ANOVA, $N = 19$ clutches, $F = 2.22$, $P = 0.12$). In addition, a multiple comparison among means (Tukey's test, $\alpha = 0.05$) found no significant difference in survival to metamorphosis between these salinities (5, 10, and 27 PSU). In contrast, salinities other than fresh water (5, 10, and 27 PSU) did have a significant effect on survival to the adult stage (ANOVA, $N = 19$ clutches, $F = 5.52$, $P = 0.0066$). A multiple comparison among means (Tukey's test, $\alpha = 0.05$) showed significant differences in survival to adulthood between 5 and 10 PSU ($q = 2.99$) and between 5 and 27 PSU ($q = 2.70$). Even though mean survival to adulthood did not differ significantly between 10 and 27 PSU (Fig. 1*A*; Tukey's $q = -14.36$), individual clutches did differ greatly in response, with some clutches having greater survival at either 10 or 27 PSU (Fig. 1*B*).

Development Time in Response to Salinity

Development time decreased with increasing salinity (Fig. 2), indicating that higher salinities (10 and 27 PSU) were more favorable for development than a lower salinity (5 PSU). Development time could not be measured in fresh water because only one clutch contained survivors to metamorphosis. Salinity had significant effects on development time to metamorphosis (ANOVA, $N = 8$ clutches, $F = 44.21$, $P < 0.0001$; Fig. 2*A*) and to adulthood (ANOVA, $N = 5$ clutches, $F = 5.97$, $P = 0.0052$; Fig. 2*A*). A multiple comparison among means (Tukey's test, $\alpha = 0.05$) found significant differences between all salinities for development time to metamorphosis (5 vs. 10 PSU, $q = 1.56$; 5 vs. 27 PSU, $q = 3.77$; 10 vs. 27 PSU, $q = 0.877$). For development time to adulthood, a multiple comparison among means (Tukey's test, $\alpha = 0.05$) yielded significant dif-

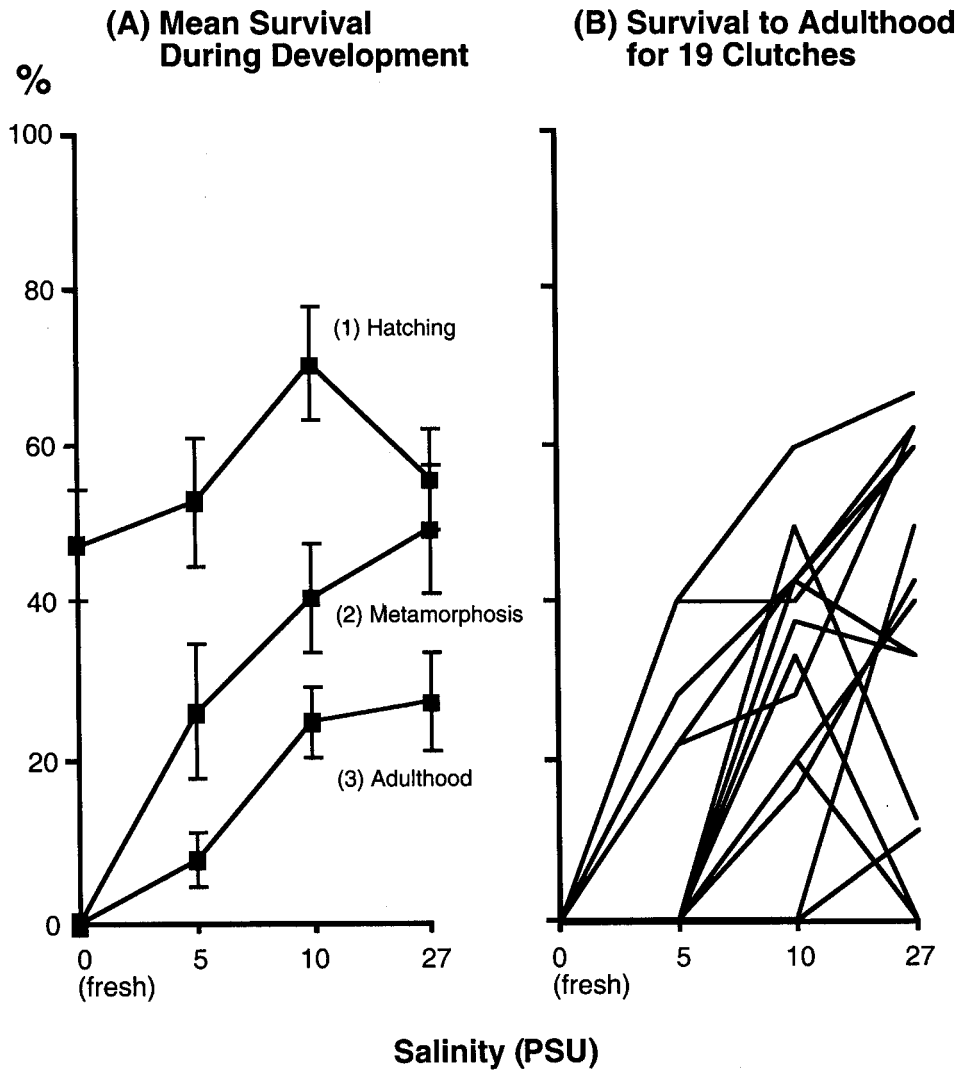

Figure 1. Hatching and survivorship in response to salinity at three life history stages. *A*, Mean hatching and survival during development at four salinities (fresh, 5, 10, and 27 PSU) in terms of (*1*) percentage of total number of eggs that hatched per replicate clutch, (*2*) percentage of total number of eggs that survived to metamorphosis, and (*3*) percentage of total number of eggs that survived to adulthood. Values are means of 19 replicate clutches ± SE (one clutch had no hatching or survivors). *B*, Percentage survival to adulthood for 19 individual clutches. Some clutches had zero survival to adulthood at all salinities.

ferences only between 5 and 27 PSU ($q = 2.74$). In spite of the general pattern of faster development at higher salinities, individual clutches varied in developmental response to salinity (Fig. 2*B*; see statistics below for clutch effects and G × E interaction).

Development in Fresh Water

The degree of developmental plasticity was not sufficient to produce adults that could survive freshwater conditions in one generation. Although proportion of larvae that hatched in fresh water was fairly high, and not significantly different from that of 10 PSU controls (Fig. 1*A*; see "Hatching and Survival in Response to Salinity"), fresh water did have adverse effects on survival and development. Larvae died on average 2.97 ± 0.55 (SE; $N = 20$ clutches) d after hatching and survived to metamorphosis in only one clutch. The diet of freshwater algae is unlikely to have prevented metamorphosis in fresh water, given that the controls (10 PSU) that were fed freshwater algae developed normally and metamorphosed after 17.5 ± 1.18 d. However, diet might still play an important role in development, given that controls fed marine algae developed signifi-

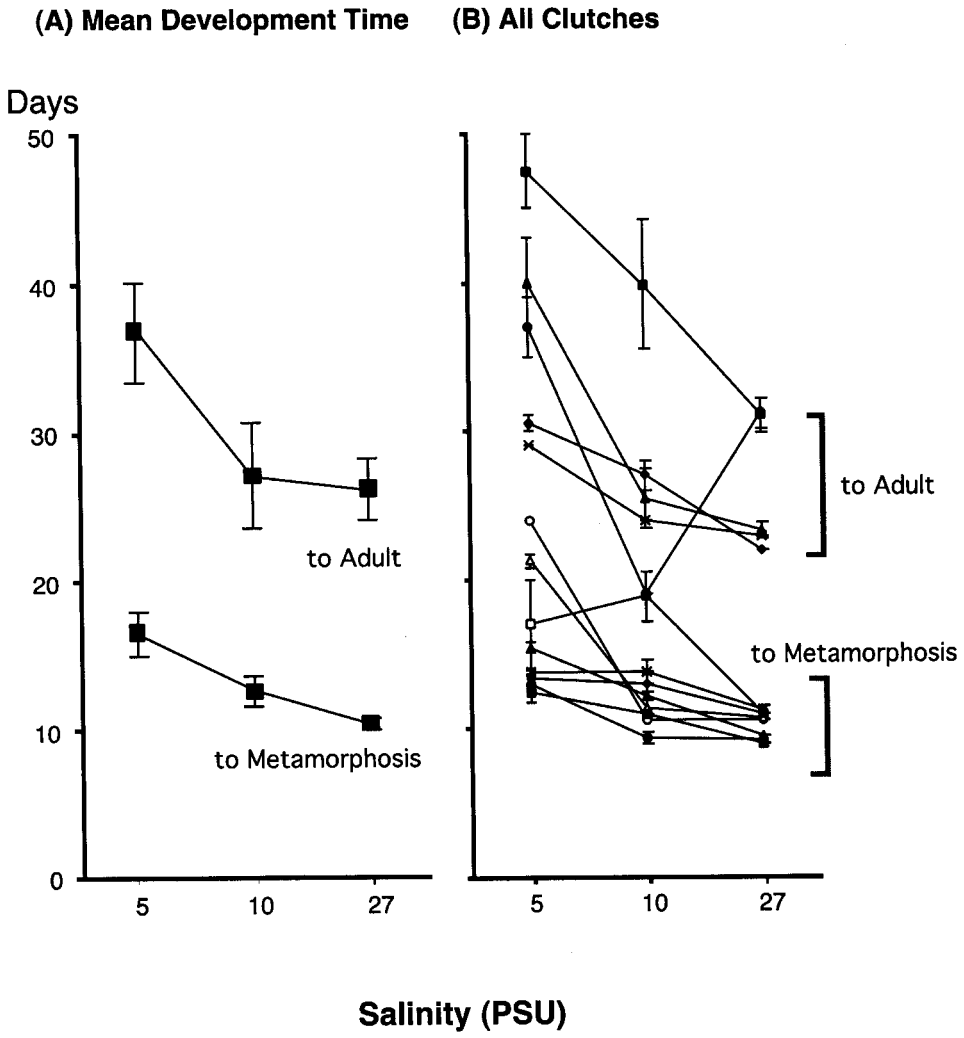

Figure 2. Development time to metamorphosis and adult stages in response to salinity. *A*, Mean development time (in days) to metamorphosis (eight clutches) and to adulthood (five clutches) at three salinities (5, 10, and 27 PSU). Values are means of replicate clutches ± SE. *B*, Development time for individual clutches. Each symbol represents a different clutch, with filled symbols corresponding to clutches for which data exist for both metamorphosis and adult stages. This figure shows results only for clutches that had survivors at all three salinities (5, 10, and 27 PSU). Values are means of individuals within clutches ± SE.

cantly faster than those fed freshwater algae and metamorphosed after 13.2 ± 0.79 d (Student's $t = -3.14$, df = 23, $P = 0.005$). This difference in developmental rate might have resulted from differences in nutritional value of the algae or from osmotic shock experienced by the freshwater algae in saline (10 PSU) water, resulting in lower availability of food.

One clutch was unusual in achieving metamorphosis in fresh water and also in having greater survival and faster development at low salinity. In this particular clutch, all 10 eggs were able to hatch in fresh water, and five reached metamorphosis quickly, after 14.4 ± 0.4 d ($N = 5$). This same clutch had the fastest development time at the low-salinity treatment (5 PSU), reaching adulthood at 12.7 ± 0.3 d ($N = 3$ individuals) in contrast to a mean development time of 29.7 ± 4.2 d for eight surviving clutches. Although these results pertain only to one clutch, the high performance at lower salinities is a stark contrast to the general trend and is consistent with a significant clutch effect and G × E interaction among clutches (see next two sections).

Clutch Effect on Salinity Tolerance and Performance

There was significant variation among clutches in development time to metamorphosis (Fig. 2*B*; ANOVA, salinity fixed, genotype random; $F = 10.24$, $P = 0.0018$) and to adulthood

(Figs. 2*B*, 3; ANOVA, salinity fixed, genotype random; $F = 7.08$, $P = 0.017$). In addition, clutches differed significantly in survival time in the freshwater treatment (ANOVA, $N = 20$, $F = 24.31$, $P < 0.0001$). The strong clutch effect suggests that salinity tolerance and performance are heritable traits.

Variation among clutches conceivably could have been induced by vial-specific environmental factors, but such effects were not apparent in this study. Clutch effects persisted across low-salinity treatments, especially in response to low-salinity stress (Figs. 3, 4). For instance, clutches that lived longer in fresh water tended to have shorter developmental times to metamorphosis at 5 PSU (Fig. 4*A*; $N = 11$ clutches, $r = -0.609$, $P = 0.047$). In addition, clutches that lived longer in fresh water also had greater survival to metamorphosis at 5 PSU ($N = 12$ clutches, $r = 0.621$, $P = 0.031$). The correlation was stronger when clutches with 0% survival were removed from the analysis (Fig. 4*B*; $N = 5$ clutches, $r = 0.956$, $P = 0.01$).

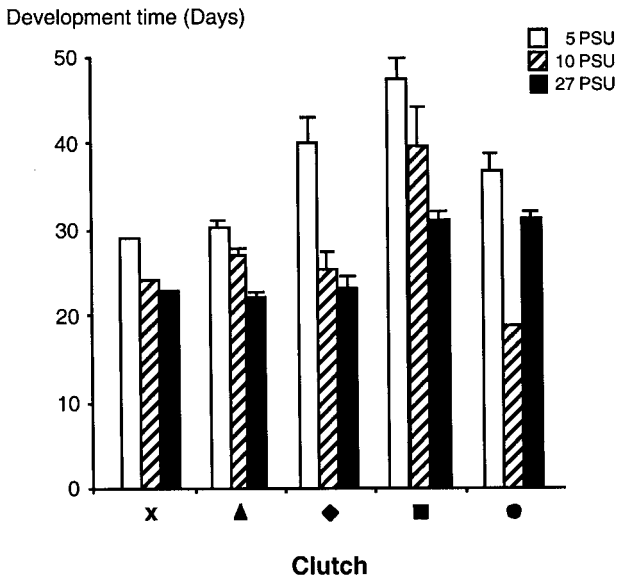

Figure 3. Development time to adulthood for five clutches at three salinities (5, 10, and 27 PSU). Values are mean development time (in days) of individuals within clutches ± SE. Symbols for clutches correspond to those in Figure 2. For bars without error shown, adulthood was reached on the same day.

Genetic Variation in Plasticity

Genetic Correlations across Salinity Treatments. In general, there were significant positive correlations for clutches between salinity treatments for survival but not for development time. Survival to metamorphosis was strongly positively correlated between salinities, between 5 and 10 PSU ($r = 0.70$, $P = 0.0009$), 5 and 27 PSU ($r = 0.50$, $P = 0.028$), and 10 and 27 PSU ($r = 0.71$, $P = 0.0007$). Correlations among different salinities were weakly positive for survival to adulthood but still significant between 5 and 10 PSU ($r = 0.52$, $P = 0.024$), 5 and 27 PSU ($r = 0.70$, $P = 0.0009$), and 10 and 27 PSU ($r = 0.46$, $P = 0.0473$). None of the correlations for mean development time to metamorphosis and adult stages were significant consistent with the divergent patterns of development time evident among clutches (Fig. 2*B*). For development time to metamorphosis (using means of eight clutches), pairwise correlations were slightly negative between 5 and 10 PSU ($r = -0.053$, $P = 0.9$), weakly positive between 5 and 27 PSU ($r = 0.34$, $P = 0.41$), and positive between 10 and 27 PSU ($r = 0.59$, $P = 0.12$). For development time to adulthood (using means of five clutches), correlations were positive but not significant between 5 and 10 PSU ($r = 0.67$, $P = 0.22$), 10 and 27 PSU ($r = 0.22$, $P = 0.72$), and 5 and 27 PSU ($r = 0.67$, $P = 0.22$). This lack of correlation for development time across salinities suggests that performance at different salinities are not related traits (Falconer and Mackey 1996) and is consistent with a significant G × E interaction (see next section).

Genotype × Environment Interaction Term. Genotype (clutch) × environment (salinity) interaction was significant for development time to metamorphosis and to adulthood. Because genotype and G × E were random effects, *F*-statistics and *P* values were not calculated (Pinheiro and Bates 2000). To test the significance of the G × E term, likelihood-ratio tests were performed between the models with and without the random interaction effects. The G × E interaction was significant for development time to both metamorphosis ($N = 8$ clutches, $\chi^2 = 65.6$, $P < 0.0001$) and adulthood ($N = 5$ clutches, $\chi^2 = 7.21$, $P = 0.0072$). The interaction was visibly evident from intersecting reaction norms of individual clutches (Fig. 2*B*). The significant G × E interaction and lack of positive correlation among salinity treatments (see previous section) for development time indicate the presence of genetic variation for plasticity in response to salinity. This result suggests that different genotypes (or gene combinations) would vary in response under different environmental conditions.

Discussion

The copepod complex *Eurytemora affinis* is considered unusual in its ability to invade habitats of different salinity (Hutchinson 1957) and is the most broadly distributed species complex within the genus *Eurytemora* (Heron 1976). Recent and rapid invasions of freshwater habitats throughout the Northern Hemisphere by *E. affinis* have been extraordinary (Lee 1999). This article addresses the question of whether this broad distribution is achieved through plasticity alone or whether natural selection is an important evolutionary force during range ex-

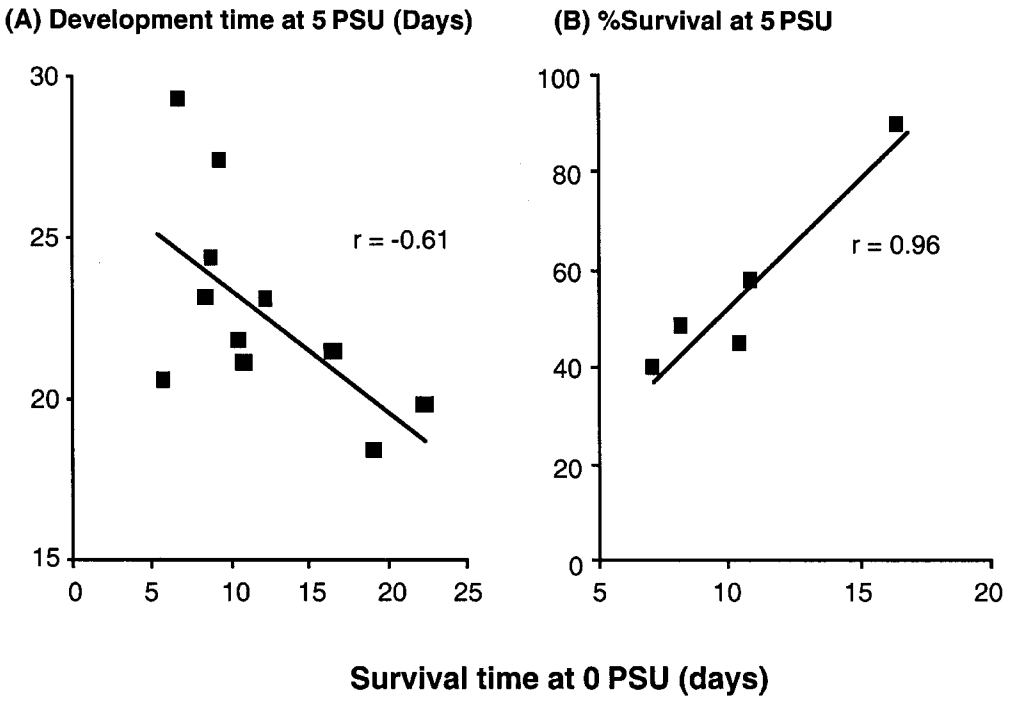

Figure 4. Performance and tolerance at fresh water versus 5 PSU for individual clutches; r = correlation coefficients. *A*, Survival time (days) at fresh water versus development time to metamorphosis at 5 PSU ($P = 0.047$). *B*, Survival time (days) at fresh water versus percentage survival to metamorphosis at 5 PSU ($P = 0.01$). Percentage data were arcsine transformed to approach a normal distribution.

pansions. Results from this study suggest the importance of natural selection during freshwater invasion events. This article presents four major conclusions: (1) survival and development were more favorable at higher (10 and 27 PSU) rather than at lower salinities (fresh water, 5 PSU) at 13°C, (2) developmental acclimation to fresh water by a brackish-water population could not be achieved in a single generation, (3) there is genetic variation for plasticity (G × E interaction) in response to salinity upon which natural selection can act, and (4) the effect of clutch (genotype) on survival and performance at low salinity is significant.

Optimal Salinity

Tolerance ranges must vary considerably within the *E. affinis* species complex, given the wide range of habitat salinity (0–40 PSU) and the inability of particular populations to tolerate the full salinity range of the species complex (Lee 1999, 2000). For the population examined in this study, higher salinities (≥10 PSU) appeared to be more favorable for survival and development than lower salinities (≤10 PSU) at 13°C (Figs. 1*A*, 2*A*). However, it is not clear where exactly the optimum resides, given that salinities within or above the 10–27 PSU range were not tested. Clearly, there is considerable variation among clutches in optimal salinity (Figs. 1*B*, 2*B*; see "Clutch (Genetic) Variation in Phenotypic Plasticity"). It was surprising that development time for many clutches was most rapid at the highest salinity treatment (27 PSU; Fig. 2*B*), which approaches the upper end of salinity experienced in Edgartown Great Pond.

Consistent with this study (Fig. 3), some previous studies on *E. affinis* have shown that lower salinities and temperatures yielded slower development time and longer life spans (Heinle and Flemer 1975; Roddie et al. 1984). Such a trend would result in fewer generations and lowered fitness for a given period of time at low salinities. For a population (or sibling species) of *E. affinis* from the Forth Estuary in Scotland, feeding rate was much higher at 15 PSU than at 5 PSU at 10°C (Powell and Berry 1990). In contrast, for a Baltic Sea population (or sibling species) of *E. affinis* found at 3 PSU, oxygen consumption was lowest at 6 PSU for adults and 3 PSU for larvae at 6°C (Gyllenberg and Lundquist 1978).

However, previous experiments on optimal salinity of *E. affinis* are difficult to evaluate because field-caught copepods reared under unknown and unpredictable conditions were used rather than those reared under standardized common garden conditions (Von Vaupel-Klein and Weber 1975; Gyllenberg and Lundquist 1978; Roddie et al. 1984; Powell and Berry 1990; Ishikawa et al. 1999). In addition, previous experiments accli-

mated and tested copepods under diverse conditions and used different measures of performance or tolerance at different temperatures (Von Vaupel-Klein and Weber 1975; Gyllenberg and Lundquist 1978; Roddie et al. 1984; Powell and Berry 1990; Ishikawa et al. 1999). There is a demonstrated temperature-salinity interaction effect on salinity and temperature tolerance, such that temperature could affect optimal salinity profoundly (Bradley 1975; Gyllenberg and Lundquist 1979; Roddie et al. 1984). Results of many of the previous experiments might not be relevant to this study because they used populations from the European clade, which is a genetically distinct sibling species (Lee 2000; Lee and Frost, in press). A common garden experiment would be required to obtain a meaningful evaluation of genetic differences among populations in salinity optima, exclusive of environmental, acclimation, and temperature effects.

Development in Fresh Water

Degree of developmental plasticity was insufficient to produce a freshwater population in a single generation for the brackish-water population used in this study. In this experiment, metamorphosis did occur in one clutch in fresh water. In a previous experiment using the same population, there was a 1.1% survival to metamorphosis over two generations in fresh water (Lee 1999). More specifically, from a starting number of 369 adults (159 females, 210 males) transferred to fresh water, 31 females and 10 males survived to produce 15 clutches of 13.33 ± 0.99 (SE) eggs, from which four larvae metamorphosed into juveniles and two survived to adulthood (Lee 1999). These results showed that only a small proportion of the population could survive and metamorphose under freshwater conditions, even over two generations.

Larvae were able to hatch in fresh water (Fig. 1*A*) and persist for several days. Similarly, a previous experiment has shown that a moderate proportion of adults (~20%) could survive in fresh water indefinitely (Lee 1999). The barrier to survival and development in fresh water appeared to be metamorphosis. In a few clutches, larval life span was excessively long, resulting in oversized larvae that died without undergoing metamorphosis. This observation suggests that low concentration of ions might be a barrier to metamorphosis in fresh water (~0.2 PSU).

Some argue that the freshwater habitats being invaded by *E. affinis* and other invertebrates (Lee and Bell 1999) are actually brackish (Wolff 2000). For example, Wolff (2000) claimed that salinity transitions did not occur during invasions because the freshwater lakes in question (Lee 1999; Lee and Bell 1999), such as the IJsselmeer, Netherlands, are brackish. This distinction boils down to an issue of semantics. The IJsselmeer has a salinity range of 0.3–0.4 PSU (Spijkerman and Coesel 1998), which is considered fresh by some definitions (Remane and Schlieper 1971) and is close in salinity to the fresh water used in this study (see "Material and Methods"). Moreover, results from this study show that, for this particular population, even 5 PSU retards development and lowers survival (Figs. 1, 2). In another study, most of the mortality is induced at salinities of 2 PSU and lower (C. E. Lee, unpublished manuscript).

Clutch (Genetic) Variation in Phenotypic Plasticity

The significant clutch (genotype)-by-salinity (environment) interaction indicates that there is genetic variation for phenotypic plasticity in response to salinity (Fig. 2*B*). A lack of correlation in development time across salinities provides additional support. A significant G × E interaction could have been an artifact of heterogeneity among vials for each treatment. However, clutch effects were consistent between low-salinity treatments, with a significant correlation between responses at 0 and 5 PSU (Fig. 4). In addition, current reaction norm studies, in which clutches were separated into replicate vials, are confirming that clutch has significant effects on survival and development (C. E. Lee and J. Remfert, unpublished data).

Significant differences among clutches in development time strongly suggest that salinity tolerance is a heritable trait. Unfortunately, limitations of using full-sib clutches (Falconer and Mackey 1996) prevented accurate estimates of heritability. Using calculations available for full-sib analysis, heritability estimates were greater than 1, suggesting dominance and environmental effects had not been removed from the analysis (C. E. Lee, unpublished data). A current study on reaction norms of saltwater ancestral and freshwater descendant populations (C. E. Lee and J. Remfert, unpublished data) is examining clutch effects over two generations, which will provide better estimates of heritability for salinity tolerance and allow us to account for maternal effects on response to salinity.

The Concept of "Euryhalinity" and the Role of Natural Selection

A "euryhaline" animal is defined as one that tolerates wide variations in salinity (Schmidt-Nielsen 1990). The current usage does not distinguish whether this term applies to individuals, populations, or species. Thus, this designation is based on diverse criteria, such as geographic distribution of the entire species, physiological tolerance at the individual level, or the range of tolerance at the population level. From an evolutionary standpoint, however, it is important to distinguish at what level variation exists, given that selection operates on polymorphism among individuals within populations.

In particular, the designations "eurytolerant" or "euryhaline" have been used to explain broad distributions and range expansions of species (Baker 1965; Strayer 1999; Wolff 2000), including that of *E. affinis* (Wolff 2000). In the case of *E. affinis*, its euryhalinity has been based on geographic distribution rather than on rigorous physiological and common garden experiments. A common problem in invasion biology is that species rather than populations are treated as units of invasion,

and entire species distributions are used to infer tolerance of particular populations (Lee 2002). It is important to recognize that populations rather than species are what invade and that these invasions often constitute evolutionary events at the population level (Lee 2002).

Results from this and a previous study (Lee 1999) suggest that plasticity alone is insufficient to account for the distribution of *E. affinis* in freshwater habitats. High levels of variation among clutches in response to low salinity, in terms of survival and development time, and the significant G × E interaction reflect ample substrate for natural selection. In a tolerance experiment on three populations of *E. affinis* (Lee 1999), a very small proportion of individuals from one population was able to survive and metamorphose in fresh water, suggesting that freshwater tolerance occurs at very low frequency in some populations. In addition, a current study is finding that a recent freshwater population has experienced a heritable shift in salinity tolerance relative to its saline ancestral population (C. E. Lee and J. Remfert, unpublished data). Given that natural selection is being implicated in the invasion process, what exactly is being selected for? Investigating genetic and physiological characteristics of those few individuals that survive the tight bottleneck into fresh water might reveal evolutionary mechanisms that allow populations within this species complex to invade.

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