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Survival of oyster larvae in different salinities depends on source population within an estuary

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ABSTRACT

The role of environmental heterogeneity in limiting connectivity and shaping population structure continues to be a major question in evolutionary biology, particularly for high-dispersal species. Many marine species have a two part life cycle comprised of a sedentary adult phase and a dispersing larval phase. For estuarine species such as *Crassostrea virginica* (eastern oyster), larvae are often carried through very distinct water masses that can affect growth and survival prior to settlement, potentially impacting population connectivity. On the mesoscale of an estuary, gene flow may be a homogenizing force; however, for genomic regions experiencing strong differential selection along estuarine gradients, gene flow may be minimal if recurrent viability selection maintains functional genetic differentiation. Estuaries are defined by salinity gradients and many taxa rely on phenotypic plasticity to thrive there. Nonetheless, even euryhaline species like eastern oysters have their physiological limits, and this study tests whether survival of *C. virginica* larvae in different salinities depends on parental source reef and/or conditioning salinity. Oysters from high, intermediate and low salinities within Delaware Bay, New Jersey, were spawned in a common garden to test for differences in larval survival that have a genotypic basis. Under the null hypothesis of functional homogeneity among adult oyster populations we expected no difference in larval survival. Broodstocks were conditioned in low and high salinity common gardens for 4–6 weeks before spawning. Larvae from 56 pair-cross families were reared in low and high salinities for 13 days. Cox proportional hazard models were used to determine significant predictors of larval survival. Population source interacted with larval salinity treatments to significantly affect larval survival. This finding suggests that the larval pool of single estuaries contains abundant genetic variation for survival across different salinities, stemming in part from functional genetic differences among source reefs. Our results can help parameterize larval connectivity models that incorporate environment-dependent survivorship.

1. Introduction

Understanding the degree of population connectivity, ranging from "closed" systems characterized by persistent genetic differentiation between populations to "open" systems showing broad-scale homogeneity, is vital to fishery management, restoration design and the designation of marine reserves (Cowen et al., 2007). Many marine species, particularly invertebrates, have a bipartite life cycle comprised of a sedentary adult phase and a dispersing larval phase. Planktonic larval stages can persist long enough for organisms to travel hundreds to thousands of kilometers; however, the connectivity of marine populations is often more restricted than predicted by the dispersal capabilities of migrants and the known hydrographic barriers (Koehn et al., 1980; Lewis and Thorpe, 1994). Two plausible and not exclusive explanations are physical barriers, such as isoclines and hydrographic fronts (Pineda et al., 2007), and biological barriers (Gaines et al., 2007; Grosberg and Cunningham, 2001). Physical explanations such as barriers to circulation have successfully predicted patterns of larval transport (Gilg and Hilbish, 2003) but the effects of physical barriers are frequently hard to determine due to interactions with larval behavior (Shanks, 2009). Biological barriers may be particularly important in systems with environmental gradients or patchiness where strong selective pressures during and after dispersal both limit connectivity and shape population genetic variation among breeders. Salinity gradients from fresh to oceanic water define estuaries and provide an excellent system for measuring biological barriers to connectivity.

Biological barriers to connectivity can occur during both larval dispersal and post-settlement. A large percentage of mortality for high fecundity marine species occurs during dispersal (Thorson, 1950). Predation and starvation are spatially unpredictable circumstances for larvae leading to potentially high mortality rates over and above intrinsic factors stemming from genetic load. In contrast, physiological stress as larvae disperse across abiotic gradients may account for spatially non-random mortality that could shape population differentiation. Apart from dispersal, the 'getting there' part of connectivity, postsettlement survivorship further determines realized connectivity between populations in terms of adult abundance, and only with successful reproduction

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do immigrants have an evolutionary impact. Phenotypic plasticity is a common adaptation to habitat heterogeneity, but every trait has tolerance thresholds beyond which plasticity is no longer sufficient to acclimate to the environment (reviewed in Auld et al., 2010). These thresholds define habitat use boundaries below the spatial scale of dispersal.

To the extent that habitat heterogeneity occurs at scales below that of dispersal, a proportion of dispersal constitutes 'migrants' across different microhabitats. Immigrants to non-parental microhabitats can experience a phenotype–environment mismatch and low relative fitness (Marshall et al., 2010) resulting in spatially balanced polymorphisms (Sanford and Kelly, 2011). Along spatially stable environmental gradients, each generation of migrants will undergo recurrent viability selection resulting in persistent population differentiation among adults when the strength of selection is strong relative to N_em (gene flow as measured by effective population size (N_e) and migration rate (m)) (Alleaume-Benharira et al., 2006; Antonovics, 1968; Barton, 2001; García-Ramos and Kirkpatrick, 1997; Holt, 2003; Kirkpatrick and Barton, 1997). For species with sedentary adults and proximity-dependent mating (e.g. broadcast spawners), the recurrent functional population differentiation among adults can be translated into greater functional diversity among larvae than what is expected under panmixia. Alternatively, where the strength of selection on a trait is less than N_em, but environmental stress is beyond plasticity thresholds, surviving immigrants can lower mean population fitness and constrain local adaptation such that no functional population differentiation would be observable (Hendry and Taylor, 2004; Nosil and Crespi, 2004; reviewed in Garant et al., 2007). The likelihood of these two outcomes depends on the degree of plasticity for a given trait, the strength of selection on that trait, and the distribution of gene effects underlying the trait (Yeaman and Whitlock, 2011). Thus, depending on the traits under investigation, populations compared across a gradient may exhibit different levels of connectivity and genetic differentiation related to these traits (Caillaud and Via, 2012).

One way to test for balancing selection is to measure population differentiation among adults for loci that are likely to be responding to selection gradients. Finding the relevant loci makes this classical population genetic approach challenging in non-model organisms, but with some luck and rigorous subsequent experiments, dramatic patterns of small scale genetic differentiation have been shown to result from post-settlement selection in several estuarine systems. One example is clinal variation at the *Lap* locus of *Mytilus edulis* (Koehn and Hilbish, 1987; Koehn et al., 1976, 1980). Among adult populations in the Atlantic Ocean and Long Island Sound a *Lap* allele decreased in frequency from 0.55 to 0.15 over a 10 mile distance with decreasing salinity (Koehn et al., 1976). In estuarine cohorts the oceanic allele was common in newly settled juveniles and progressively declined to the characteristic frequency found in local adults, consistent with recurrent post-settlement selection. Similarly, a strong selection gradient across the intertidal zone filters genotypes from the mixed larval pool in *Semibalanus balanoides* and maintains a stable polymorphism (Schmidt et al., 2000). These examples, along with other studies (rainbow smelt: Saint-Laurent et al., 2003; three-spined stickleback: Hendry et al., 2002, McCairns and Bernatchez, 2010), demonstrate the impact of a strong selection gradient on population differentiation in high gene flow systems.

An alternative approach is to experimentally test for genetically-based differences in survival limits for larvae derived from breeding populations experiencing different environments within a single estuary. If adults from different habitats are functionally differentiated as a result of recurrent selection then, after controlling for maternal effects, they should produce larval cohorts with distinct genotype-by-environment patterns of viability. Previous studies experimentally testing for genotype-by-environment effects on survival and growth of larvae have found phenotype–environment mismatches that suggest better survival and growth in the natal habitat than in other environments (eastern oyster: Newkirk et al., 1977; Newkirk, 1978; European oyster: Newkirk, 1986; hard clam: Knaub and Eversole, 1988; Manzi et al., 1991). In fact, larvae have been shown to have narrower physiological tolerances than adults in several bivalve species (Bayne et al., 1976) facilitating this experimental approach. The strength of this approach is that no a priori knowledge of candidate loci or markers for population differentiation are needed. Additionally, differentiation is identified directly at the phenotypic level after accounting for plasticity and maternal effects, explicitly demonstrating the extent of phenotype–environment mismatch at the dispersal stage.

The goal of this study was to identify functional differentiation in *Crassostrea virginica* (eastern oyster) adults along a salinity gradient within a single estuary by experimentally measuring the impact of source location and broodstock conditioning salinity on larval progeny survivorship at low and high salinity treatments. In western North Atlantic estuaries the eastern oysters are ecosystem engineers (Lenihan and Peterson, 1998) whose complex reef systems provide habitat for over 300 species (Beck et al., 2011). Due to its diverse ecosystem services (reviewed in Constanza et al., 1997), the oyster is considered a keystone estuarine species (Barnes et al., 2007; Coen et al., 1999). With historic loss of 90% of eastern oyster reefs in North America (Jackson et al., 2001; Kirby, 2004), restoration of oyster populations is needed to realize these ecosystem services again. Many states are engaged in efforts to restore oysters (Beck et al., 2011), often through reef construction and planting of hatchery-produced oysters. It is this restoration objective that motivates a more rigorous examination of larval tolerances and the mechanisms that determine them.

A potentially valuable methodological advance in restoration planning is to couple hydrodynamic models with larval particle tracking and habitat heterogeneity to project the efficacy of different management and restoration procedures. The Oyster Restoration Optimization model (North et al., 2010) designed for the Chesapeake Bay and a model of oyster larval dispersal in the Delaware Bay (Narváez et al., 2012) are two such models. The integration of larval swimming behavior and environment-dependent mortality potentially increases the accuracy of source and sink relationships inferred from the models. By identifying sources and sinks, organizations can focus on the appropriate sites for their particular restoration goals such as constructing reefs at sink locations or enhancing stock at source locations. Currently, environment-dependent larval mortality is modeled based on species-specific thresholds. If functional genetic differentiation occurs among breeding oyster populations within single estuaries, and especially given that dispersal is predicted to be strongly asymmetric and downstream based on these models (North et al., 2010), then implementing population specific thresholds may improve the ability of models to accurately predict the realized connectivity resulting from differential larval and post-settlement survival.

2. Materials and methods

2.1. Sample collection

Two hundred adult oysters were collected from each of three sites with distinct salinity regimes within the Delaware Bay on April 18, 2011 (Fig. 1). Oysters from Cape Shore (39° 04.10′ N, 74° 54.77′ W; salinity range of 20–25; Narváez et al., 2012) were hand collected from intertidal reefs. Oysters from Arnolds reef (39° 23.055′ N, 75° 27.002′ W; salinity range of 6.5–14.5; Bushek et al., 2012) and New Beds reef (39° 14.518′ N, 75° 15.071′ W; salinity range of 9–16.5; Bushek et al., 2012) were collected by dredge from the NJ Fish and Wildlife vessel *Zephyrus*.

2.2. Adult oyster conditioning

The main objectives of adult oyster conditioning were to minimize the impact of maternal effects on larval survival and to have adults undergo gametogenesis under two different salinities (10 and 30). Half of the oysters were conditioned in recirculating tanks at Haskin Shellfish Research Laboratory (HSRL) of Rutgers University while the

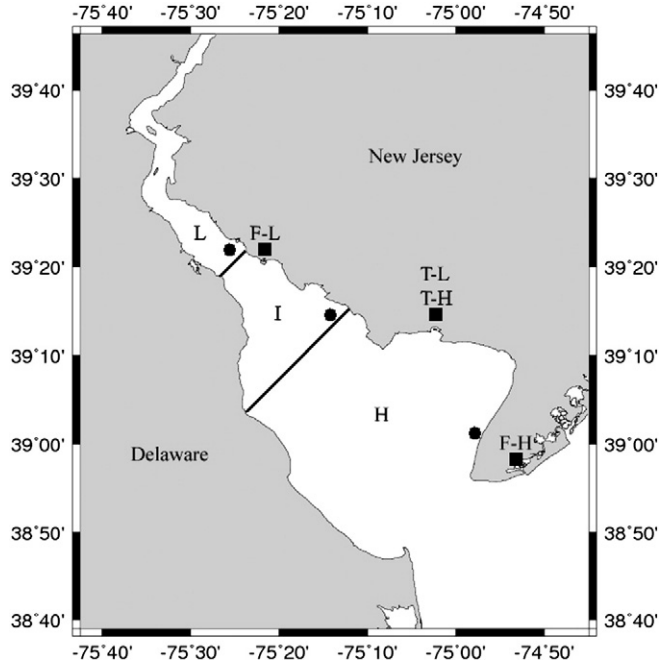

Fig. 1. The Delaware Bay is divided into three salinity regimes: L — low salinity (6.5–14.5), I — intermediate salinity (9–16.5), and H — high salinity (20–25). Circles represent 3 oyster broodstock collection sites. Squares are conditioning locations with T representing tank conditioning at a hatchery with low (L, 10) or high (H, 30) salinity and F representing field conditioning in open water field sites with low (L, ~5) and high (H, ~25–30) salinity.

other half were conditioned in the field. For the tank-conditioned oysters at the hatchery, fifty de-fouled oysters from each of the three populations were placed in a tank of UV-irradiated 1 mm filtered seawater (salinity = 30) and fifty from each were in a separate tank with the seawater diluted to a salinity of 10 with distilled freshwater. Total tank volume was 500 l. Temperature for the first three weeks of tank conditioning was 15 °C. Water heaters were then used to slowly increase the temperature to 20 °C and maintained there until spawning. The broodstocks were fed a 2:2:1 mixture of *Pavlova lutheri*, *Chaetoceros muelleri* and *Tetraselmis chui* twice a day. For a slow release of algae during feeding, a bucket of the mixture was siphoned via an airline into the tank. Broodstocks were conditioned in tanks for 14 weeks.

Previous experiences in an unpublished pilot study indicated that tank conditioning can be challenging with oysters collected from low-salinity wild stocks. Therefore, in this study half of the oysters were conditioned at field sites in the Delaware Bay. Fifty oysters from each source population were outplanted on racks in Cape May harbor (38° 56.73′ N, 74° 53.98′ W) for the high salinity conditioning (25–30; Narváez et al., 2012) and the remaining fifty oysters from each population were outplanted in bags off of a dock in the Cohansey River (39° 22.75′ N, 75° 21.32′ W) for the low salinity conditioning (~5; Narváez et al., 2012). Because of the geographic distance between the two field sites, the locations may have differed in other biologically important ways such as food availability. The oysters remained outplanted in the field for 10 weeks.

2.3. Oyster strip spawning

When field and tank broodstocks became ripe, gametes were stripped from the oysters using standard hatchery methods (e.g. Allen and Bushek, 1992). Field oyster condition was monitored by microscopically examining subsamples of oyster gonads and when they appeared to be ripe, moving all oysters to a 10 salinity tank for Cohansey-conditioned oysters and a 30 salinity tank for Cape May-conditioned oysters on the day before the spawn. Females were considered ripe when their follicles were filled with large, round oocytes and males were considered ripe when the follicles were densely packed with moving spermatozoa. All ripe males and females were used for spawning. Eggs were passed through an 80 μm sieve and retained on a 20 μm sieve in order to clean the eggs. The eggs were then resuspended in 200 ml filtered sea water at the conditioning salinity of the female. Sperm were passed through a 20 μm sieve to remove gonad tissue. Sperm from a single male was then slowly added to the eggs of a single female to produce a single family. Sperm–egg mixtures were examined on a Sedgewick-Rafter slide to ensure that sperm density was approximately 7–10 sperms per egg (determined by counting the average number of sperm surrounding an egg) and sperm was added until this density was reached (similar to Eudeline et al., 2000). After 2 h fertilization was confirmed based on observation of polar bodies.

2.4. Larval cultures

Two hours after fertilization, embryos were added to 1000 ml beakers of water at a density of 10 embryos/ml, one beaker per family per experimental treatment salinity. Embryos were maintained at the parental conditioning salinity until 24 h after fertilization. Then, to reduce osmoregulatory shock, the water was changed and salinities were adjusted to a midway salinity between conditioning and experimental treatment salinities. At 48 h, 25 D-stage larvae from each beaker were transferred to separate small glass dishes with 50 ml of water at experimental salinities of 10 and 30. Initiation of the treatment was counted as Day 1 and survival data were recorded every other day when all surviving larvae were pipetted into a new watchglass with clean water. Larvae were fed with T-*Isochrysis galbana* initially and a 1:1 ratio with *P. lutheri* starting Day 7 of the experiment. Feeding was daily and the quantity increased as the larvae grew. Larvae were kept in a temperature controlled room (temp = 25 °C) and eyed larvae began to develop on Day 11. Final counts were taken on Day 13.

2.5. Data analysis

Data from tank and field-conditioned oysters were analyzed separately. Each combination of population (H, I or L) and conditioning salinities (H or L) was replicated by having multiple families. The twenty-five larvae from each family replicated the time to mortality for a family (population and conditioning combination) at a specific salinity treatment (H or L). We attempted to make ten pair-cross families for each source population (H, I and L) at each conditioning salinity (H or L). Eyed larvae first appeared on Day 11 and many families went extinct before Day 13. Day 11 was therefore used as the time point for calculating mean number of surviving larvae and standard deviation as well as for comparisons between model-predicted reaction norms.

A Cox proportional hazards regression model (survival model) was used to compare survival of larvae over the course of the 13 day experiment based on population source (P), conditioning salinity (C) and treatment salinity (T). Coefficient subscripts distinguished their association to a particular factor and the subscript *i* indicated the individual larvae. The model, where $h_i(t)$ is the instantaneous risk of demise for individual larvae (i) at time t, leaves the baseline hazard distribution unspecified:

$$\log h_i(t) = \alpha(t) + \beta_j * \mathrm{P}_i + \beta_k * \mathrm{C}_i + \beta_l * \mathrm{T}_i.$$

Larvae from tank-conditioned and field-conditioned oysters were analyzed separately. In the survival model, time dependent variables are incorporated through a counting process that accounts for the time of mortality for each larva in the experiment or for survival until the end of the experiment (Table 1) (Andersen and Gill, 1982). The model was implemented with the *survival* package (Therneau, 2011) in R (R Development Core Team, 2011). The best models were identified using Akaike information criterion (AIC) to evaluate relative model fit in

Table 1
Number of independent pair-cross families and Day 11 surviving larvae per family in the larval survival experiment. P is the population source and C is the conditioning salinity. The % mor. is the percentage of mortality experienced by adult oysters during conditioning, not including random oysters killed to inspect gonad and gamete condition. The n_1 value is the number of surviving Day 11 families. Mean (mean number of surviving larvae) and sd (standard deviation) are for the families at Day 11. The n_2 value is the sample size (number of individual larvae) for the Cox proportional hazards regression model. The * indicates a family not used in the analysis.

				Treatment							
				High				Low			
Location	P	C	% mor.	n_1	mean	sd	n_2	n_1	mean	sd	n_2
Tank	High	High	2.7	7	14.00	7.66	175	7	10.29	5.74	175
		Low	4.0	10	12.20	6.09	250	10	16.1	5.70	250
	Intermediate	High	5.1	6	1.83	2.71	150	6	0.50	0.84	150
		Low	4.6	9	7.22	3.99	225	9	4.44	5.27	225
	Low	High	32.0	0	–	–	–	0	–	–	–
		Low	8.0	1*	–	–	–	1*	–	–	–
Field	High	High	8.5	0	–	–	–	0	–	–	–
		Low	50.0	0	–	–	–	0	–	–	–
	Intermediate	High	2.0	4	9.75	7.76	100	4	16.75	6.18	100
		Low	0.0	6	0.33	0.52	150	6	5.67	3.27	150
	Low	High	8.2	5	7.80	3.11	125	5	19.80	3.11	125
		Low	5.5	10	1.78	3.35	250	10	15.78	5.21	250

relation to the number of parameters and the model with the lowest AIC was selected.

3. Results

3.1. Oyster conditioning and spawning

The field-conditioned oysters developed mature gametes earlier than the tank-conditioned oysters as determined visually from gonad and gamete characteristics. Therefore, the spawning and larval culture of field-conditioned families preceded that of tank-conditioned oysters by four weeks. The high salinity population conditioned at the high salinity field site was spawned first, followed by the low and intermediate populations two weeks later and finally all three populations at the low salinity field site three weeks after the high salinity spawn. Additionally, for tank-conditioned broodstocks, gonad maturation and spawning were one week earlier for the high salinity population than for the low and intermediate populations.

Larval families were only successfully produced from a subset of the source populations. Oysters conditioned in tanks yielded multiple families from both high and intermediate salinity source populations (Table 1), but eggs did not fertilize in most pair crosses from the low

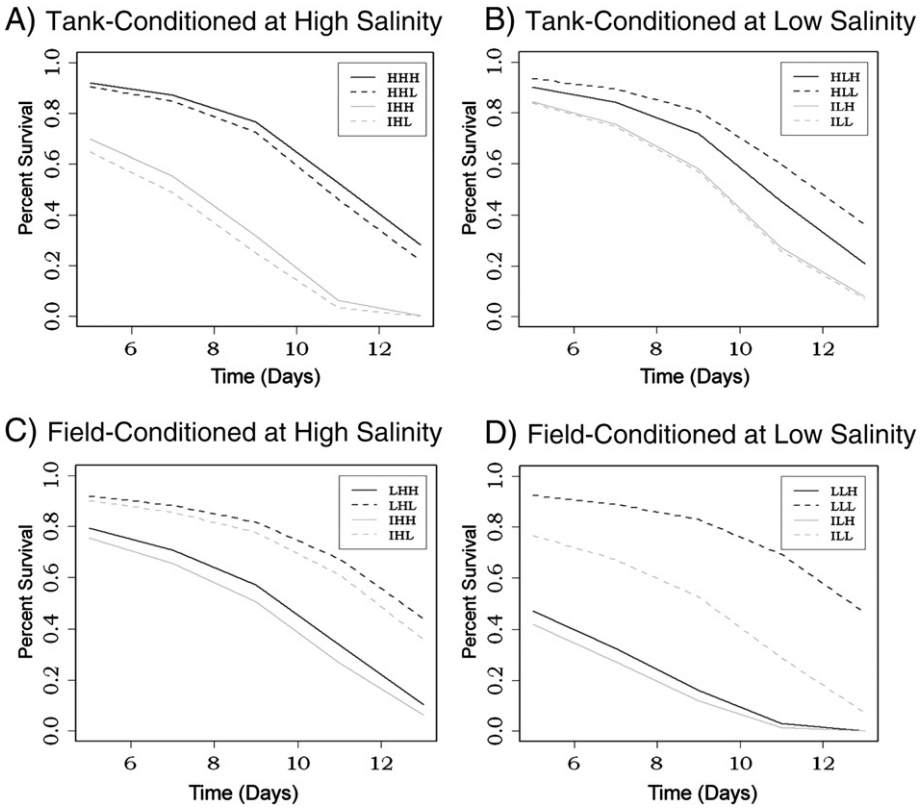

Fig. 2. Survival curves for tank-conditioned (A and B) and field-conditioned (C and D) oyster larvae over time predicted from two independent Cox proportional hazards regression models. The three letter legend abbreviations indicate the population source with the first letter, conditioning salinity with the second and treatment salinity with the third. Dashed lines are low (L) salinity treatments and solid lines are high (H) salinity treatments. Black and gray lines indicate population source with gray always indicating intermediate (I) population source, whereas black lines represent results from high source oysters with tank-conditioning in graphs A and B and represent results from low source oysters with field-conditioning in graphs C and D.

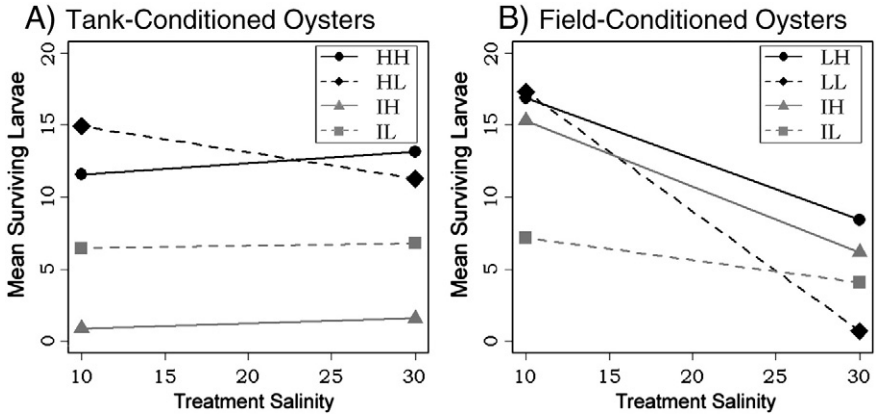

Fig. 3. Reaction norms of predicted larval survival from the Cox proportional hazards models for each experimental group: (A) Tank-conditioned oysters and (B) field-conditioned oysters. The two letter legend abbreviations indicate population source (low, intermediate or high salinity) with the first letter and the conditioning salinity (low ~ 10, high ~ 30) with the second letter. For tank-conditioned broodstocks (A) the only larvae experiencing 'home' conditions were those from the high salinity source at a treatment salinity of 30 in which case mean survival was slightly better than at a salinity of 10, but only when conditioned at high salinity (HH). Mean survival was better at a salinity of 10 when conditioned at low salinity (HL). For field-conditioned broodstocks (B) the only larvae experiencing 'home' conditions were those from low salinity source oysters. The L population larvae survived better at 10 than 30, regardless of conditioning.

salinity population. The gonads and gametes for low salinity source oysters appeared fully developed relative to oysters from intermediate and high salinities, but the lack of successful fertilization suggests that the eggs were not fully mature. Oysters conditioned in the field yielded multiple families from both intermediate and low salinity source populations (Table 1). For oysters from the high salinity source, ten out of ten spawned pairs conditioned at the high salinity location resulted in successful fertilization but the embryos did not develop to the D-stage for unknown reasons. This was the earliest population in which spawning was attempted, so it is possible that eggs were not fully mature. High-salinity source oysters conditioned at the low salinity location had high mortality while outplanted and the surviving oysters did not develop gonads.

3.2. Survival model

Analysis for the Cox proportional hazards models began on Day 5 when the first mortality event occurred. The survival curves predicted by the best models are presented in Fig. 2 and reaction norms of the number of surviving larvae at Day 11, predicted from the models, are presented in Fig. 3. For the tank-conditioned oysters from high and intermediate salinities, significant predictors of survivorship in the best regression model included population source (P), conditioning salinity (C), treatment salinity (T), and pairwise interactions of these factors, including the P × T interaction ($p = 0.012$) (Table 2). The largest model coefficient was population source (P: $p < 0.0001$) and the strongest contrast in survivorship curves showed higher overall survivorship in the high salinity source families relative to intermediate source families regardless of conditioning and larval culture treatments (Fig. 2A & B).

For the field-conditioned oysters from low and intermediate salinity populations, statistically significant predictors of survival in the best model included C, T, C × T and P × C × T terms ($p < 0.0001$ for each; Table 3). The low salinity source population larvae reared in the low salinity 'home' treatment had the greatest overall survival and lowest among-family coefficient of variation for survival (CV = 0.244) of any field experimental group (Figs. 2C & D, 3B, Table 1). In this field conditioning experiment the best survivorship model showed the location/salinity conditioning factor having the largest model coefficient ($p < 0.0001$, Table 3). Both source populations showed a relatively large change in larval survivorship in response to conditioning treatments, best illustrated by the predicted reaction norms at Day 11 (Fig. 3B). Predicted reaction norms from the field conditioning experiment illustrate the significant treatment (T) effect as steep reaction norm slopes (Fig. 3B).

To determine what factors were driving the higher-order interaction term of P × C × T for the field-conditioned oysters, the two conditioning locations were analyzed separately for the four possible models: P, T, P + T and P × T. For the high salinity conditioning, the best model was P + T where the P term was not significant but the T term was highly significant ($p < 0.001$) (Fig. 2C). For the low salinity conditioning, the best model was P × T where the main effect P term was not significant but both T ($p < 0.0001$) and P × T ($p < 0.0001$) were highly significant (Fig. 2D and dashed lines of Fig. 3B).

Table 2
Cox proportional hazards regression results for the tank-conditioned experiment involving broodstocks from high and intermediate salinity populations (P), conditioned at low and high salinities (C), with survival estimated for larvae at low and high treatment salinities (T). The Δ AIC is the difference in Akaike's information criterion between the best-performing model (top row) and the model being compared. Significant model coefficients ($\alpha = 0.05$, df = 6) are indicated in bold italics. Negative values increase survival and positive values decrease survival relative to that of larvae from the intermediate salinity population conditioned at high salinity and reared in the high salinity treatment.

	Model coefficient							
Model	P	C	T	P * C	P * T	C * T	P * C * T	Δ AIC
P * C + P * T + C * T	***−1.32***	***−0.64***	***0.32***	***0.74***	***−0.28***	***−0.37***		0
P * C * T	***−1.46***	***−0.75***	0.19	***0.97***	0.00	−0.15	−0.47	7.62
C + P * T	***−0.91***	***−0.46***	0.09		***−0.23***			51.21
C + P + T	***−1.02***	***−0.46***	−0.02					53.76
P * C	***−1.45***	***−0.82***		***0.73***				11.51
P * T	***−0.80***		0.08		***−0.26***			112.2
C * T		0.10	0.15			***−0.37***		349.5
P	***−0.93***							114.4
C		***−0.28***						358.3
T			−0.08					380.1

Table 3
Cox proportional hazards regression results for the field-conditioned experiment involving broodstocks from low and intermediate salinity populations (P), conditioned at low and high salinities (C), with survival estimated for larvae at low and high treatment salinities (T). The Δ AIC is the difference in Akaike's information criterion between the best-performing model and the model being compared. Significant model coefficients ($\alpha = 0.05$, df = 7) are indicated in bold italics. Negative values increase survival time and positive values decrease survival time relative to that of larvae from the low salinity population conditioned at high salinity and reared in the high salinity treatment.

	Model coefficient							
Model	P	C	T	P * C	P * T	C * T	P * C * T	Δ AIC
P * C * T	1.22	***3.27***	***0.36***	0.94	1.02	***0.29***	***2.89***	0
P * C + P * T + C * T	0.93	***2.73***	***0.26***	***1.46***	***2.00***	***0.47***		13.17
C + P * T	1.16	***2.43***	***0.15***		***2.19***			41.88
C + P + T	***1.58***	***2.38***	***0.22***					75.53
P * C	1.22	***1.36***		***1.61***				525.1
P * T	1.11		***0.20***		***1.94***			209.6
C * T		***3.11***	***0.37***			***0.44***		87.9
P	***1.53***							599.6
C		***1.60***						590.1
T			***0.27***					263.8

4. Discussion

Some recent studies have suggested that marine populations are not as homogenized as larval planktonic duration and potential dispersal distance would predict (Sanford and Kelly, 2011; Schmidt et al., 2008). Until the major mechanisms limiting gene flow are identified, it will remain difficult to generalize guidelines for spatially explicit management and restoration plans. One potentially important biological barrier to connectivity is phenotype–environment mismatches during dispersal or at settlement when habitat heterogeneity exceeds the tolerances and plasticity of individuals. For euryhaline species adapted to tidally variable estuaries, plasticity is the assumed primary mechanism by which individuals cope with both temporal and spatial variation in salinity. Phenotypic plasticity incurs an energetic cost, so species are expected to experience environmental margins where plasticity is stressful and beyond which environmental variation may be lethal, depending on genotype. For any particular species and environmental gradient there is presumably a zone of marginal habitat where differential viability selection becomes relatively important, relative to phenotypic plasticity, for population persistence. To the extent this is true, and mating is local, offspring from parents in marginal environments may have genotypes that are quite distinct from the species' norm. The demographic and evolutionary consequences of these marginal populations depend on their extent and patterns of connectivity. High fecundity and broad scale dispersal are life history traits that may jointly increase the likelihood that differential viability selection has spatially broad effects. Not only will broad dispersal make phenotype mismatches common, but the large effective population size associated with these life history traits will increase the efficacy of selection relative to drift so that more moderate habitat heterogeneities have consequences in terms of a selective cost.

This conceptual model requires quantitative theoretical development and empirical systems conducive to hypothesis testing. One fundamental prediction is that spatially proximate populations exchanging many migrants across a steep environmental gradient will show functional genetic differentiation. Previous studies have demonstrated that recurrent post-settlement viability selection produces spatially balanced polymorphisms at one or a few loci responding to fine-scale estuarine habitat gradients (Day, 1990; Johannesson et al., 1995; Koehn et al., 1976, 1980; Schmidt et al., 2000). Transplant experiments have also been used to demonstrate local adaptation at various scales for marine species with larval dispersal (reviewed in Sanford and Kelly, 2011).

Here we took an alternative approach to test for phenotype–environment mismatches that could generate a biological barrier to dispersal. We experimentally tested whether limits to salinity tolerance differentially affect survivorship for larvae derived from a 'common garden' of local populations that had settled and survived in different salinity regimes. The approach taken here focuses on the complex physiological phenotype of salinity tolerance, crudely in terms of survivorship, and measures the fitness impact of phenotype–environment mismatches within a single estuary where larvae are well mixed (Milbury et al., 2008).

Under the null hypothesis of phenotypic plasticity, reaction norms should have no slope and there should be no P × T interaction effects. Alternatively, if broodstock populations are genetically adapted to the salinity regime of their home reef, then their larvae should survive better at that 'home' salinity relative to larvae from other broodstock source locations (population [P] effect), and better relative to cultures from the same population reared at non-natal salinities (treatment [T] effect) for a combined P × T effect. This effect was clear in the survival model for larvae from the more experimentally-controlled tank-conditioned oysters collected at high and intermediate salinities within Delaware Bay. For tank-conditioning, the P × T term had a significant effect on larval survivorship ($p = 0.012$), although it was not the most significant predictor (population: $p < 0.0001$). The significance of the population main effect was driven by overall low survival in the intermediate population (the population that was not environmentally matched by either 'high' or 'low' conditioning or treatments).

For the field-conditioned oysters, conditioning location/salinity had a large effect, perhaps not surprisingly given the potential for confounding with other environmental factors such as temperature and primary productivity that may have co-varied with salinity between the conditioning locations. A P × T effect was apparent in the larvae from broodstocks conditioned up-bay at low salinity (Fig. 2D), and was likely subsumed in the overall field experiment model within the higher-order interaction of P × C × T ($p < 0.0001$). When the two conditioning locations were analyzed separately a highly significant P × T effect ($p < 0.0001$) was indeed found for larvae from low and intermediate source broodstocks conditioned up-bay at low salinity (Fig. 2D). With low-salinity field conditioning, larvae from the low salinity population had as much as four-fold better survival in low salinity versus high salinity cultures, whereas intermediate-source larvae showed only a two-fold difference (Fig. 2D).

Intermediate source broodstocks were not conditioned nor were their larvae cultured under 'home' conditions in either experiment so predictions were ambiguous. Nonetheless, all else being equal we expected larvae from the intermediate broodstocks to respond to salinity treatments similarly in the tank and field experiments. This was true with the reaction norm slope produced by the intermediate source larvae after low-salinity conditioning, suggesting comparability of results across the experiments, but not for high-salinity conditioning (compare Fig. 3A and B). If the two experiments are comparable they imply that low salinity broodstock populations are more genetically differentiated with respect to alleles influencing osmoregulatory tolerance and produce larvae with narrower phenotypic plasticity than intermediate or high salinity populations under most conditions.

Across these analyses a population by treatment effect is evident both statistically and graphically, either alone or in interaction with conditioning location/salinity. An unfortunate constellation of experimental factors eliminated our ability to make many of the direct comparisons sought, complicating interpretation of the results. However, the experimentally cleanest (tank) experiment generated results consistent with a significant P × T interaction with respect to high and intermediate salinity source populations. Interestingly, the prediction of home-environment advantage, testable in this experiment only with the high population source larvae tested under 'home' and 'away' conditions, was only seen with conditioning at high (home) salinity (Fig. 2A). Home-environment advantage was also found for larvae from low-salinity source broodstocks (Fig. 2C & D) with a steep reaction norm no matter what conditioning location/salinity was experienced by broodstocks (Fig. 3B). Overall these results provide tentative support for

a model in which selection across the salinity gradient in Delaware Bay was strong enough to generate functional genetic differences among low, intermediate and high salinity adults such that they produced larvae with different survival probabilities at different salinities.

An intriguing pattern that emerges from these results is that the low salinity population has much greater survival in low salinity than in high salinity treatments whereas the high salinity population shows more similar survival rates across salinity treatments. Some biophysical models, such as the Oyster Restoration Model (North et al., 2010), suggest a predominantly downstream movement of larvae from low salinity regions to high salinity regions of the estuary. Our results suggest a greater potential for genetic differentiation in the upstream reaches of the estuary and this is consistent with limited up-estuary dispersal. Furthermore, the more plastic phenotypic response (lower reaction norm slope) observed in families from high salinity regions was potentially due to downstream transport generating recruitment from a more diverse larval pool. Further research should investigate the degree of asymmetric gene flow within estuaries and its consequences for functional genetic differentiation.

To draw inferences about genotypic differentiation, we minimized maternal effects on larval survival with the one exception of conditioning salinity, a variable that in principle could be manipulated in the hatchery if there were strong justifications to do so. To minimize general maternal effects two approaches were taken. In the more controlled experiment using tank-conditioned broodstocks to generate larvae, broodstocks were collected early in gametogenesis and maintained in common garden tanks where temperature, water volume and water change frequency were uniform and only salinity differed. Oysters in each tank were also fed equal densities of algae relative to the mass of oysters in the tank. The second experiment using field-conditioned broodstocks was an attempt to test larvae based on a common garden broodstock design, while using a more natural 'garden'. However, environmental conditions other than salinity may have differed between the two conditioning locations in Delaware Bay. Production of and experimentation with F2 progeny from the original broodstocks is a more thorough method of controlling maternal effects, but captive propagation of oysters typically entails reductions in genotypic diversity (Boudry et al., 2002) and invites inadvertent artificial selection (Christie et al., 2012). Many maternal effects are expected to wane during larval development, particularly after metamorphosis to a feeding veliger. Newkirk et al. (1977) reported that significant maternal effects on the survival of *C. virginica* larvae ended after day 6. Thus, in this study general maternal effects were further minimized by starting the larval survival experiment 48 h after fertilization and measuring larval survival out to 13 days.

Our experimental focus on larvae allows us to directly relate results to the dispersing phase of oysters and concomitant selection in the plankton, and as such will help parameterize dispersal and recruitment models. The relevance of these results to post-settlement selection is less clear, especially given that salinity tolerances are somewhat narrower in oyster larvae than in adults (Kennedy, 1996). Nonetheless, given other examples of strong post-settlement selection (e.g., Koehn et al., 1976, 1980; Schmidt et al., 2008) we can expect that functional genetic differentiation among adults from different salinity regimes was produced by a combination of pre- and post-settlement selection.

The genetic patterns demonstrated here lead to several recommendations for restoration practice and modeling. For hatchery-based restoration methods, survival of outplanted juveniles may be improved by collecting broodstocks from the region of the estuary where the outplanting will occur, or from an environmentally similar region within the estuary. Additionally, larval survival in the hatchery can be maximized by conditioning broodstocks at a salinity that falls within the source location range of variation. Environmental matching between broodstock source location and outplant site can maximize post-outplant survival but does not necessarily improve the success of subsequent larvae. Success of subsequent larval cohorts will depend on their dispersal patterns relative to salinity gradients, among other factors. For modeling, realized dispersal may be more accurately estimated if larval survivorship is parameterized as a function of parental environment. Because broadcast spawning enforces local mating at the scale of individual reefs (Levitan et al., 1991), our results imply that the larval pool is not just a product of generalist parents, but includes contributions from assortative mating among physiological specialist genotypes along the habitat margins. Depending on the demographic extent of contributions, this interpretation of the larval pool suggests a dramatically different source–sink dynamic for oyster recruitment than would be presumed for a homogeneous habitat.

Our study has demonstrated greater larval survival at salinities that more closely match the parental source salinity, consistent with pre-settlement selection contributing to functional genetic differentiation of osmoregulatory genes in adults spanning the estuarine salinity gradient. In order to quantify the combined pre- and postsettlement effects of selection on functional connectivity, future research should compare estimates of neutral marker gene flow to that realized in functional genes under selective pressure across habitat heterogeneities.

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