

Evolution of Physiological Tolerance and Performance During Freshwater Invasions¹

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SYNOPSIS. Invasive species that penetrate habitat boundaries are likely to experience strong selection and rapid evolution. This study documents evolutionary shifts in tolerance and performance following the invasion of fresh water by the predominantly estuarine and salt marsh copepod *Eurytemora affinis*. Common-garden experiments were performed on freshwater-invading (Lake Michigan) and ancestral saline (St. Lawrence marsh) populations to measure shifts in adult survival (at 0, 5, and 25 PSU), and survival during development and development time (both using full-sib clutches split across 0, 5, 15, and 25 PSU). Results showed clear evidence of heritable shifts in tolerance and performance associated with freshwater invasions. The freshwater population exhibited a gain in low-salinity tolerance and a reduction in high-salinity tolerance relative to the saline population, suggesting tradeoffs. These tradeoffs were supported by negative genetic correlations between survival at fresh (0 PSU) versus higher salinities. Mortality in response to salinity occurred primarily before metamorphosis, suggesting that selection in response to salinity had acted primarily on the early life-history stages. The freshwater population exhibited curious patterns of life-history evolution across salinities, relative to the saline population, of retarded development to metamorphosis but accelerated development from metamorphosis to adulthood. This pattern might reflect tradeoffs between development rate and survival in fresh water at the early life-history stages, but some other selective force acting on later life-history stages. Significant effects of clutch (genotype) and clutch-by-salinity interaction ($G \times E$) on survival and development time in both populations indicated ample genetic variation as substrate for natural selection. Variation for high-salinity tolerance was present in the freshwater population despite negative genetic correlations between high- and low-salinity tolerance. Results implicate the importance of natural selection and document the evolution of reaction norms during freshwater invasions.

INTRODUCTION

Invasive species pose among the greatest threats to biodiversity, ecosystem integrity, agriculture, fisheries, and public health. Economic costs associated with the more publicized invaders, such as weeds, agricultural pests, zebra mussels, and plant pathogens, are estimated at 137 billion dollars per year in the United States alone (Pimentel *et al.*, 2000). The rapid spread of exotics has received considerable attention within the international community, and has mobilized substantial ecological research. However, evolutionary mechanisms of invasions have remained largely unexplored, leaving fundamental gaps in our understanding (Lee, 2002).

Invasive species are often assumed to penetrate habitat boundaries through their broad tolerance (eurytolerance) or phenotypic plasticity (Baker and Stebbins, 1965; Ricciardi and MacIsaac, 2000; Wolff, 2000; Reid and Orlova, 2002), with few rigorous tests of such assumptions (Lee, 1999, 2002; Agrawal, 2001). Both phenotypic plasticity and selection could have profound effects on invasion success (Lee, 2002), but aside from a few notable case studies (Weinig, 2000a, b; Weinig and Delph, 2001), their relative importance and fitness tradeoffs remain unclear.

Mounting evidence suggests that acclimation alone might be insufficient to account for invasion success

of many species, but that response to selection and “evolubility” of invasive species might be important (Lee, 2002). Several studies document strong selection and rapid evolution of phenotypic traits following invasions (Hendry and Kinnison, 1999; Huey *et al.*, 2000; Carroll *et al.*, 2001; Reznick and Ghalambor, 2001; Lee, 2002). However, studies that examine physiological shifts accompanying invasion events are still rare, though more common in the plant literature (Hakam and Simon, 2000; references cited in Lee, 2002; Sexton *et al.*, 2002). The purpose of this study was to examine the evolution of physiological reaction norms and potential tradeoffs in response to habitat invasions.

The copepod (species complex) *Eurytemora affinis* provides an ideal model for examining evolutionary shifts in physiological reaction norms during habitat invasions. This species (complex) is broadly distributed in saline estuaries and salt marshes in the Northern Hemisphere, but has invaded fresh water independently in North America, Asia, and Europe within the past century (Figs. 1, 2; Lee, 1999). These invasions occurred through various means, such as through impoundment of saline water bodies followed by reduction in salinity (De Beaufort, 1954; Miller, 1958) and human-mediated introduction into reservoirs and lakes, possibly through the introduction of fish (Ban and Minoda, 1989; Ishikawa *et al.*, 1999; Orsi, 2001) or bilge and ballast water (Faber *et al.*, 1966). Such transitions from saline to freshwater habitats characterize invasion pathways of many recent invertebrate invaders (Jazdzewski, 1980; Taylor and Harris, 1986; Dermott *et al.*, 1998; Lee and Bell, 1999; Väinölä and Oulasvirta, 2001; Smith *et al.*, 2002).

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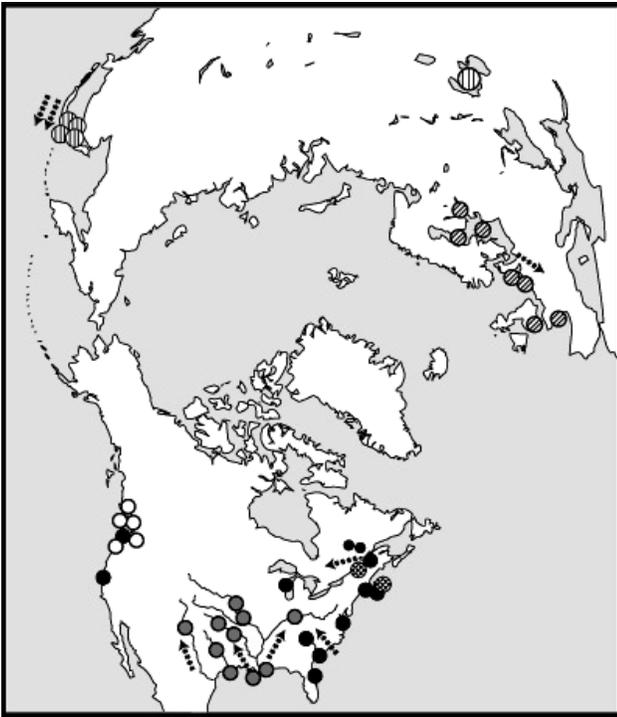


FIG. 1. Geographic pattern of invasions by *Eurytemora affinis* from saline sources into freshwater lakes and reservoirs. Dashed arrows indicate pathways of freshwater invasions. Patterned circles represent distinct clades, shown in Figure 2. Details on locations and timing of invasions are presented in Lee (1999).

The broad habitat range of *E. affinis* and its recent invasions into freshwater habitats (Lee, 1999) have often been attributed to broad tolerance (Wolff, 2000). However, experimental results showed that individuals from saline populations could not gain freshwater (0 PSU) tolerance through either short-term or developmental acclimation alone (Lee, 1999; Lee and Petersen, 2002, 2003). Rather, the results suggested the importance of selection on physiological tolerance and performance during habitat invasions.

Analyzing patterns of reaction norm evolution provides a powerful approach for examining response to environmental change. Exploring reaction norm evolution yields insights into how selection in one environment could affect performance in another, and the tradeoffs and costs involved in adaptation. In addition, genetic variation in reaction norms within populations (genotype by environment interaction, $G \times E$) reveals the potential for selection to act on tradeoffs in performance in different environments (*i.e.*, disruptive selection) (Via and Lande, 1985). Yet, relatively few studies have applied a reaction norm approach to species invasions. Most examples are from the plant literature (Weber and Schmid, 1998; Weinig and Delph, 2001; Bastlova and Kvet, 2002; Sexton *et al.*, 2002). A reaction norm is the range of phenotypes that a genotype produces when exposed to different environments, and is the result of phenotypic plasticity (Schmalhausen, 1949). Therefore, reaction norms de-

scribe the relationship between genotype, phenotype, and the environment (Schlichting and Pigliucci, 1998).

Reaction norm evolution during freshwater invasions could follow the pattern of (1) an increase in performance in fresh water, with no sacrifice of high-salinity performance, (2) an increase in performance breadth but reduction of optimal performance (generalist-specialist tradeoff; *e.g.*, generalized stress response), or (3) a gain in freshwater tolerance accompanied by a loss of saltwater tolerance (high- vs. low-salinity tradeoff) (Huey and Kingsolver, 1993; Kingsolver *et al.*, 2001). The first scenario would result if responses to low- and high-salinity were genetically independent of one another, while the third would occur if the responses were genetically negatively correlated.

Thus, our goals were to determine (1) the pattern of reaction norm evolution and tradeoffs associated with freshwater invasions and (2) genetic variation in reaction norms ($G \times E$) in source and invading populations. In order to accomplish these goals, we compared physiological reaction norms for a saline source population in the St. Lawrence River drainage and a freshwater population that invaded Lake Michigan *ca.* 45 years ago (Anderson and Clayton, 1959; Faber *et al.*, 1966). For both populations, we measured adult and developmental tolerance and development time for full-sib clutches (genotypes) split across a range of salinities.

METHODS AND MATERIALS

Ancestral saline and descendent freshwater populations

Common-garden experiments were performed on two populations from the St. Lawrence River drainage: a saline ancestral population from the Bay of L'Isle Verte in the St. Lawrence River drainage and a freshwater invasive population in Lake Michigan near Racine, Wisconsin (Fig. 2, Atlantic clade). The L'Isle Verte population occurs in saline pools of a floodplain, where salinity can range from 5 to 40 PSU seasonally. *Eurytemora affinis* was first discovered in the Great Lakes about 45 years ago (Engel, 1962; Faber *et al.*, 1966), and probably originated from one or several saline populations in the St. Lawrence River drainage, based on genetic and geographic proximity (Lee, 1999). Although the L'Isle Verte population might not be the direct ancestor of the Lake Michigan population, it is probably closely related to the original source population. The estuarine population in the main basin of the St. Lawrence estuary belongs to a genetically divergent clade (Figs. 1, 2) that is reproductively isolated from the populations used in this study (Lee, 2000; Lee and Frost, 2002). The saline population was collected on 23 May 2001, whereas the freshwater population was collected in October and November of 2001. Several hundred individuals were maintained for each population in culture. Water of different salinity was made from mixtures of water from Lake Michigan

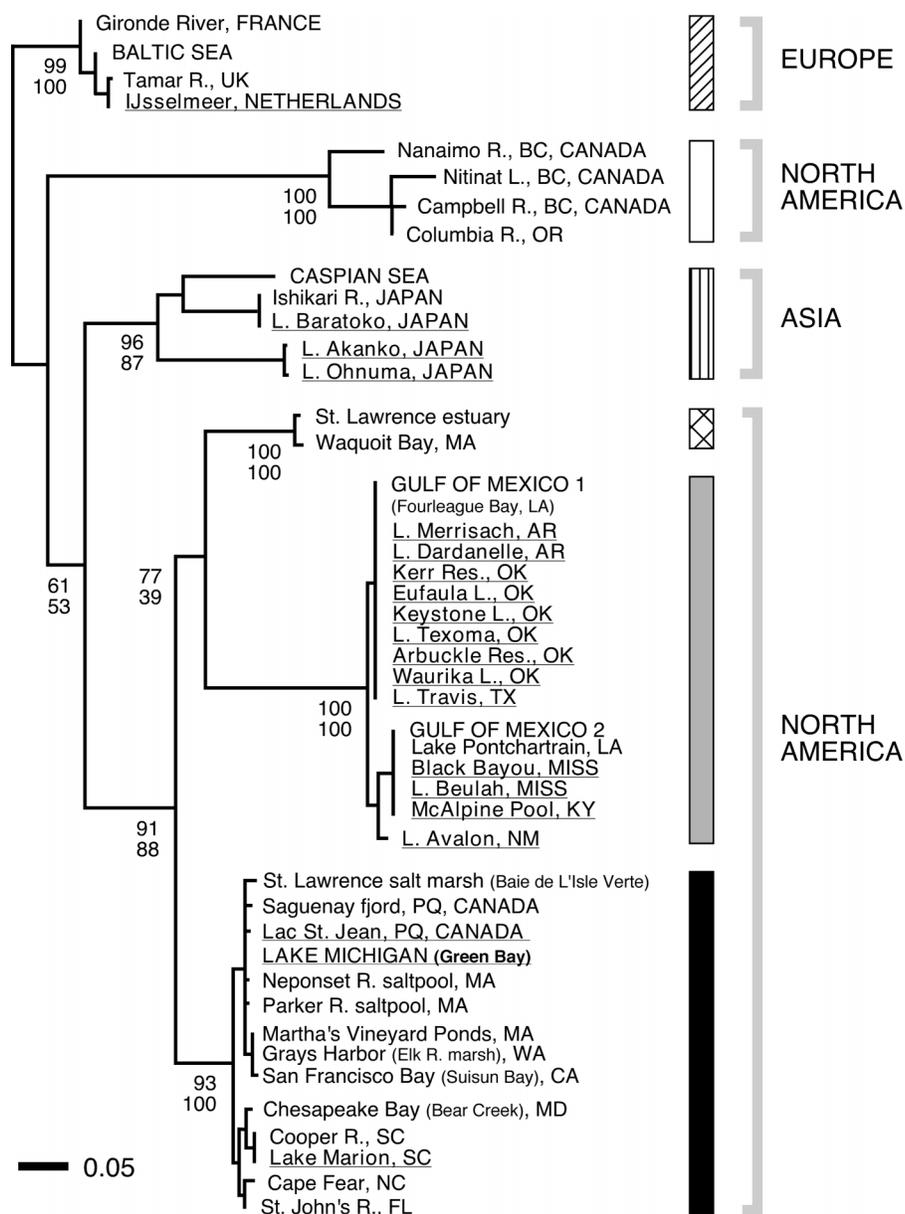


FIG. 2. Unrooted phylogeny of the *Eurytemora affinis* species complex based on cytochrome oxidase I sequences (652 base pairs). Populations are shown at branch tips. Numbers at nodes are bootstrap values for 100 replicates (Felsenstein, 1985) using distance matrix (upper number) and parsimony (lower number) approaches. Underlined locations represent populations that have recently invaded freshwater sites (see Lee, 1999 for approximate dates). Patterened bars represent clades (also shown in Fig. 1). For additional information on locations of populations and phylogenetic methods, see Lee (1999, 2000) and Lee and Frost (2002).

and Instant Ocean®. Throughout this paper, the saline L'Isle Verte population is referred to as "St. Lawrence salt marsh (LAW)," while the freshwater population is referred to as "Lake Michigan (MICH)."

Short-term salinity tolerance of adults

Adult survival in response to salinity was compared between the saline and freshwater populations to determine evolutionary shifts in tolerance. Prior to the experiment, both populations were reared under common-garden conditions for 2–3 generations (generation

time = 20 days at 13°C) at a salinity of 5 PSU, light cycle of 9D:15L, temperature of 13°C, and a mixture of salt and freshwater *Rhodomonas* sp. as the food source. Adult females from both populations were then placed into three treatment salinities of 0 (fresh), 5, and 25 PSU, with three replicates each, and 15 individuals per replicate. These treatments were maintained under the light, temperature, and food conditions described above. Percentage survival was measured after three days. Results were analyzed using likelihood-ratio tests under a binomial model. Where

necessary, block effects across the three replicates were accommodated by including additional model parameters for each replicate.

Salinity tolerance during development and development time

Tolerance during development was examined because past results revealed that larvae are more susceptible to osmotic stress than adults (Lee, 1999; Lee and Petersen, 2002). For instance, larvae from saline populations had high mortalities and difficulty achieving metamorphosis in fresh water (Lee, 1999; Lee and Petersen, 2002). The experiment reported here is the first of two, where the first examined development of clutches taken from parental populations raised at native salinities (0, 15 PSU) for at least two generations, whereas the second used clutches taken from parental populations raised at a common salinity (5 PSU; C. E. Lee and J. L. Remfert, in preparation). The design included both experiments because each could potentially introduce artifacts (see Discussion).

Each population was reared in the laboratory at 13°C at their native salinities (fresh, 15 PSU) for at least two generations prior to the reaction norm experiment. Cultures were fed a combination of salt- and freshwater *Rhodomonas* sp. For each population, eight full-sib clutches were excised from adult females with a pin and split into four salinity treatments of 0 (lake water), 5, 15, and 25 PSU, with 6–10 eggs per treatment. Each treatment was split again into two replicates per treatment. For the Lake Michigan population, eggs were transferred gradually to treatment salinities to avoid osmotic shock, because none could hatch at 25 PSU without prior acclimation. Similarly, a freshwater population from Lake Ohnuma in Japan could not hatch at higher salinities without prior acclimation (Ishikawa *et al.*, 1999). Eggs from 0 PSU were transferred to 5 PSU for a day, and then to the 15 PSU treatment. After an additional day, a subset was transferred from 15 PSU to the 25 PSU treatment. Gradual transfer of eggs was unnecessary for the saline St. Lawrence population because salinity did not affect hatching rate.

Eggs were placed in 20-ml scintillation vials maintained half-full with caps left ajar to allow for oxygen exchange. Vials were kept at 13°C on a 9D:15L light cycle. To avoid confounding effects of vial position, vials were placed in racks such that treatment, clutch, and replicates within clutch were spatially interspersed and distributed evenly. Developing copepods were fed in excess every day with a mixture of fresh and salt-water *Rhodomonas* sp. Every 12 days, 75% of the water volume was replaced. Visual inspection of vials was performed daily. Metamorphosis occurred during the transition between the nauplius VI and copepodid I stages. Individuals were classified as adults when males developed geniculate right antennules, and when females developed large wing-like processes on the posterior end of their prosomes (body).

Differences in developmental tolerance between

the two populations were analyzed using generalized linear mixed-models (implemented with PROC NLMIXED) using SAS (version 8; 1999), whereas differences in development time were analyzed using linear mixed-models in R (version 1.6.1; <http://cran.r-project.org/>). Likelihood-ratio tests were used to test significance of models for both datasets. Tests for differences in means were modified to accommodate unequal population variances (heteroskedasticity), when present.

Effects of clutch (genotype) and clutch-by-salinity interaction ($G \times E$) on survival during development and development time

Mixed-models were used to determine effects of genotype (G , clutch) and environment (E , salinity) and genotype-by-environment interaction ($G \times E$) on survival during development and development time. Clutch and $G \times E$ were designated as random factors, whereas salinity was designated as a fixed factor. To determine significance of the models with random factors, likelihood-ratio tests were performed between the models with and without the random effects. In mixed models, likelihood-ratio tests of the significance of variance components are conservative because the null hypothesis lies at the boundary of a variance component of zero (Crainiceanu *et al.*, in revision). Survival was treated as binary data (dead or alive) for each clutch.

For survival data, models were fit to the data using maximum likelihood with PROC NLMIXED in SAS. Initial parameters were estimated using the functions glmmPQL (generalized linear mixed-model penalized quasi-likelihood) and glm (generalized linear model) in R. A logistic link function was used for the generalized linear models. For development time data, models were fit to the data using maximum likelihood with the function lme in R. Likelihood-ratio tests were performed to compare nested models: (1) a model with fixed effects alone nested within a model containing genotype (G) as a random effect, (2) a model with G as a random effect nested within a model with both G and $G \times E$ as random effects, and (3) a model with fixed effects alone nested within a model with both G and $G \times E$ as random effects. Salinities were treated as ordered factors. Replication was not equal because survivorship of hatchlings varied among clutches. Full-sib clutches were assumed to represent a set of closely-related genotypes.

To determine whether differences in survival or development time were actually due to clutch rather than to stochastic vial effects, clutches were split into replicate vials for each treatment salinity. The null hypothesis that apparent clutch effect resulted from stochastic vial effects was tested by determining whether clutch was a significant predictor of vial outcome. Such a test is equivalent to testing whether the two vials within clutch within salinity were correlated. If differences among clutches were simply due to stochastic vial effects, the pairs should not be correlated.

For survival during development, a generalized linear model (function `glm`) in *R* was used with the quasibinomial family option to account for overdispersion across vials. An *F*-test was used to compare a model where clutch was included as predictor of vial outcome *versus* a model where clutch was not a predictor. For development time, an analogous test was performed except using a linear model (function `lm`) in *R*. Here an *F*-test was used to determine whether clutch was a significant predictor of vial means of development time.

A ratio of the variance component due to clutch relative to that due to vial was computed to assess their relative magnitudes for survival (using the function `glmmPQR` in *R*) and development time data (using `lme` in *R* with restricted maximum likelihood). For these analyses, vial was nested within clutch. For the development time data, treatment salinities with insufficient sample size (due to high mortality; 0 PSU for LAW, 25 PSU for MICH) were dropped from the analyses.

Genetic correlations for survival across different salinities

Correlations for mean survival within clutches at different salinities were obtained from variance-covariance matrices calculated from the data using generalized linear mixed-models (function `glmmPQL`) in *R*. The variances were parameters of the random effects due to clutch. Significance of covariances between 0 PSU and higher salinities were tested using a likelihood-ratio test, performed between unconstrained (covariance can be negative) and constrained (covariance constrained ≥ 0) models. Likelihoods were obtained using PROC NLMIXED in SAS using starting parameter values from `glmmPQL` in *R*. A one-tailed χ^2 test was employed (using a $\bar{\chi}^2$ distribution). Twelve tests were performed (3 salinities, survival to metamorphosis and adult, and 2 populations) to determine whether covariances between 0 PSU and the three other salinities were significantly less than zero.

Heritabilities for survival and development time

Broad-sense heritability was determined for survival to metamorphosis and development time for both populations. For development time, heritability was calculated using intra-class correlations using the function `lme` in *R* (Falconer and Mackey, 1996). In contrast, heritability for survival was calculated by treating survival as binary data (dead or alive) for full-sibs within eight clutches. Variance components were calculated using the `glmmPQL` (generalized linear mixed-model penalized quasi-likelihood) function in *R*. Variance partition coefficients (“intra-class correlations”) for binary data were calculated using method “A” of Goldstein *et al.* (2002), which generates an approximate variance partition coefficient using model linearization through a Taylor series expansion. Alterations to the usual procedure of using simple intra-class correlations (calculated via linear mixed models) were necessary

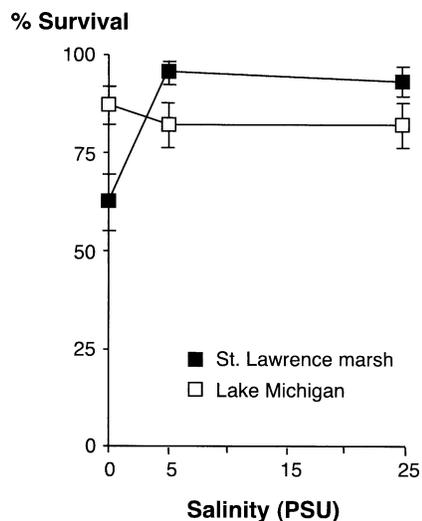


FIG. 3. Survival of adults in response to salinity (0, 5, 25 PSU) after three days. Percentage survival is shown for the St. Lawrence saltmarsh and Lake Michigan populations. Sample size is 45 individuals per treatment. Data shown are Means \pm SE for binomial data. Adults were taken from populations reared at a common salinity of 5 PSU for at least two generations.

in order to accommodate binary data. Replicate treatments within clutches were used to partition out variance components due to vial effect, which were removed from heritability estimates. Estimates using full-sib clutches yield upper-bound values for heritability (Falconer and Mackey, 1996).

RESULTS

Short-term adult tolerance

After a 3-day assay at three salinities, adults from the freshwater Lake Michigan population exhibited significantly higher survival at 0 PSU relative to the saline St. Lawrence marsh population, significantly lower survival at 5 PSU only when replicates were pooled, and no significant difference in survival at 25 PSU (Fig. 3). Survival data for adult tolerance were analyzed for replicate treatments that were both unpooled (to allow for block effects) and pooled (when significant differences between replicates were absent). Overall, survival in response to salinity differed significantly between the saline St. Lawrence marsh (LAW) and freshwater Lake Michigan (MICH) populations (Fig. 3; likelihood ratio $T = 27.38$, $df = 9$, $P = 0.0012$, replicates not pooled). Adults from the freshwater population had significantly higher survival at 0 PSU relative to the saline population (Fig. 3; $T = 13.85$, $df = 3$, $P = 0.031$), and no significant difference at 5 and 25 PSU when replicates were not pooled (5 PSU: $T = 6.68$, $df = 3$, $P = 0.083$; 25 PSU: $T = 6.86$, $df = 3$, $P = 0.077$). However, the 5 PSU treatment, which had no significant block effects, did reveal significant differences when replicates were pooled (likelihood ratio $T = 4.31$, $df = 1$, $P = 0.038$; Fisher's Exact Test, $P = 0.090$, two-tail, $P = 0.045$, one-tail).

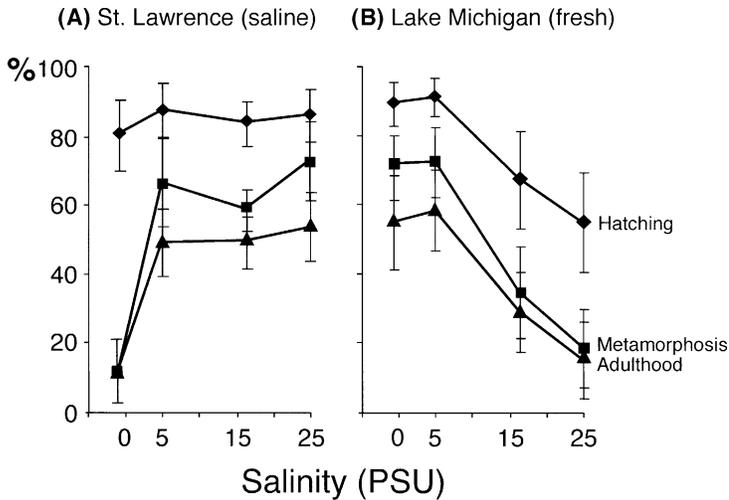


FIG. 4. Survival during development in response to salinity (0, 5, 15, 25 PSU) of populations from (A) St. Lawrence marsh and (B) Lake Michigan. Curves represent percentage hatching, percentage survival to metamorphosis, and to adulthood. Data shown are Mean \pm SE for 8 clutches. Replicate treatments per clutch are pooled in this graph. Clutches were taken from parental populations reared at their native salinities (0, 15 PSU) in the laboratory for at least two generations.

Salinity tolerance during development

Survival during development differed dramatically between the two populations, with the freshwater population exhibiting greater survival at lower salinity (0 PSU) and lower survival at higher salinity (25 PSU) relative to the saline population (Fig. 4). Most of the mortality in response to salinity occurred prior to metamorphosis (nauplius to copepodid) in both populations, indicating that the larval (nauplius) stages were susceptible to osmotic stress (Table 1). For both populations, salinity had overall significant effects on survival up to metamorphosis (Fig. 4; LAW: likelihood ratio $T = 10.32$, $df = 3$, $P = 0.016$; MICH: $T = 12.27$, $df = 3$, $P = 0.0065$), but no significant effects for the interval from metamorphosis to adulthood (LAW: $T = 3.44$, $df = 3$, $P = 0.33$; MICH: $T = 0.65$, $df = 3$, $P = 0.89$). Comparisons at each salinity between the two populations revealed that the Lake Michigan population had significantly higher survival to metamorphosis at 0 PSU ($T = 16.87$, $df = 1$, $P = 4.01E-05$), significantly lower survival at 25 PSU ($T = 8.31$, $df = 1$, $P = 0.0039$), and no significant difference at the intermediate salinities of 5 PSU ($T = 0.13$, $df = 1$, $P = 0.71$) or 15 PSU ($T = 2.72$, $df = 1$, $P = 0.099$). In contrast, survival from metamorphosis to adulthood was not significantly different between the two populations ($T = 0.014$, $df = 1$, $P = 0.91$).

Pairwise comparisons of survival at each salinity for the St. Lawrence population revealed significant differences between survival at 0 PSU and all other salinities to metamorphosis (pairwise likelihood-ratio tests, $P < 0.005$) and to adulthood ($P < 0.01$). Differences in survival between the other salinities (5, 15, and 25 PSU) were not significant. Pairwise differences

TABLE 1. Comparisons between survival (%) during larval (hatching to metamorphosis) and juvenile (metamorphosis to adulthood) life-history stages.*

	Salinity	%Survival	
		Hatching to metamorphosis	Metamorphosis to adulthood
St. Lawrence marsh	0	12.5	100
	5	66.2	76.7
	15	59.7	83.8
	25	74.2	73.9
Lake Michigan	0	69.8	78.4
	5	71.2	81.1
	15	32.1	82.4
	25	14.0	71.4

* Data are pooled for the replicate treatments.

for the Lake Michigan population were significant ($P < 0.05$) except for a few cases (metamorphosis: 0 vs. 5 PSU, 15 vs. 25 PSU, ns; adult: 0 vs. 5 PSU, 0 vs. 15 PSU, 15 vs. 25 PSU, ns). The dip in survival observed for the saline population at 15 PSU (Fig. 4A) resulted from an accidental introduction of polluted water from a dredged harbor (Racine, WI) in Lake Michigan.

Genetic correlations across clutches for survival at different salinities

Tradeoffs between high- and low-salinity tolerance were apparent from the negative genetic correlations between survival at 0 PSU versus higher salinities (Table 2). In general, correlations between 0 PSU and higher salinities for mean survival within clutches, based on a likelihood estimation of the variance/covariance matrix, were negative or low (Table 2). Pearson correlations of clutch means were consistent with the likelihood estimates (9 of 12 Pearson correlations between 0 PSU and higher salinities were negative). Three negative correlations between 0 PSU and other salinities were significant based on likelihood-ratio tests (Table 2). Although significance would not withstand Bonferroni correction, the number of significant tests clearly exceeded that expected by chance alone.

Life-history evolution (development time)

The freshwater Lake Michigan population exhibited a curious pattern of life-history evolution relative to the saline St. Lawrence population, of retarded development to metamorphosis, but accelerated development from metamorphosis to adulthood (Fig. 5). Across all salinities, development time was significantly longer in the Lake Michigan population up to metamorphosis relative to the St. Lawrence population (Fig. 5; likelihood ratio $T = 7.12$, $df = 1$, $P = 0.0076$, when testing the addition of a fixed effect of population to a model containing fixed and random effects of salinity), but significantly shorter to adulthood ($T = 43.15$, $df = 1$, $P = 5.08E-11$). For the Lake Michigan population, salinity had an overall fixed effect on development time to metamorphosis (Fig. 5; $T = 9.88$,

TABLE 2. Correlations between clutch means (8 clutches) for survival at different salinities calculated from a variance/covariance matrix.

St. Lawrence marsh (saline)		(A) Survival to metamorphosis			(B) Survival to adult		
		5 PSU	15 PSU	25 PSU	5 PSU	15 PSU	25 PSU
0 PSU		-0.797 ^a	-0.433	-0.486	-0.516	0.125	0.382
25 PSU		0.844	0.770		0.376	0.928	
15 PSU		0.869			0.683		
Lake Michigan (fresh)		(C) Survival to metamorphosis			(D) Survival to adult		
		5 PSU	15 PSU	25 PSU	5 PSU	15 PSU	25 PSU
0 PSU		-0.363	-0.369	0.016	0.289	-0.766 ^b	-0.914 ^c
25 PSU		0.921	0.921		0.036	0.958	
15 PSU		0.995			0.243		

Values that are significant from a likelihood-ratio test: ^a $T = 3.22$, $P = 0.036$, ^b $T = 3.15$, $P = 0.038$, ^c $T = 5.09$, $P = 0.012$.

df = 3, $P = 0.020$) and to adulthood ($T = 10.22$, df = 3, $P = 0.017$). In contrast, for the St. Lawrence population, salinity had no significant fixed effect on development time to metamorphosis ($T = 1.61$, df = 3, $P = 0.68$) or to adulthood ($T = 1.66$, df = 3, $P = 0.65$).

Effect of clutch (genotype) and $G \times E$ on survival

Effect of clutch (G) on survival at different salinities (E) was computed from the mixed-model analysis (see Methods). Clutch had a highly significant main effect on survival to metamorphosis for both populations (LAW: likelihood ratio $T = 15.15$, df = 1, $P = 0.0001$; MICH: $T = 10.05$, df = 1, $P = 0.0015$). Clutch effect was significant for survival to adulthood for the St.

Lawrence ($T = 11.35$, df = 1, $P = 0.00075$), but not for the Lake Michigan population ($T = 1.73$, df = 1, $P = 0.19$). However, clutch effect for the Lake Michigan population was significant when both mean effect of clutch and $G \times E$ were tested ($T = 28.25$, df = 10, $P = 0.0016$). For the St. Lawrence population, the $G \times E$ term was highly significant for survival to metamorphosis (likelihood ratio $T = 46.08$, df = 9, $P = 5.81E-07$) and to adulthood ($T = 21.79$, df = 9, $P = 0.00096$). For the Lake Michigan population, the $G \times E$ term was marginally significant for survival to metamorphosis ($T = 17.85$, df = 9, $P = 0.037$) and significant for survival to adulthood ($T = 26.52$, df = 3, $P = 0.0017$).

The null hypothesis that differences in survival among clutches were due merely to stochastic vial effects was rejected as replicate vials within clutches were strongly correlated (LAW metamorphosis: $F = 2.78$, numdf = 28, dendif = 32, $P = 0.0029$; MICH metamorphosis: $F = 2.91$, numdf = 28, dendif = 32, $P = 0.0020$; MICH adult: $F = 2.40$, numdf = 28, dendif = 32, $P = 0.0090$; LAW adult results were affected by differential survival among vials due to accidental introduction of polluted water). In addition, the variance component for clutch typically exceeded that for vial, as indicated by ratio of variance contributed by clutch relative to that contributed by vial (LAW metamorphosis: 1.79; MICH metamorphosis: 4.52).

Effect of clutch (genotype) and $G \times E$ on development time

For both populations, clutch had a significant main effect on development time to metamorphosis (LAW: likelihood ratio $T = 16.04$, df = 1, $P = 6.22E-05$; MICH: $T = 24.89$, df = 1, $P = 6.07E-07$) and to adulthood (LAW: $T = 51.09$, df = 1, $P = 8.82E-13$; MICH: $T = 34.88$, df = 1, $P = 3.51E-09$). Clutch-by-salinity interaction ($G \times E$) was significant for development time to metamorphosis for both populations (LAW: $T = 52.84$, df = 9, $P = 3.13E-08$; MICH: $T = 51.30$, df = 9, $P = 6.12E-08$) and to adulthood for the Lake Michigan ($T = 26.23$, df = 9, $P = 0.0019$)

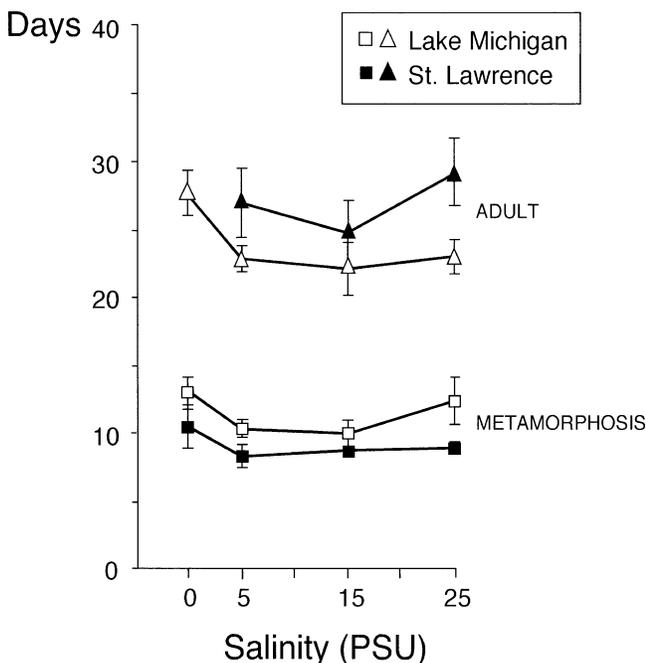


FIG. 5. Development time at 13°C in response to salinity (0, 5, 15, 25 PSU) for the St. Lawrence and Lake Michigan populations to metamorphosis and adulthood. Data shown are Mean \pm SE for 8 clutches.

but not for the St. Lawrence marsh population ($T = 1.94$, $df = 9$, $P = 0.99$).

The null hypothesis that differences in development time among clutches were due merely to stochastic vial effects was rejected because replicate vials for clutches were strongly correlated (using a model that includes both additive and $G \times E$ effects of clutch; LAW metamorphosis: $F = 2.50$, $numdf = 15$, $dendf = 18$, $P = 0.033$; MICH metamorphosis: $F = 6.58$, $numdf = 13$, $dendf = 16$, $P = 0.00033$; MICH adult: $F = 5.90$, $numdf = 16$, $dendf = 23$, $P = 7.31E-05$). Because the $G \times E$ term was not significant for development time to adult for the saline population, we tested a simple additive model, which likewise revealed a significant correlation between vials within clutches ($F = 4.75$, $numdf = 7$, $dendf = 29$, $P = 0.0012$). In addition, the variance component for clutch typically exceeded that for vial, as indicated by the ratio of variance contributed by clutch relative to that contributed by vial (LAW metamorphosis: 2.77; LAW adult: 2.81; MICH metamorphosis: 4.44; MICH adult: 16.00).

Heritabilities for salinity tolerance and development time

Broad-sense heritabilities for survival, based on calculations from full-sib clutches, were high. Survival to metamorphosis was used because sample sizes for survival to adulthood were low and because selection on tolerance probably had acted prior to metamorphosis (Table 1). Heritability for survival at high salinity (15 PSU) for the Lake Michigan population was 0.79, whereas heritability for survival at low salinity (5 PSU) was 0.83 for the St. Lawrence marsh population. Heritability for survival at 25 PSU for the St. Lawrence marsh population was 0.57. Heritabilities could not be calculated in some cases because survival was either too low (e.g., survival in 3 clutches for Lake Michigan at 25 PSU, 2 clutches for St. Lawrence marsh at 0 PSU) or too high (100% survival in half the clutches at 0 and 5 PSU for Lake Michigan). In addition, heritability was not calculated for the 15 PSU treatment for the St. Lawrence marsh population for reasons stated above (see *Salinity tolerance during development*). Heritabilities based on intra-class correlations from full-sib clutches for development time were excessively high, exceeding 1 in most cases, suggesting that dominance or maternal effects inflated the estimates.

DISCUSSION

The saline to freshwater interface represents a formidable barrier that relatively few invertebrate species have been able to penetrate (Hutchinson, 1957; Lee and Bell, 1999). Yet, many successful invaders in freshwater habitats are immigrants from brackish or marine habitats (Jazdzewski, 1980; Taylor and Harris, 1986; Lee and Bell, 1999; Ricciardi and MacIsaac, 2000; Reid and Orlova, 2002). Such invaders are often assumed to be broadly tolerant generalists (Baker,

1965; Ricciardi and MacIsaac, 2000; Wolff, 2000; Reid and Orlova, 2002), but few rigorous tests confirm such assumptions (Davis and Shaw, 2001; Lee, 2002). This study documents evolutionary shifts in tolerance and performance following such invasion events, by directly comparing physiological tolerance and performance of saline ancestral and freshwater descendant populations. Studies that document evolutionary shifts in physiological reaction norms during habitat transitions are still rare (references cited in Lee, 2002).

Evolution of freshwater tolerance

For copepods, 5 to 8 PSU serves as a biogeographic and physiological boundary between fresh and saline species (Khlebovich and Abramova, 2000). The rare ability of *E. affinis* to span both saline and freshwater habitats has been attributed to broad tolerance (Wolff, 2000). Consequently, the discovery of heritable shifts in adult survival, survival during development, and development time (Figs. 3–5) is important for implicating the role of natural selection. High heritabilities for salinity tolerance and performance, as witnessed in this study, are required for efficient and rapid responses to natural selection.

Selection in response to salinity appears to act primarily on life-history stages prior to metamorphosis (nauplius; Table 1). The minor effect of salinity on survival after metamorphosis (Table 1) might be due to greater tolerance of post-metamorphic stages, or to culling of less-tolerant individuals during development. Both are probably factors, as adults do have much greater tolerance than larvae, with relatively high survival across all salinities, but still display a significant response to salinity (Fig. 3, see Results).

The reduction in high-salinity tolerance and gain in freshwater tolerance in the freshwater population (Lake Michigan) suggests evolved tradeoffs between high- and low-salinity tolerance (Fig. 4). The striking negative genetic correlations between survival at 0 PSU *versus* higher salinities (Table 2) support these tradeoffs. Significant effects of clutch (genotype, G) and clutch-by-salinity interaction ($G \times E$) on survival and development time were concordant with previous studies (Lee and Petersen, 2002, 2003). Without significant $G \times E$, indicating genetic variation in tradeoffs, divergent physiological evolution between the two populations would not be possible (Via and Lande, 1985; Via, 1994). Given the tradeoffs and the relatively constant low salinity of the freshwater environment, the maintenance of high levels of genetic variation for salinity tolerance and plasticity in the Lake Michigan population is puzzling. This maintenance of genetic variance might have resulted from selection due to other unmeasured factors (see citations in Reznick and Ghalambor, 2001), antagonistic pleiotropy, overdominance, or insufficient time for selection to proceed to fixation. Genetic variation for both low- and high-salinity tolerance is understandable for the St. Lawrence marsh population, given its occurrence in shallow pools with seasonal and daily fluctuations in salinity.

The particular tradeoffs and the traits under selection during freshwater invasion events are worthy of further investigation.

There is no salinity at which both populations are at physiologically identical states, such that stress (and selection) is not imposed on either population. Thus, parental populations were reared at both native salinities (0 and 15 PSU; this study) and a common (5 PSU) salinity (C. E. Lee and J. L. Remfert, in preparation) prior to the reaction norm experiments. Rearing at native salinities might introduce effects of maternal environment, and the common salinity imposes selection against freshwater tolerance (C. E. Lee and J. L. Remfert, in preparation). Maternal effects are unlikely to have been solely responsible for the observed population differences in this study (Fig. 4).

Life-history evolution

Reaction norms for development time revealed curious patterns of life history evolution in the freshwater population relative to the saline population, of retarded development prior to metamorphosis but accelerated development from metamorphosis to adulthood (Fig. 5). Such contrasting patterns of life-history evolution suggest that different selective forces might be operating on early *versus* later stages. This pattern was replicated in a subsequent experiment in which parental populations were reared at 5 PSU for five generations prior to the reaction norm experiment (C. E. Lee and J. L. Remfert, in preparation).

Given that most of the mortality due to osmotic (or ionic) stress occurs at the early life-history stages (Table 2), retarded development might reflect a tradeoff between osmotic tolerance and development rate. This tradeoff might have resulted from greater energetic requirements and allocation of resources for osmoregulation (Hart, 1998). Retarded development across all salinities might reflect lack of plasticity (canalization). The retarded development is concordant with studies that show increases in development time in response to selection for stress tolerance (Barrera and Medialdea, 1996; Chippindale *et al.*, 1998; Harshman *et al.*, 1999).

In contrast, accelerated development at later stages might reflect the action of other selective forces. While tolerance of low-salinity is essential for immediate survival in fresh water, other factors might also be important, and some might impose weaker but continuous selection over time. For instance, freshwater environments tend to have high concentrations of parasitic species of bacteria, microsporidia, oomycetes, and fungi (Ebert, 1995; Stirnadel and Ebert, 1997), many of which are absent in the marine environment. In addition, physical factors, such as greater temperature fluctuations, lower pH, and lower oxygen concentrations, might also impose selection. Accelerated development at later life-history stages might have evolved as a means to achieve reproductive maturity sooner and escape high parasite loads in fresh water.

Despite its retarded development to metamorphosis,

the freshwater Lake Michigan population still had faster development rate than those of calanoid copepods native to freshwater habitats (Kamps, 1978; Peterson, 2001). For example, development time to adult was 31.31 ± 5.47 days at 15°C for *Diaptomus pallidus* (Kamps, 1978), 37.9 days at 12°C for *Metadiaptomus meridianus* (Hart, 1998), and 61.4 days at 12°C for *Tropodiptomus spectabilis* (Hart, 1998). In general, development rate is 1.5 to 2 times slower in freshwater calanoid copepods relative to their brackish or marine counterparts (Peterson, 2001). The relatively rapid development rate of the Lake Michigan population and other freshwater populations of *E. affinis* (Vijverberg, 1980; Ban, 1994) might reflect incomplete adaptation to freshwater habitats due to its recent derivation from saline sources. Relative to previous experiments using other saline populations of *E. affinis* (Lee and Petersen, 2002), the populations in this study had relatively faster development at 5 PSU. Differences in results might reflect either real physiological differences between the populations or differences in experimental conditions, such as food source.

CONCLUSIONS AND FUTURE DIRECTIONS

This study revealed that *E. affinis* is not as broadly tolerant as previously assumed (Wolff, 2000), but experiences evolutionary adaptations during freshwater invasions. Tradeoffs between low- and high-salinity tolerance suggest that there could be costs involved in adaptations to both fresh and saline (+25 PSU) environments. Questions remain on the nature of the tradeoffs and why genetic variation for high-salinity tolerance is maintained in the freshwater populations. Given that selection is occurring on physiological tolerance and performance, what genes are under selection? Also, are the same or different genes under selection during independent invasion events?

Independent invasions into fresh water (Figs. 1, 2) provide replicate tests of adaptation for addressing these questions. Repeatability of evolution of quantitative traits is influenced by the genetic substrate for selection and genetic correlations among traits (Houle, 1991; Garland and Carter, 1994; Gromko, 1995). At a reductionist level, what appears to constitute the same response to independent freshwater invasions (such as similar reaction norms) might actually have arisen through different mutations, proteins, or gene networks, reflecting convergent evolution (Rifkin *et al.*, 2003; Townsend *et al.*, 2003). Determining evolutionary mechanisms of independent invasion events within and among clades is a long-term goal of this study.

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