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Differential Fertilization Success Between Two Populations of Eastern Oyster, *Crassostrea virginica*

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Abstract. Identification of mechanisms promoting prezygotic reproductive isolation and their prevalence are key goals in evolutionary biology because of their potential role in speciation. In marine broadcast-spawning species, molecular interactions between gamete surface proteins are more important than mating behavior for determining reproductive compatibility. Evidence for differential fertilization capacity has been reported from experiments utilizing competing sperm from two males sampled within populations and between species, but to our knowledge conspecific populations that might have diverged in allopatry have never been tested on the basis of sperm competition. In the present study, the gametic compatibility and embryo survivorship from matings between two allopatric populations of *Crassostrea virginica*, the eastern oyster, on either side of a genetic step cline were investigated. Fertilization success, embryo survival, and paternity data all indicated an absence of strong reproductive barriers between the two oyster populations, implicating other mechanisms for maintenance of the cline step. Sperm from northern male oysters showed a tendency to produce more larvae than expected when competing with sperm from southern male oysters. Although the northern male advantage was not strong, the trend implies that long-distance dispersal across the step cline might more successfully result in north-to-south gene flow than the reverse, providing a mechanistic hypothesis explaining the asymmetric cline shape.

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Introduction

Mechanisms that promote prezygotic reproductive isolation are thought to contribute to speciation. Identification of such mechanisms and their prevalence thus contributes significantly to evolutionary biology. Genetic population structure exists at surprisingly small scales in many marine organisms given their high dispersal capacity (Hellberg *et al.*, 2002). The fate of long-distance dispersers, and therefore the scale of population homogenization by gene flow, is potentially influenced by habitat selection, mate preference, spawning synchrony, and preferential fertilization. When selectively neutral markers provide evidence for limited gene flow, inferences about barriers to dispersal are too often made without testing for barriers at fertilization that act after dispersal (Palumbi, 1994). Because adults of most broadcast-spawning marine invertebrates lack mating behaviors that contribute to reproductive isolation, the gamete recognition and fertilization capacity mediated by the surface proteins of eggs and sperm are likely to be an important post-dispersal, prezygotic factor that limits gene flow (Palumbi and Metz, 1991; Palumbi, 2009).

Fertilization efficiency depends on compatibility between egg and sperm surface proteins (gamete recognition proteins, GRP). In theory, sexual selection and sexual conflict can cause rapid co-evolution of these proteins, generating a diversity of fertilization capacities among individuals within populations and reproductive isolation between allopatric populations (Palumbi, 2009). In support of this theory, positive selection and rapid interspecific divergence usually occur at the molecular level for GRPs (Swanson and Vacquier, 2002). Also, differential fertilization capacity has been documented among individuals within populations (Gaffney *et al*., 1993) and correlated to the GRP allele carried by the uniting gametes in sea urchins (Palumbi,

1999). Almost no data exist on fertilization barriers across allopatric populations within species. As a result, it is unknown whether these powerful co-evolutionary forces act on scales of time and space that are commensurate with intraspecific gene flow restrictions, or if local co-evolutionary arms races promote a geographic mosaic of fertilization compatibilities (Biermann and Marks, 2000; Styan *et al*., 2008). Species with geographically clinal genetic variation are ideal for testing differential fertilization capacity because spatially proximate populations experiencing similar environments have a documented history of limited gene exchange, at least for some portions of the genome. The mechanisms maintaining genetic clines in broadcast-spawning species are unknown in most cases (oysters, Hare and Avise, 1996; mussels, Gilg and Hilbish, 2003; barnacles, Sotka *et al*., 2004; Zakas *et al*., 2009). Relatively low fertilization capacity across a genetic cline could be either a cause or a consequence of low gene flow, but either way it is an unexplored and potentially important factor maintaining clines.

Crassostrea virginica (Gmelin), the eastern oyster, is a broadcast spawner with external fertilization and a planktonic larval duration of 2 to 3 weeks before settlement (Galtsoff, 1964). On the mid-Atlantic coast of Florida, continuously distributed eastern oyster populations show sharply stepped clinal variation at several genetic markers, both mitochondrial and nuclear (Reeb and Avise, 1990; Hare and Avise, 1996). The cline (Fig. 1) has been stable for the past dozen years (MPH, unpubl. data) and is hypothesized to result from secondary contact of historically isolated populations (Hare and Avise, 1996). The mechanisms maintaining the oyster cline are unknown; dispersal barriers or spatially heterogeneous selection acting pre- or postzygotically could be contributing. If dispersal establishes contact between the genetically divergent populations, interbreeding and admixture will depend on the relative gametic compatibility between migrants and local individuals.

Experiments with gamete competition or choice are required to test for differential fertilization capacity (sometimes referred to as egg preference or sperm precedence) and can provide a much more sensitive indicator of reproductive barriers than no-choice experiments (Howard, 1999). Although fertilization preferences have been demonstrated in free-spawning species through gamete competition experiments at the interspecific level (Bierne *et al*., 2002; Geyer and Palumbi, 2005; Harper and Hart, 2005; Bushek *et al*., 2008) and among individuals within a population (Palumbi, 1999; Boudry *et al*., 2002; Marshall and Evans, 2005), we are unaware of any previous study that has incorporated gamete competition into a test for differential fertilization among populations of a broadcast-spawning species.

In this study, we investigated the gametic compatibility between two allopatric populations of *C. virginica*, on either side of a genetic step cline, using *in vitro* tests of differential fertilization efficiency between sperm from competing males. We found no strong fertilization barriers capable of maintaining the oyster cline, but we discuss subtle fertilization preferences in terms of cline shape as well as intraspecific mechanisms that potentially generate incompatibilities.

Materials and Methods

Oysters were collected from two Florida populations (Fig 1a). The North broodstock (N) was collected near the University of Florida Whitney Laboratory, Marineland (29°40.209′, 81°12.940′); the South broodstock (S) was from Vero Beach (27°39.274′, 80°22.166′). Broodstock oysters were collected between 20 March and 21 April in 2008 and maintained on a standard microalgal diet (Krantz, 1982; Creswell *et al.,* 1991) in filtered seawater (FSW) from a well located near the Indian River Lagoon at Harbor Branch Oceanographic Institute at Florida Atlantic University, Fort Pierce.

For each experiment, four individuals (S♂, S♀, N♂, and N♀) were chosen on the basis of gamete quality (high motility of sperm or shape of eggs) after shucking about 12 to 20 individual oysters. Sperm were fixed in 4% formalin before concentration was estimated by averaging two independent counts from a hemacytometer. Eggs were counted by using a Sedgewick-Rafter counting slide.

Sperm from the two males were mixed in different ratios, 1:9, 1:1, and 9:1, in 1000-ml beakers with 200 ml of FSW (19.5–24.5 °C, 31–32 parts per thousand salinity) at total concentrations of 2×10^5 sperm/ml and used to fertilize each female in parallel. Eggs were added to the sperm at a final concentration of 100/ml, producing a 1000:1 ratio of sperm to egg (as in Gaffney *et al*., 1993). In the two control crosses the male and female from the same population were paired, making a total of eight crosses in each experiment (Table 1). Different broodstock oysters were used in each experiment conducted at about the same time on different days. Fertilization success was calculated as the proportion of fertilized eggs (as indicated by a polar body or cell cleavage under 40–100× magnification) after 2 h. To ensure that good-quality gametes were used, experiments proceeded only if fertilization success of both controls was above 70%. After the 2-h sampling, embryo density was lowered 4-fold, aeration was initiated, and the temperature was maintained at 27–28.5 °C by placing the beakers in a heated water bath. After 24 h, all D-stage larvae were collected by filtration through a 20-μm screen and preserved in 95% ethanol. Embryo survivorship through the developmental transition to larvae was calculated by dividing the total number of D-stage larvae at 24 h by the total number of fertilized eggs at 2 h.

DNA was extracted from broodstock oyster mantle tissue

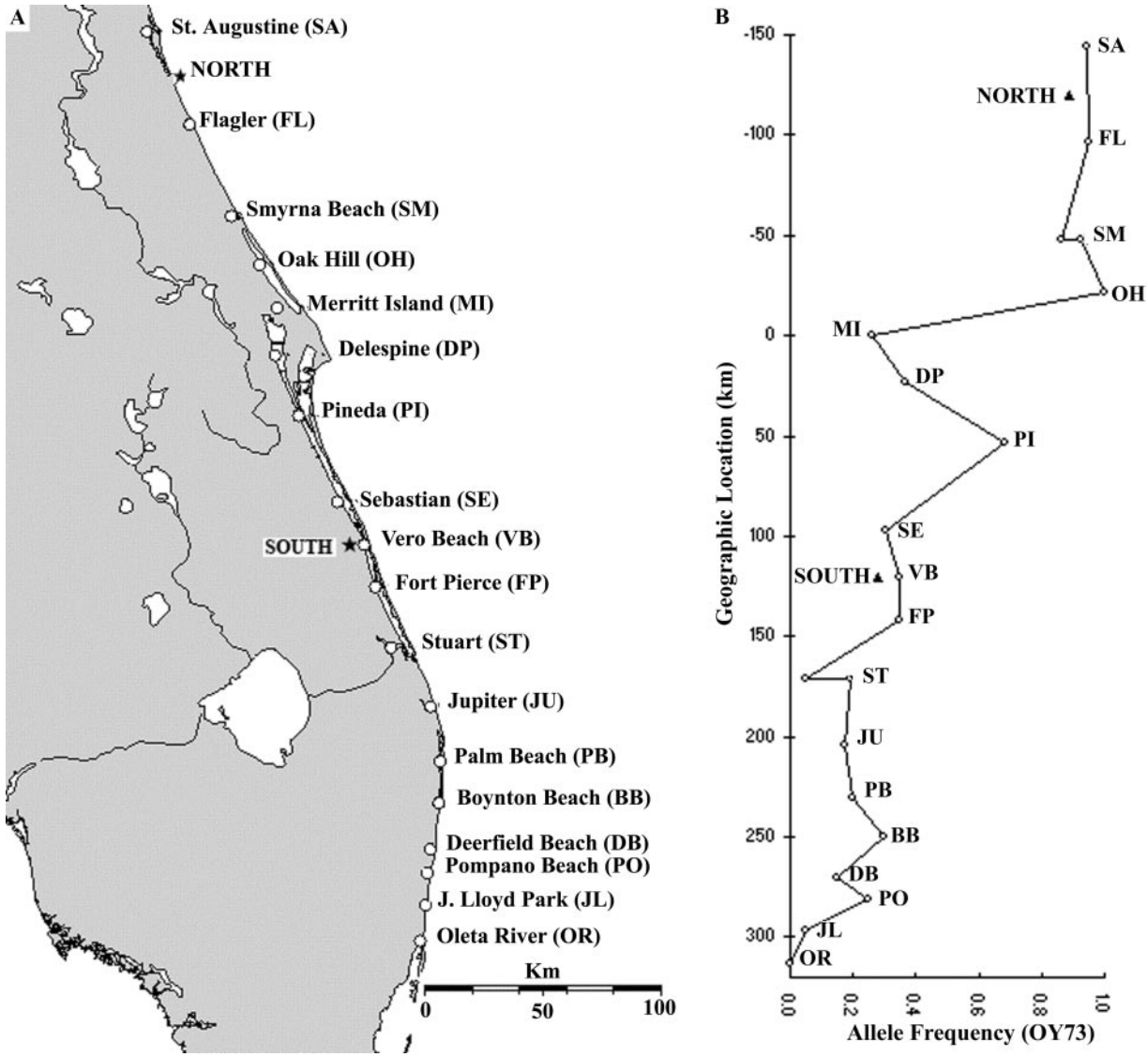

Figure 1. Collection sites (stars on map) and mitochondrial OY73 RFLP frequencies for North and South oyster broodstock used in present study (closed triangles on graph, $n = 36$ each) shown relative to (A) Florida map indicating 1993 oyster collection sites and previously published (Hare and Avise, 1996) site acronyms (one site, GA, from the previous publication is not included) and (B) 1993 OY73 RFLP allele frequencies (open circles; Hare and Avise, 1996). Vertical axis in (B) shows the distance (km) of each site from the approximate cline center at Cape Canaveral. The North broodstock sample had no corresponding sample in 1993.

using DNeasy Spin-Columns (Qiagen Inc., Valencia, CA). Larval oyster DNA extraction followed Hubert and Hedgecock (2004), but 20-μl volumes were used. To confirm genetic differentiation of source populations for oyster broodstock, a mitochondrial restriction fragment length polymorphism (RFLP) was assayed in the broodstock oysters used in the present study (Hare and Avise, 1996) and tested for significant geographic differentiation using Fisher's exact test (Sokal and Rohlf, 1995, p. 734). Broodstock oysters were also genotyped for four microsatellite loci (*Cvi*9, *Cvi*12, *Cvi*2g14, *Cvi*2i23) as in Rose *et al*. (2006). Each oyster larva was genotyped with 1–2 microsatellite loci to produce definitive paternity assignments. Genotyping was done using an ABI-Prism 3730 genetic analyzer, and all automated allele assignments made using GENEMAPPER 4.0 (Applied Biosystems) were confirmed by eye.

Statistical analyses were performed using SPSS 13.0, and results were considered significant if $P \leq 0.05$; sequential Bonferonni analysis was used to control for multiple tests. Percent fertilization and embryo survival data were arcsine transformed before applying parametric tests. A mixed model was used to test fertilization success and embryo survival; family nested within source was used as a random effect (there are two families in each experiment), with

Table 1

Mating design for each experiment

Experiment	Sperm ratio: (S♂:N♂)
South: all eggs from South females	
#1	1:0 (Control)
#2	9:1
#3	1:1
#4	1:9
North: all eggs from North females	
#5	0:1 (Control)
#6	9:1
#7	1:1
#8	1:9

broodstock source and sperm ratio treatment (TMT) and their interaction as fixed effects:

$$Y = \text{family(source)} + \text{source} + \text{TMT} + \text{source} \times \text{TMT}$$

Within each treatment (9:1, 1:1, 1:9) and egg source (North, South), a homogeneity test was applied to paternity results to evaluate variation in fertilization across experiments (McDonald, 2009, pp. 64–69).

To determine if one male had a fertilization advantage over the other male relative to the expectation based on the sperm mixture ratio, observed and expected larval paternities were compared using exact goodness of fit tests (McDonald, 2009, pp. 24–32). To check if differential fertilization by males depended on egg source, the Fisher exact test of independence was used to compare the observed paternity of larvae produced for the same sperm mixture applied to eggs from a North *versus* South female (Sokal and Rohlf, 1995, p. 734).

Results

We performed 12 independent experiments using different individuals. Three experiments were eliminated from analysis because of low fertilization in the control cross (0%–30% fertilization success). For the remaining nine experiments, mean fertilization success in control crosses was 92.63% for the South population (S♂ × S♀) and 86.36% for the North population (N♂ × N♀). Two independent sperm counts for each male in each experiment had a mean coefficient of variation of 14.78% (range 1.96%–47.14%) for northern males and 11.12% (1.77%–27.08%) for southern males.

Genetic differentiation of broodstock source populations

Mitochondrial RFLP frequencies in the broodstock oyster collections from North (0.889) and South (0.278) populations were significantly different. Figure 1 shows the sample locations and mitochondrial allele frequencies of broodstock oysters in comparison with the mitochondrial DNA cline reported by Hare and Avise (1996). Broodstock oyster RFLP frequencies in 2008 also were not significantly different from those in 1993 samples (Hare and Avise, 1996) at adjacent sites in the north or at the same site in the south, indicating temporal stability.

Table 2

Effect of female source location (Source) and sperm ratio treatment (TMT) on fertilization success (%)

	Degrees of freedom			
Source	Numerator	Denominator	*F*	*P*
Intercept	1	16	2029.040	0.000
TMT	3	48	0.936	0.431
Source	1	16	0.100	0.756
Source × TMT	3	48	2.636	0.060

All fertilization success data were arcsine transformed.

Fertilization success and embryo survival

Several mechanisms can result in skewed paternities, including preferential fertilization or differential survival following uniform fertilization success. We used a mixed model to test whether fixed effects of sperm mixture treatment or geographic egg source had a significant effect on fertilization success or embryo survival after accounting for random variation among families. No significant effect was found for either fixed factor or their interaction (Tables 2 and 3), indicating an absence of strong reproductive barriers for allopatric sperm/egg mixtures.

Paternity analysis

A single microsatellite locus was sufficient to distinguish competing males in eight experiments. In the remaining experiment, conclusive paternity assignments were based on genotypes at two loci. Larvae from the 1:1 sperm ratio treatment were not genotyped for three experiments either

Table 3

Effect of female source location (Source) and sperm ratio treatment (TMT) on embryo survival (%)

	Degrees of freedom			
Source	Numerator	Denominator	*F*	*P*
Intercept	1	16	444.278	0.000
TMT	3	48	0.860	0.468
Source	1	16	0.263	0.615
Source × TMT	3	48	0.992	0.405

All survival data were arcsine transformed.

because they degraded (experiment 2) or because they were deemed uninformative (experiments 4 and 8, see below). Thus, paternity was tested in 48 crosses from nine experiments based on genotyping a total of 3858 larvae. An average of 80 larvae were genotyped in each cross (treatment × egg source). At this sample size the null hypothesis of fair-raffle fertilization leads to an expectation of eight embryos fathered by the rare male in the 1:9 or 9:1 treatments; an observation of ≤ 2 or ≥ 14 embryos from that male would reject the null hypothesis at $P \leq 0.05$. Homogeneity results within treatments and egg source indicated significant variation in paternity results among experiments ($P \leq 0.05$). Two experiments in particular (4, 8) showed strong and opposite preferential fertilization of one male over the other regardless of egg source (Fig. 2).

Figure 2 shows the percent North *versus* South paternity results from all three treatments across nine experiments. Compared to fair-raffle paternity expectations based on sperm mixture ratios, 21 out of 48 crosses (44%) showed significant deviations ($P \leq 0.05$). Among these 21 cases, superiority of the northern male occurred 17 times (81%). All four cases in which South sperm achieved greater than expected paternity occurred in only one experiment (#4), whereas the 17 cases of northern male superiority were found across seven experiments. After sequential Bonferroni correction, 13 deviations (27% of 48 crosses) remained significant overall; nine cases of northern male superiority occurred among five experiments, and the four cases of southern male superiority remained restricted to experiment 4.

Among the nine cases of Bonferroni-corrected significant northern male advantage, four were from pairings with a South female and five from a North female. To test for an effect of egg source on differential paternity using all the data, Fisher's exact tests of independence were used to compare paternity between egg sources. Among 24 crosses (nine experiments and three treatments, three missing observations), six had significantly different paternity for the two males depending on the source of eggs they competed for (Table 4). After sequential Bonferroni correction, one case in each of two experiments remained significant, both showing stronger northern sperm advantage when competing for northern eggs (Table 4).

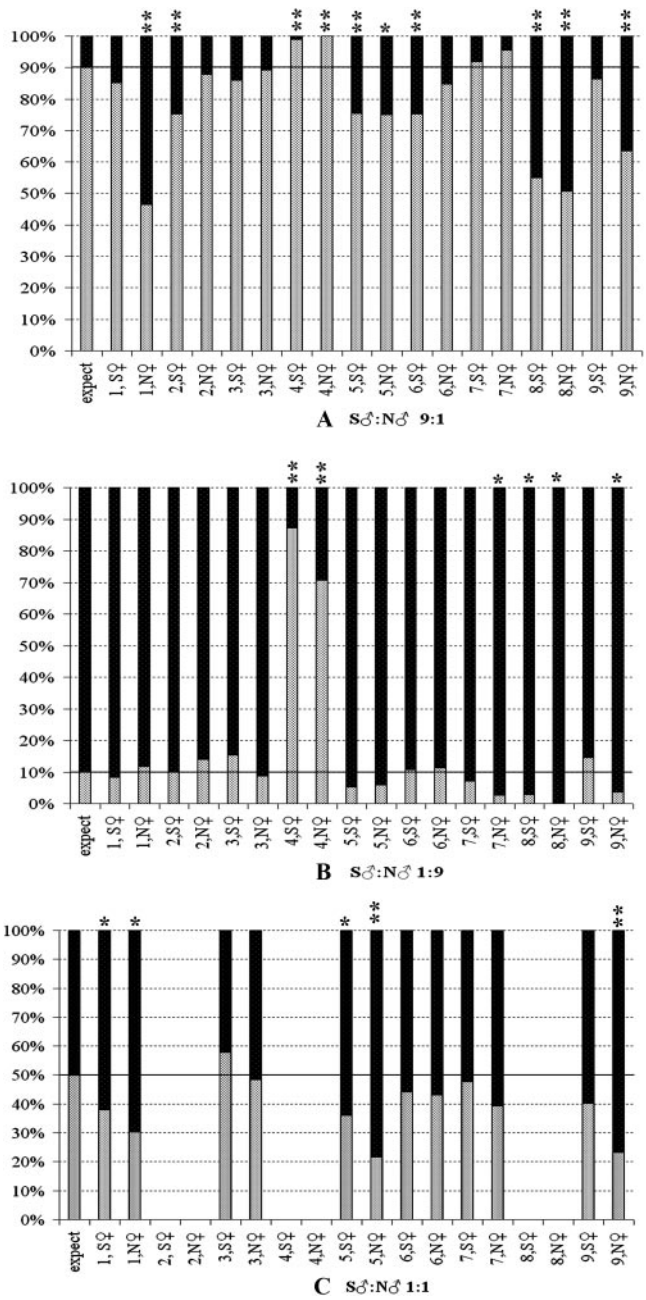

Figure 2. Percent paternity results from nine fertilization experiments using sperm mixtures from two males, presented by sperm ratio treatment (S:N = 9:1, 1:1, 1:9). Different oysters were used in each of the nine experiments. Exact goodness of fit tests were used to compare the difference between observed paternity and expected fair-raffle paternity within treatments and by egg source (S or N). Solid columns show the percent paternity from the northern male (N♂); stippled columns show percent paternity from the southern male (S♂). First column of each graph ('expect') and the horizontal line indicate expected paternity based on fair-raffle probabilities between the competing males given the estimated sperm mixture ratio. Legends under other columns show experiment number and egg source. Single asterisks indicate significance at $P < 0.05$; double asterisks indicate results that remain significant after sequential Bonferroni correction.

Discussion

Given that the sharp genetic cline in Florida oysters is stable despite broad dispersal potential in this species, our first hypothesis proposed prezygotic reproductive barriers as a mechanism capable of limiting gene flow between northern and southern oyster populations. No significant effect of geographic source of sperm was found for fertilization success or embryo survivorship, indicating a lack of strong reproductive barriers acting at these levels. In addition, paternity analysis of larvae produced under sperm compe-

Table 4

Comparison of observed paternity between egg sources (Southern egg, S♀, Northern egg, N♀), by microsatellite DNA data from D-stage oyster larvae in nine experiments with different broodstock oysters

Experiment	Sperm ratio (S:N)	Observed paternity				Fisher's exact test *P* value
		S♀		N♀		
		S♂	N♂	S♂	N♂	
1	9:1	63	11	33	38	**0.001***
	1:1	30	49	19	44	0.377
	1:9	7	77	5	38	0.538
2	9:1	73	24	65	9	**0.050**
	1:9	6	55	13	80	0.617
3	9:1	68	11	67	8	0.628
	1:1	52	38	47	50	0.241
	1:9	12	66	7	73	0.228
4	9:1	89	1	104	0	0.464
	1:9	75	11	63	26	**0.009**
5	9:1	77	25	27	9	1.000
	1:1	37	66	13	47	0.078
	1:9	4	72	3	48	1.000
6	9:1	61	20	84	15	0.131
	1:1	37	47	34	45	1.000
	1:9	9	75	11	86	1.000
7	9:1	80	7	64	3	0.515
	1:1	41	45	33	51	0.283
	1:9	6	78	2	74	0.282
8	9:1	49	40	34	33	0.629
	1:9	2	71	0	64	0.498
9	9:1	64	10	54	31	**0.001***
	1:1	35	52	19	63	**0.021**
	1:9	12	71	4	107	**0.008**

Sperm were quantified for each of two males (S♂, N♂) in each experiment and mixed at 9:1, 1:1, and 1:9 ratios. Each sperm ratio treatment was used for independent simultaneous crosses with eggs from a northern female and a southern female. Bold *P* values are ≤ 0.05, and an asterisk indicates significance after sequential Bonferroni correction.

tition all indicated an absence of strong barriers to reproduction between oyster populations on either side of the genetic cline. Clearly, other mechanisms such as physical dispersal barriers or environmentally mediated selection, both currently under investigation, are more important deterrents to the mixing of oyster populations along the Atlantic Florida coast.

Nonetheless, the genetic cline highlights a legacy of separate population histories for oysters in the north and south. Depending on the specifics of this history, more subtle fertilization preferences or genomic incompatibilities might have evolved that can influence the degree or pattern of population mixing. Using mixtures of sperm from competing males, we measured the degree of successful fertilization as an index of barriers to molecular gamete recognition, embryo survival as an index of genomic compatibility through development to the larval stage, and larval paternity as a test for differential reproductive compatibility that integrates over these two factors. Paternity results provided some indication that North and South males have differential capacities to produce larvae when competing for the same female. Not surprisingly, given the subtle and inconsistent effects demonstrated with paternity analysis, the methods used here did not detect overall reproductive barriers (egg source or egg source by sperm ratio effect) with respect to percent fertilization success or embryo survival. Allopatric sperm mixtures as well as sympatric control crosses produced embryos.

Paternity analyses need to be conducted on early embryos to positively discriminate between fertilization and embryo viability effects (*e.g.,* Huvet *et al*., 2001). Almost all studies of sperm competition require growth and development of offspring before genotyping (Harper and Hart, 2005). Here, for continuity with many sperm competition studies on broadcast spawners that genotyped ≥36-h larvae (Bierne *et al*., 2002; Harper and Hart, 2005; Bushek *et al*., 2008), we refer to our results in terms of "fertilization capacity" but will discuss the possibility of embryo viability effects in larvae 24-h post-fertilization.

Within sperm mixture treatments and egg source, the

observed ratio of northern and southern paternities varied among experiments, indicating variation in fertilization capacity at the level of the individual, the family, or both. Within this heterogeneity there was a strong asymmetry, with 73% of significant deviations from fair-raffle paternity showing a fertilization advantage for northern males. The exceptions were restricted to a single experiment (#4) in which all four crosses showed significant opposite (southern advantage) effects. Thus, differential fertilization capacity was common when sperm from different oyster populations were forced to compete for eggs from a single female, and our results suggest a trend toward northern male advantage.

Egg source effects on paternity were similarly heterogeneous, but they showed a subtle tendency for sperm advantages to be expressed more strongly with local mates than with non-local mates. Both Bonferroni-corrected cases of an egg source effect showed a consistent advantage to the North male but with a stronger advantage in the North egg cross (Table 4). Among the marginally significant results, the northern male advantage was sometimes expressed more strongly with the southern eggs (experiment 2, treatment 9:1) or else each male showed an advantage only with the same-population female (experiment 9, treatment 1:9). Interestingly, the experiment showing strong southern male advantage (experiment 4) also showed this advantage more strongly in the competition for the same-source female, the South female. Thus, we tentatively conclude that male fertilization advantages in *Crassostrea virginica* are expressed by sperm more strongly with eggs from the same geographic source than with eggs from a different source.

Accurate measurement of sperm concentration is one of the most challenging aspects of sperm competition experiments and can be difficult to control for. Thus, it is possible that random error in sperm concentration measurements contributed to among-experiment heterogeneity, but these errors are equally likely to favor northern and southern males. For the eight experiments that showed a significant deviation from fair-raffle expectations, the binomial probability of finding a fertilization advantage by sperm from the same source population seven out of eight times is 0.03 under the null hypothesis of equal fertilization capacity and random measurement error. Thus, while sperm measurement errors may have contributed to random variation, they are not a likely explanation for the trend toward northern male advantage.

Given that there were deviations from fair-raffle expectations, we expected and observed stronger deviations in the extreme treatments where the superior sperm were relatively rare. Among the nine crosses showing statistically significant northern sperm advantage there were no cases in the 1S:9N treatment, two in the 1S:1N treatment (average chi square = 24), and seven in the 9S:1N treatment (average chi square = 74). Experiment 4 showed southern sperm advantage so strongly that we declined to analyze parentage in the 1:1 treatment, deeming it relatively uninformative. The southern advantage in experiment 4 was significant in one 9S:1N treatment cross (chi square = 9.7) and both 1S:9N treatment crosses (average chi square = 468). Thus, the number of significant crosses and the magnitude of deviation from expected both show a sperm mixture effect that does not create an advantage but can increase the impact of an advantage. The apparent minority advantage emerges in sperm mixtures as a simple result of head-to-head competition between sperm: when advantaged sperm are relatively common in the two-male mixture, they will compete for mates primarily against other advantaged sperm from the same male; but when relatively rare they compete primarily against more numerous disadvantaged sperm (Bryant *et al*., 1980). Thus, assuming an advantage and imagining pairs of competing sperm for illustration, the deviation from fair-raffle expectations using a 1:1 sperm mixture will result from the 50% of advantaged sperm that on average compete with sperm from the other male (while all other sperm are competing with their sibling sperm). In treatments where advantaged sperm are so rare as to always be head-to-head with sperm from the other male, then on average 100% of sperm contribute to a fair-raffle deviation. This mixture effect is distinct from the "rare male effect" reported for some internal fertilizers (Partridge, 1988) and from theoretical predictions of frequency dependence from gamete-interaction arms races (Levitan and Ferrell, 2006) because these latter evolutionary phenomena are both predicted and observed within populations.

We tentatively conclude that sperm from northern Florida oysters often have greater fertilization capacity when competing against sperm from southern Florida males. This conclusion is based solely on a comparison between populations from two regions separated by the genetic step cline. To better understand the geographic scale and pattern of nonrandom reproductive capacities, and its relationship to historical gene flow, it will be necessary to compare variation in fertilization capacity within populations and within regions in addition to across regions with independent evolutionary histories. Multiple populations of eastern oyster were tested for gametic incompatibility by Gaffney *et al.* (1993) using single-pair crosses (no sperm competition) in a 2×2 design. The mid-Atlantic populations compared were farther north than the "north" population studied here, but corresponded to the same genetically homogeneous North Atlantic region north of the Florida cline (Hare and Avise, 1996). They found no population-specific pattern to fertilization success, but there was a slight overall advantage for within-population pairings over between-population matings. The strongest pattern was a dependence of fertilization success on the specific parents involved in a cross (Gaffney *et al.,* 1993), a phenomenon that presumably contributes to the variation seen among crosses in this study.

Our conclusion remains tentative partly because we are not

able to completely rule out environmental factors that could elevate overall sperm vigor in northern males relative to southern males, although the uniformly high fertilization success in our experiments (nearly 90% of control crosses had ≥ 80% fertilization success) and identical hatchery conditioning of broodstock make this a less likely mechanism. Both in terms of temperature and salinity, hatchery conditions used for fertilization and culture did not clearly mimic (*i.e.,* potentially favor) one or the other broodstock source. In fact, at the time of collection in 2008 the environmental differences between broodstock sources were minimal, about 31 to 33 parts per thousand salinity and 26 to 22 °C, south to north.

The generality of our conclusions may also be limited because they are based on a single overall sperm-to-egg ratio even though this ratio is expected to vary widely with ecological context (*e.g.,* variation in adult density, gamete production, or spawning time). Robustness of our conclusions based on the 1000:1 sperm-to-egg ratio (chosen for comparability with Gaffney *et al.,* 1993, and maximum fertilization rate based on Lyu and Allen, 1999) is suggested by two studies in which the rank order of fertilization advantage in reciprocal crosses between two *Crassostrea* species was consistent across experiments that used a wide range of sperm/egg ratios (Lyu and Allen, 1999; Bushek *et al.,* 2008). Nonetheless, the importance of relative sperm and egg density to the asymmetric fertilization reported here remains to be tested.

The sperm mixture effect observed in the present study is a nonequilibrium artifact of competing sperm from different populations and would not be stable within a population. However, episodic migration is a nonequilibrium process in which mixture effects could be relevant for determining the population-level impacts of differential fertilization capacities. If gene flow across the oyster step cline is episodic or otherwise limited, the evolutionary impact of northern male fertilization advantages could make introgression more likely in a north-to-south direction because rare migrant males arriving in the south are predicted to have reproductive success above fair-raffle expectations. The jagged southern tail in the oyster cline (Fig. 1) is consistent with this predicted directionality, but other evolutionary cline models are equally suitable to explain this pattern and discriminating among them requires additional data. There are currently no data supporting low fitness of north-south *C. virginica* hybrids (M. Burford, Department of Natural Resources, Cornell University; unpubl. data), so reinforcement of these subtle differences in fertilization capacity is not the most likely outcome of population admixture.

In principle, the trend for northern male advantage shown here could stem from lowered embryo viability due to outbreeding depression or differential fertilization capacity stemming from divergence of gamete recognition systems. Outbreeding depression is suggested by the fact that sperm advantage shown by males from each population, North and South, was usually stronger in matings with eggs from the local female as opposed to the non-local female. Thus, the effect could have resulted from relatively poor viability of inter-population embryos during development. Intrinsic genetic incompatibilites can lower fitness in crosses between divergent populations due to underdominance or deleterious epistatic interactions in F1 progeny (Edmands, 2002). Results consistent with this mechanism were reported for dry shell weight in *Crassostrea gigas* (Garnier-Géré *et al*., 2002).

There are several models describing evolutionary mechanisms by which differential fertilization capacity can evolve within populations of broadcast-spawning species. These processes may be acting independently and dynamically within *C. virginica* populations, producing variation in fertilization capacity across populations such that our allopatric population comparison captured a contemporary difference. Potential within-population processes generating variation in fertilization capacity include a gamete compatibility arms race between the sexes due to conflicts between polyspermy avoidance by females and maximization of fertilizations by males (Vacquier *et al*., 1997; Gavrilets, 2000; Gavrilets and Waxman, 2002). This mechanism may include frequency-dependent selection driven by spatial/temporal heterogeneity in effective sperm densities (Levitan and Ferrell, 2006; Palumbi, 2009). Any within-population process leading to variation in fertilization capacity over time will also have the potential to create differential fertilization abilities between independently evolving allopatric populations.

Molecular gamete recognition systems are known to produce asymmetric intraspecific barriers (Biermann and Marks, 2000). The same is true of genomic incompatibilities that reduce hybrid fitness between divergent populations (Edmands, 2002). Given the extraordinary diversity documented in *C. gigas* for the expressed sperm bindin protein (Moy *et al*., 2008) and its likely evolution as a result of sexual conflict in that species, we speculate that similar processes may be active within *C. virginica* populations and responsible for the differential fertilization capacity found here when males from allopatric populations competed experimentally for fertilizations. Alternatively, the phenotypic differences we report could have arisen by genetic drift in the absence of gene flow. Regardless of the mechanism, the differential fertilization capacities across allopatric populations presumably represent a consequence, not a cause, of cline steepness and stability for eastern oysters along Atlantic Florida. We are aware of only a few other studies that have tested for differential fertilization capacity across populations potentially connected by gene flow (Gaffney *et al.,* 1993; Biermann and Marks, 2000; Styan *et al*., 2008), but none that utilized mate-choice experiments to directly test relative fertilization capacity. This study, therefore, provides the first direct evidence for the allopatric evolution of differential fertilization capacities within a broadcast-spawning species.

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