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Comparison of Gamete Compatibility Between Two Blue Mussel Species in Sympatry and in Allopatry

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Abstract. Recent demonstrations of positive selection on genes controlling gamete compatibility have resulted in a proliferation of hypotheses concerning the sources of selection. We tested a prediction of one prominent hypothesis, selection to avoid hybridization (*i.e.,* reinforcement), by comparing heterospecific gamete compatibility in two *Mytilus edulis* populations: one population in Cobscook Bay, Maine, in which the close congener, *M. trossulus,* is abundant (a region of sympatry), and one population in Kittery, Maine, in which *M. trossulus* is absent (a region of allopatry). Three diagnostic nuclear DNA markers were used to identify mussels to species and to estimate the frequency of both species and their hybrids in the two populations. Controlled crosses were then conducted by combining eggs of *M. edulis* females with a range of *M.edulis* and *M. trossulus* sperm concentrations. Results were not consistent with the reinforcement hypothesis. *M. edulis* females collected from the region of sympatry were no more incompatible with *M. trossulus* males than were *M. edulis* females collected from the region of allopatry. A trend in the opposite direction, toward greater compatibility in sympatry, suggests that introgression of *M. trossulus* genes that control egg compatibility, such as those encoding receptors for sperm, may influence evolution of gametic isolation in hybridizing populations.

Introduction

Recent demonstrations of positive selection on genes encoding gamete recognition proteins in free-spawning marine animals have sparked considerable interest in the evolutionary dynamics of these systems. DNA sequences of proteins controlling gamete interactions in sea urchins and both gastropod and bivalve molluscs show strong signals of positive selection evidenced as amino acid–altering DNA substitutions that exceed silent changes (Lee *et al.*, 1995; Metz and Palumbi, 1996; Biermann, 1998; Hellberg and Vacquier, 1999; Swanson and Vacquier, 2002; Riginos and McDonald, 2003; Zigler and Lessios, 2003; McCartney and Lessios, 2004; Zigler *et al.*, 2005). Because these data conflict with traditional expectations that stabilizing selection will maintain sperm-egg recognition within species, they have spawned an array of hypotheses about the sources of diversifying selection on fertilization proteins.

Proposed selective forces range from selection against hybridization, to sperm competition, female choice, and polyspermy avoidance (Rice and Holland, 1997; Vacquier *et al.*, 1997; Howard *et al.*, 1998; Palumbi, 1998; Howard, 1999; Clark *et al.*, 2006). However, experimental tests of these hypotheses are rare (but see Palumbi, 1999; Geyer and Palumbi, 2003). Avoidance of hybridization (as in the hypothesis of "reinforcement") is frequently assumed to be the main fitness benefit of gamete incompatibility (Coyne and Orr, 2004). But in sea urchin and abalone model systems for work on gamete incompatibility in marine invertebrates, hybrids are uncommon in natural populations (*e.g.,* Owen *et al.*, 1971; Palumbi and Metz, 1991; Lessios and Pearse, 1996), and the published tests of reinforcement have been limited so far to comparisons of DNA sequences that code for sperm proteins in sea urchins (Geyer and Palumbi, 2003) and mussels (Springer and Crespi, 2007). Remarkably, no studies have yet compared gamete compatibility between populations with and without coexisting congeneric species.

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Opportunities for such work abound in the *Mytilus edulis* (Linnaeus, 1758) blue mussel species complex, in which species exist in multiple zones of sympatry and allopatry and frequently hybridize where they are sympatric (*e.g.,* Gardner, 1994; Hilbish *et al.*, 2000; Riginos and Cunningham, 2005). A recent study explored gamete compatibility in the hybrid zone between *Mytilus edulis* and *Mytilus trossulus* (Gould 1850) in the Gulf of Maine (Rawson *et al.*, 2003). Although a strong reciprocal block to fertilization between these two species was shown to exist, eggs from female *M. edulis* varied broadly in their compatibility with sperm from *M. trossulus* males, and variation in compatibility was a property of specific females, rather than of males (Rawson *et al.,* 2003). Yet in all of these experiments, *M. edulis* females were collected from within the hybrid zone, where this species is sympatric with *M. trossulus*. A pattern of reinforcement would predict that these females would show higher levels of cross-species incompatibility than would females collected from a zone of allopatry.

In this report, we test the reinforcement hypothesis by comparing heterospecific gamete compatibility in *M. edulis* females collected from two populations, one in allopatry and one in sympatry with *M. trossulus*. We used molecular markers to identify individuals of the two species and characterize the composition of the populations, and then quantified gamete compatibility through controlled laboratory crosses.

Materials and Methods

Study system

Blue mussels are free-spawning bivalves found throughout temperate and subpolar regions in both the northern and southern hemispheres (Hilbish *et al.*, 2000; Rawson *et al.*, 2001). The *Mytilus edulis* complex consists of three species (McDonald *et al.*, 1991): *M. edulis* (Linnaeus 1758), *M. galloprovincialis* (Lamark 1819), and *M. trossulus* (Gould 1850). Analysis of nuclear and mitochondrial DNA sequences indicate that *M. edulis* and *M. galloprovincialis* are sister taxa, with a divergence date of about 2 million years ago (mya; Rawson and Hilbish, 1998; Wilhelm and Hilbish, 1998; Hilbish *et al.*, 2000). *Mytilus trossulus* is more distantly related, having diverged from the other two species about 3.5 mya (Vermeij, 1991; Rawson and Hilbish, 1995, 1998; Beynon and Skibinski, 1996; Hilbish *et al.*, 2000).

Mytilus edulis and *M. trossulus* are sympatric throughout the Canadian Maritimes and eastern Gulf of Maine, and they display temporal overlap in gametogenesis and spawning (Maloy *et al.*, 2003a). The frequency of hybrids ranges from 2% to 26% in different populations (Bates and Innes, 1995; Mallet and Carver, 1995; Saavedra *et al.*, 1996; Comesaña *et al.*, 1999; Innes and Bates, 1999; Gardner and Thompson, 2001). Few hybrids are from the F1 generation; most represent F2 or later-generation backcrosses (Rawson *et al.*, 2001; Toro *et al.*, 2004). This bimodality in genetic composition suggests that selection against hybrids and strong assortative mating are maintaining species boundaries (Jiggins and Mallet, 2000).

Although *M. edulis* is distributed throughout the northeastern United States, the Gulf of Maine represents the southern end of the *M. trossulus* distribution (Rawson *et al.,* 2001). *Mytilus trossulus* individuals are abundant in some locations along the northeastern coast of Maine, near the Canadian border, but decrease in frequency to the southwest (Rawson *et al.,* 2001, 2007). *Mytilus trossulus* alleles are rare, and no pure *M. trossulus* have been found in intertidal populations southwest of midcoast Maine (Rawson *et al.,* 2001), where *M. edulis* can be considered to be allopatric.

Collection, species identification, and population characterization

Mussels used in fertilization assays were collected from two locations in the Gulf of Maine from 6 to 19 June in 2003 and 2004, when visual inspection of gonads indicated that both males and females were prepared to spawn. Collections were made from the East Bay portion of Cobscook Bay in northeastern Maine (latitude 44°52′50½N; longitude 67°07′13½W) where *M. edulis* and *M. trossulus* co-occur and hybrids have constituted 12%–13% of the population in previous years (Rawson *et al.,* 2001), and in Kittery in southwestern Maine (latitude 43°04′04½N; longitude 70°41′20½W). At each site, about 200 adult mussels (50–80-mm shell length) were randomly collected along two 10-m transect lines placed parallel to the shoreline at lowest tide. Following collection, mussels were transported to either the University of Maine's Darling Marine Center, in Walpole, Maine (2003), or the University of New England's Marine Science Center, in Biddeford, Maine (2004), where they were separated by site and maintained in a static seawater system at 9° C for species identification and subsequent spawning. Mussels were fed daily with a commercial phytoplankton mixture composed of *Isochrysis galbana, Pavlova lutheri,* and *Nannochloropsis oculata* (Algal paste; Innovative Aquaculture, Lasqueti Island, British Columbia, Canada).

Mussels were individually labeled with bee tags (Graze Inc., Germany). Tissue was collected for genetic assays by inserting a wooden peg between each mussel's valves and clipping a small piece of the mantle frill. Genomic DNA was extracted using a modification of the "Rapid Isolation of Mammalian DNA" protocol (Sambrook and Russell, 2000). Individuals spawned for experiments were initially identified to species or to hybrid genotype, using three nuclear DNA PCR-based markers that are diagnostic for *M. edulis* and *M. trossulus: Glu 5′* (Rawson *et al.*, 1996), *ITS* (Heath *et al.*, 1995), and *Mal I* (Rawson *et al.*, 2001). Although hybrids were regularly encountered, all individu-

als chosen for fertilization experiments were three-locus homozygotes; that is, they typed as "pure" *M. edulis* and *M. trossulus* at these loci. After spawning, the genetic identity of all individuals was re-confirmed using the three nuclear DNA markers. To characterize the genetic composition of both source populations, 93 Cobscook individuals and 35 Kittery individuals collected from transects in 2003 were also typed using the same three markers.

Experimental crosses

About one month after collection (both years), mussels were induced to spawn by the application of a series of thermal shocks. Females that began to release eggs were moved to room temperature water; males that released sperm were immediately removed from the container, wrapped in damp paper towels, and placed on ice. Sperm collection from males was subsequently augmented by strip spawning (Rawson *et al.*, 2003) as follows: Valves were pried open, and a small incision was made in the mantle tissue to allow the sperm to flow into a 1.5-ml centrifuge tube, which was capped and stored on ice. This "dry sperm" was stored no longer than the time necessary to set up and execute all of the fertilization assays, which ranged from 6 h to 13 h 45 min (mean of 7h 18 min). Analysis of homospecific crosses *post hoc* (not shown) showed no relationship between fertility of males and storage duration within this time range. Eggs obtained from each female were washed in aged seawater (ASW), allowed to settle, measured volumetrically, and re-suspended at 2% by volume in ASW in a 15-ml conical vial, then held on ice for 5–28 h (mean 12 $\pm$ 6 h) while sperm was prepared for the cross. Previous work has detected no decrease in egg viability over this time frame (Slaughter, 2005).

Each cross was performed by pipetting 0.5 ml of the egg/ASW suspension into six scintillation vials each containing 4 ml of ASW. The sperm solution was diluted as follows: 100 μl of the sperm suspension was added to 900 μl of ASW in a 1-dram vial, mixed with a pipet, transferred to the next vial containing 900 μl of ASW, and so on to obtain five 10-fold serial dilutions. Then 100 μl of dry sperm, or 100 μl from each of the diluted sperm suspensions, was added to each egg-containing scintillation vial and gently swirled. Two 50-μl subsamples of the third sperm dilution from each cross were preserved in an equal volume of 2% gluteraldehyde for subsequent sperm counts with a Neubauer hemacytometer. Embryos were allowed to develop to the 4–16-cell stage, then development was stopped by the addition of 1 ml of 37% formaldehyde to each vial. Fertilization in each vial was quantified as the percentage of successfully cleaving embryos from a random sample of 200 eggs.

Sperm-free controls were also created (0.5 ml of egg suspension into 4 ml of ASW) and fixed with 1 ml of 37% formaldehyde when the corresponding experimental crosses were fixed. Crosses were excluded from analysis if fertilized embryos were present in these control vials. In addition, in a small number of cases, egg suspensions were apparently contaminated by exogenous sperm sometime during handling, because heterospecific trials showed an appreciable number of fertilized eggs that did not increase with increasing concentrations of added sperm. All of the crosses involving any female that showed evidence of contamination, in any single trial, were excluded from further analysis and are not included in the mating design described below.

Mating design

Each *M. edulis* female tested was crossed with 1–4 different *M. trossulus* males (mean = 2.8). With one exception, each of these females was also tested in conspecific crosses (1–3 males, mean = 1.9) to control for potential variation in egg quality *(i.e.,* to ensure that apparent heterospecific incompatibility was not simply a by-product of defective eggs). A total of 50 heterospecific and 33 conspecific crosses were performed for six females from the Cobscook population and 12 females from the Kittery population. To augment our sample size of females from the Cobscook population, we also include F_{20} data from a previously published study (Rawson *et al.,* 2003). This study was conducted by two of the same authors (Yund and Slaughter) in 2001, following exactly the same methods, with a similar experimental design. Six *M. edulis* females were crossed with 1–3 *M. trossulus* males (mean = 1.8), and to check for egg viability, 1–3 *M. edulis* males (mean = 1.5), for a total of 11 heterospecific and 9 conspecific crosses. Data from all *M. edulis* females crossed with both *M. edulis* and *M. trossulus* males in this earlier study were included in our analysis. Combined sample sizes for the reported data are 12 females from each population, with 61 heterospecific and 42 conspecific crosses.

Data analysis

To quantify the representation of species-diagnostic alleles within individuals in both populations, and therefore to check for the degree to which both sympatric and allopatric *M. edulis* populations experience introgression of *M. trossulus* alleles, our three-locus genotype data were used to calculate a hybrid index (HI). For this index, each *M. edulis* allele was assigned a value of 1 and each *M. trossulus* allele was assigned a value of 0. A pure *M. edulis* is thus represented by a value of 6, while a pure *M. trossulus* is represented by a value of 0. A first generation hybrid would have a value of 3, while other intermediate values represent various backcross hybrid combinations.

To quantify gamete compatibility in each cross, we calculated the F_{20}, or sperm concentration necessary to fertilize 20% of the eggs (Levitan, 2002b; McCartney and Lessios,

2002; Rawson *et al.*, 2003). These values exhibit an inverse relationship with compatibility, so that a lower F_{20} indicates a more compatible cross. F_{20} was employed rather than the F_{50} used in many studies because most heterospecific crosses failed to achieve 50% fertilization at the highest sperm concentration, and estimating F_{50} would have required extrapolation beyond the data range (McCartney and Lessios, 2002; Levitan, 2002a, b). Because fertilization exhibits a logistic relationship with sperm concentration, the raw proportional fertilization data (P) were logit transformed, where logit (P) = ln ($P/1 - P$) (McCartney and Lessios, 2002; Rawson *et al.,* 2003). A value of 1 was added to each raw count of fertilized and unfertilized eggs, which together sum to 200, because logit (P) where $P = 0$ or 1 is undefined. Least-squares linear regressions were then used to fit logit (P) to sperm concentration, and the resulting regression equations were used to calculate a single value for F_{20} for each cross (assuming no error in estimated regression parameters). Because values for F_{20} varied across so many orders of magnitude, we present the data as the log of F_{20}.

Several incompatible heterospecific crosses were found, in which zero or near-zero fertilization occurred at the highest sperm concentration tested, and in which the slope of the sperm dilution curve was not significantly different from zero (*i.e.*, fertilization showed no dependence on sperm concentration). These females were considered to be "completely blocked" to fertilization by heterospecific sperm, and we compared their frequency between the allopatric and sympatric population using a G-test with Williams' correction (Sokal and Rohlf, 1994). The reinforcement hypothesis would predict a higher frequency of blocked females in sympatry.

However, the existence of these individuals presented a challenge for quantitative analysis. Simply omitting them would have eliminated 5 of 12 females from the allopatric population, so we used the following approach. If the calculated F_{20} value was either undefined (slope = 0) or if it exceeded the highest sperm concentration used in the cross, we set F_{20} to equal the concentration of undiluted dry sperm in that cross. This amounts to a conservative attempt to quantify compatibility that may be either not quantifiable or biologically irrelevant, but we did this so that all crosses could be included in a single quantitative analysis of variation in F_{20}. We recognize the disadvantage, that females whose eggs are completely blocked to fertilization with heterospecific sperm are being treated in this analysis as if they were fertilizable, albeit at very high sperm concentrations.

With the exception of one female, all females in the heterospecific mating experiments were crossed with multiple males, but due to the vagaries of success with induction of spawning on a given day, the number of males varied. Since compatibility varied among females (see results), those females that were tested on more males would have been granted greater leverage if we treated individual crosses as independent data. An alternative approach, and one that is not prone to lack of independence among crosses performed with the same female, was adopted, in which mean F_{20} values for each female were calculated and these means were compared. However, the reader should recognize that mean F_{20} values are based on variable numbers of crosses. For all quantitative analyses, we used ANOVA models that are described further in the text of the results.

Results

Species composition of study populations

Consistent with expectations from previous studies, *Mytilus edulis* and *M. trossulus* were sympatric at the Cobscook Bay site, with 38% of the individuals typing as pure *M. trossulus,* 47% as pure *M. edulis,* and 15 % as hybrids (Fig. 1). Few F_1 hybrids (hybrid index = 3) were detected, and most hybrid genotypes were consistent with F_2 or greater backcrosses to either *M. edulis* or *M. trossulus* (Fig. 1). By contrast, all members of the Kittery population typed as pure *M. edulis*, confirming our use of this as an allopatric population.

Gamete compatibility in sympatric vs. *allopatric populations*

All conspecific crosses showed the sigmoid relationship between sperm concentration and fertilization (Fig. 2A) predicted by theory (Vogel *et al.*, 1982) and supported by data from several species (*e.g.,* Levitan, 1996, 1998, 2002a, b; McCartney and Lessios, 2002). The log of F_{20} estimated

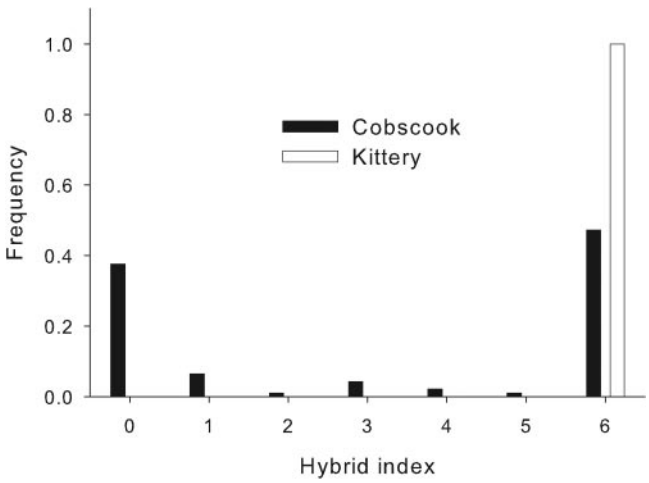
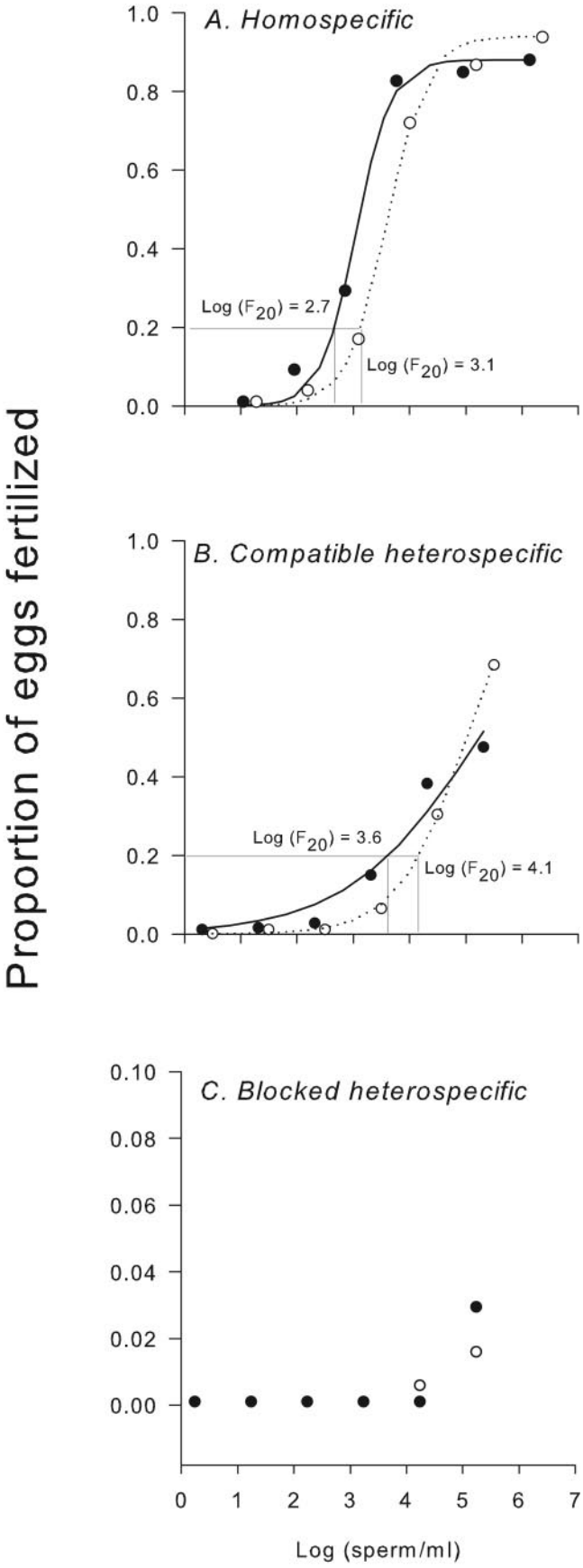


Figure 1. Frequency distribution of *Mytilus edulis* and *M. trossulus* genotypes in the Cobscook and Kittery populations (Cobscook, $n = 93$; Kittery, $n = 35$). The hybrid index is a numerical representation of the three-locus genotype of each mussel, with each *M. trossulus* allele assigned a value of 0, and each *M. edulis* allele assigned a value of 1. Pure *M. trossulus* are represented by a value of 0, while pure *M. edulis* are represented by a value of 6, and hybrids have intermediate values.



for conspecific crosses was consistently low and exhibited relatively little variation among crosses, and there was no evidence that compatibility of *M. edulis* eggs with conspecific sperm differed between the two populations. Mean (± S.E.) conspecific log (F_{20}) values were 2.04 (± 0.16) for crosses involving Kittery (allopatric) females and 1.88 (± 0.15) for crosses involving Cobscook (sympatric) females, a difference that was not significant by one-way ANOVA ($F = 0.521$; df = 1, 40; $P > 0.05$). All *M. edulis* females, regardless of heterospecific compatibility, were highly compatible with *M. edulis* males, as evidenced by the lack of significant correlation between mean conspecific and mean heterospecific F_{20} values estimated for individual females (Kendall's $\tau = -0.181$, $P > 0.05$). A significant positive correlation might indicate that greater cross-species compatibility was positively related to "fertilizability" of eggs within species, as has been shown for sea urchins (*e.g.,* Levitan, 2002b). Instead, neither any biologically relevant variation such as this nor any variation in egg quality that affected fertilization kinetics, appeared to be responsible for variation in heterospecific compatibility.

Results from heterospecific crosses were much more variable than results within species (Fig. 2B, C; Fig. 3). Eggs of some female *M. edulis* showed steep increases in percent fertilization with increasing concentrations of *M. trossulus* sperm, yielding sperm dilution curves with similar shape and only slightly depressed slope, compared to curves produced with *M. edulis* sperm (*e.g.,* Fig. 2B). Other *M. edulis* females produced eggs that were either strongly incompatible with (Fig. 2C) or completely blocked to fertilization by heterospecific sperm (*i.e.,* showed no increase in percent fertilization at increasing sperm concentrations). Five out of 12 females from the allopatric Kittery population but only 2 out of 12 females from the sympatric Cobscook population were blocked. Despite this trend in the direction opposite to that predicted by the reinforcement hypothesis, contingency table analysis showed no difference in frequency of blocked

Figure 2. Sample fertilization curves illustrating the percentage of eggs fertilized as a function of the log of sperm concentration. In all three panels, sample data are presented for a single *Mytilus edulis* female crossed with two different males (represented by the open and closed symbols). For visualization purposes, the curves represent logistic functions fit to the raw data, instead of linear functions fit to logit-transformed data (as used in the actual analysis). Calculation of the log of the F_{20} is represented in the first two panels by the lines indicating the log of sperm concentration for each curve that corresponds to 20% fertilization. (A) Kittery female F4 crossed to Kittery *M. edulis* males M2 and M3. (B) Cobscook female B20 crossed with *M. trossulus* males M1 and M2. (C) Cobscook female F1 crossed with *M. trossulus* males M1 and M3. The log F_{20} cannot be calculated in panel C without extrapolating well beyond the data range (to biologically impossible sperm concentrations), so the crosses were classified as completely blocked and the logs of the dry sperm concentrations were substituted for quantitative analysis purposes. Note that the incompatible crosses in panel C are very strongly blocked—the *y*-axis is scaled 2 orders of magnitude lower than in the other two panels.

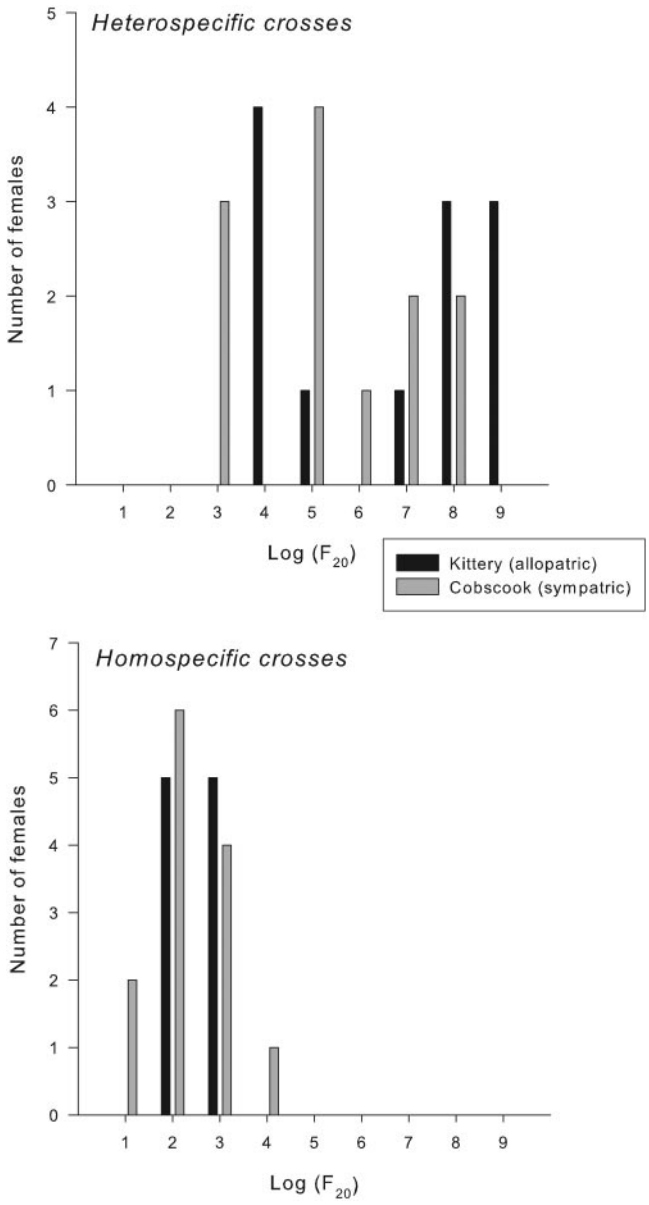


Figure 3. Frequency distributions of log F_{20} for heterospecific crosses (top panel) and homospecific crosses (bottom panel), calculated as means of females from sympatric and allopatric populations as indicated.

females between the two populations ($G_{adj} = 0.851$, $P > 0.05$). For the remaining majority (17/24) of females in the two populations together, cross-species fertilization was possible over a range of sperm concentrations that are likely to be ecologically relevant. This conclusion is based on the close proximity of heterospecific mussels in natural mussel beds (pers. obs.), and on results from advection/diffusion models (Denny, 1988; Denny and Shibata, 1989) that predict that sperm concentrations will drop only 2–3 orders of magnitude over the first 10 cm from a spawning male, under prevailing flow conditions.

Comparisons of con- and heterospecific crosses, pairwise for each female, showed that F_{20} values were elevated in heterospecific combinations in every case. Mean (±S.D.) log (F_{20}) was 5.99 (± 0.27) and 1.95 (± 0.11) for heterospecific and conspecific crosses, respectively; meaning, on average, that 20% fertilization of *M. edulis* eggs required 10,000 times more *M. trossulus* than *M. edulis* sperm. Analysis by randomized blocks ANOVA, with repeatedly tested females as blocks, showed this difference to be highly significant ($F = 189.01$; df = 1, 77; $P < 0.001$), and also supported highly significant variation in F_{20} among females ($F = 3.835$; df = 25, 77; $P < 0.001$). The degree of cross-species incompatibility varied widely among *M. edulis* females; some females produced values that approached conspecific compatibility. Females that, for any single cross with an *M. trossulus* male, produced a log (F_{20}) less than 3.373 (two standard deviations above the conspecific mean), can be considered to be "highly compatible"; these females showed steep increases in fertilization with increasing concentrations of *M. trossulus* sperm (*e.g.,* Fig 2B). Six of 24 females (25%) showed this highly compatible phenotype in cross-species crosses, and contrary to expectations from reproductive character displacement, 3 of these were from the sympatric population.

Quantitative comparisons of cross-species compatibility between females from the sympatric and the allopatric population (Fig. 3) showed no evidence of reproductive character displacement, manifest as greater incompatibility in sympatry; in fact, the trend suggests the converse pattern. Mean log F_{20} was higher in allopatric females (5.942 ± 0.623) than in sympatric females (5.010 ± 0.550) for $n = 12$ females in both groups. Two-way ANOVA showed this effect of geographic source of the female to not be significant, while cross type remained highly significant, and showed no significant interaction between these main effects (Table 1). This analysis again provided no evidence that *M. edulis* females from within the hybrid zone have evolved a higher level of gamete incompatibility than they have in an allopatric population, in which they would not have been exposed to natural selection to avoid heterospecific fertilizations.

Table 1

Two-way analysis of variance on the main effects of cross type and geographic source of the female, on gamete compatibility

Source of variation	df	SS	MS	F_s
Cross type (homo/heterospecific)	1	138.06	138.06	60.35***
Female source (allopatric/sympatric)	1	3.436	3.436	1.502 *ns*
Error	44	100.65	2.287	
Interaction MS = 1.873 ($F = 0.815$; df = 1,43; $P = 0.372$)				

Since the interaction term was not significant, interaction and error MS were pooled in this Model I ANOVA. ***$P < 0.001$.

Discussion

We found that 5 out of 12 *Mytilus edulis* females from Kittery, Maine, were completely blocked to fertilization by *M. trossulus* sperm, despite the fact that they were collected some 320 km away from populations with high frequencies of *M. trossulus* (Rawson *et al.*, 2007). Selection arising from present-day exposure to the hazards of hybridization cannot explain the strong incompatibility of these allopatric females. Conversely, we found partial compatibility in 10 of 12 *M. edulis* females collected from a Cobscook Bay population in which about 40% of the individuals are *M. trossulus*, and quantitative analysis showed these sympatic females to be no less compatible with sperm of heterospecifics than were allopatric females. Hence in this first study in which character displacement was tested directly using assays of gamete interactions in blue mussels, we find no signal of a pattern consistent with reinforcement.

Coyne and Orr (2004) point out that while reinforcement is often considered the most likely cause of the evolution of gamete incompatibility, the force of selection is apt to be weaker than it would be on premating isolation controlled by courtship and mating behavior. They argue that selection should be stronger in females than in males, because a sperm that contacts a heterospecific egg is unlikely to survive long enough to detach and search for homospecific eggs; hence avoidance of hybrid fertilizations will be selected for most strongly in eggs. Nevertheless, it is striking that the two previous demonstrations of character displacement of sperm-egg compatibility, one on sea urchin bindins (Geyer and Palumbi, 2003) and another on blue mussel lysins (Springer and Crespi, 2007), have involved proteins expressed in sperm. Selection on sperm to adapt to changing egg receptors, the latter changing due to reinforcement, is one explanation for these findings (Coyne and Orr, 2004). The present results stand in contrast to the expectation that eggs with "less promiscuous" receptors for sperm should be selected in sympatric populations.

Are conditions in place that should favor reinforcement in this hybrid zone? Examination of its genetic features suggests the answer is yes. Throughout the Canadian Maritimes (Bates and Innes, 1995; Saavedra *et al.*, 1996; Comesaña *et al.*, 1999) and in Cobscook Bay, Maine (Rawson *et al.*, 2001), the hybrid zone is highly "bimodal", which is also evident in the survey we conducted. Pure parentals dominate, hybrid individuals are nearly all late-generation backcrosses, and F_1 hybrids are rare. Jiggins and Mallet (2000) suggest that reinforcement should be common in bimodal hybrid zones, because recombination between species genomes is low. The expected strong linkage disequilibrium between loci controlling gamete recognition and those controlling hybrid fitness are exactly the conditions thought to promote reinforcement (Felsenstein, 1981). Whether hybrids show low fitness has unfortunately received very little study in the western Atlantic (see Riginos and Cunningham, 2005). The single study to experimentally cross *M. edulis* and *M. trossulus* and follow offspring traits showed some evidence of postzygotic incompatibilities, in that the typical transmission of the paternal lineage of *Mytilus* mitochondrial DNA (mtDNA) from father to male offspring breaks down, with several hybrid male offspring lacking the paternal mtDNA (Zouros *et al.*, 1994). Clearly, more work is needed in this direction.

Servedio and Noor (2003) encourage caution in equating a lack of a "signal" of reproductive character displacement with the absence of the process of reinforcement. One point they stress is that biologists must be careful to consider all prezygotic reproductive interactions that could exhibit displacement in sympatry. For broadcast-spawning animals like mussels, the options are few—most likely limited to spatial and temporal overlap of gamete release, chemical communication controlling gamete interactions, and compatibility at fertilization. No obvious displacement of spawning synchrony occurs within the Gulf of Maine hybrid zone. Maloy *et al.* (2003b) used histological examinations of gonad tissue to follow the timing of gametogenesis monthly from January through April and October through December, and semimonthly from 4 May through 14 September 2003, in the same Cobscook Bay (East Bay) population from which we obtained mussels for the present study. Plots of changing mass of spermatogenic and oogenic tissue were identical in *M. edulis* and *M. trossulus*: building continuously to early June, remaining constant to mid July, then declining precipitously. While asynchrony on a small temporal scale (*e.g.,* hours or days) could play a role, the evidence so far suggests that the two species overlap in spawning when sympatric.

Two issues may limit the implications of the present study: (1) the potential for migration between allopatric and sympatric *M. edulis* populations and (2) the contribution of conspecific sperm precedence. High migration from areas outside of a contact zone generally lessens the likelihood that reinforcement occurs in evolutionary models, yet reinforcement remains plausible under a range of conditions in which gene flow is ongoing (Felsenstein, 1981; Liou and Price, 1994; Servedio and Kirkpatrick, 1997). Gene flow will, moreover, interfere with detection of any signature of reinforcement. Any prezygotic incompatibilities that are adaptive within a hybrid zone, for instance, might be swamped by gene flow from areas outside the zone (Bigelow, 1965; Howard, 1993), and the evidence for reproductive character displacement would be blurred.

Blue mussels certainly possess the potential for broad-ranging dispersal. The duration of the feeding larval stage prior to settlement is from 22 to as many as 55 days post-fertilization (Bayne, 1965). Results of earlier, extensive allozyme surveys of populations along the east coast of North America, when interpreted in light of more recent

work that extends the range of genetically differentiated, sympatric *M. trossulus* into Maine at Cobscook Bay (Rawson *et al.*, 2001), show *M. edulis* populations to be genetically uniform from midcoast Maine and southwest to Cape Cod (Koehn *et al.*, 1976, 1984). Southwest of Cape Cod there is an abrupt change in allele frequencies. Some gene flow is therefore expected to occur between populations in Kittery and Cobscook Bay, since both sites lie to the northeast of the Cape Cod discontinuity. Export of adaptive egg incompatibility alleles outside of the zone, particularly if they were selectively neutral in allopatry, may be more likely here than transport into the hybrid zone, as the prevailing Maine coastal current flows southwest from Cobscook Bay (Pettigrew *et al.,* 1998), though it is often forced offshore at Penobscot Bay (between Cobscook Bay and Kittery; Geyer *et al.,* 2005). For logistical reasons (*i.e.,* similarity of spawning dates, and issues of permitting) we chose the Kittery population, but there are valid arguments for extending the current study to allopatric populations south of Cape Cod.

Conspecific sperm precedence (CSP) is the favoring of conspecific over heterospecific sperm when females are offered a choice (Howard, 1999). If CSP evolves in allopatry, it may reduce the likelihood that reinforcement would act upon gamete recognition in a hybrid zone. Marshall *et al.* (2002) argue that in the presence of strong CSP, reinforcement of mating discrimination in females will be weak, essentially because the fitness costs of heterospecific gamete fusion are avoided by preferential sperm usage. Discrimination can be interpreted in a free-spawning organism as the compatibility of a female's eggs with heterospecific sperm, and several cases of marine invertebrate gamete incompatibility are listed as examples of CSP (Marshall *et al.*, 2002). Yet all results were obtained from fertilization trials in which females were tested separately with conspecific and with heterospecific sperm, as in the present study. Geyer and Palumbi (2005) correctly noted that the concept would be more appropriately applied to free-spawners if fertilization trials offered sperm mixtures to females, and they provided evidence that in *Echinometra* sea urchins, CSP is stronger in choice trials than in no-choice trials. Choice experiments in which *Mytilus* females were offered sperm mixtures, while technically more challenging, would be very valuable. Our "highly compatible" females might show greater discrimination when tested in choice trials, which would lower the cost of heterospecific fertilization and lessen the force of selection against hybridization. Alternatively, it is possible for CSP to be stronger in sympatric than in allopatric populations; an interesting and ecological relevant signal of reinforcement that we do not address in the present study.

In contrast to the prediction of the reinforcement hypothesis for the evolution of gamete compatibility, we found evidence for increased, rather than decreased, heterospecific compatibility in a sympatric population of *M. edulis.* Eggs of *M. edulis* females from a population with a history of exposure to sperm from *M. trossulus* males appeared to be more likely to be fertilized by heterospecific sperm than were eggs from allopatric *M. edulis* populations that have historically been isolated. An alternative hypothesis suggests that heterospecific compatibility in *M. edulis* may have arisen from the introgression of *M. trossulus* genes involved in sperm receptivity.

Although the animals used in our experiments typed as "pure" at three marker loci, they still may carry heterospecific alleles at loci controlling gamete interactions. Other blue mussel hybrid zones are characterized by introgression that varies extensively across loci. This is true for genes that are not involved in reproduction (Gosling, 1992; Gardner, 1994; Riginos and Cunningham, 2005), but we are just beginning to examine introgression of genes and proteins involved in gamete recognition. Nevertheless, other evidence supports the introgression of gamete-recognition genes and proteins within this hybrid zone. Although we do not know the actual gene or genes involved in sperm recognition on bivalve eggs, *Mytilus* eggs spawned from females from the hybrid zone have been used to isolate proteins from the vitelline envelope that are separable on SDS PAGE gels. *M. edulis* and *M. trossulus* eggs differ in protein composition, and hybrids express proteins from both species (Harper *et al.*, 2005). Introgression of gamete recognition genes may also operate in the opposite direction. Many individuals from Cobscook Bay typed as pure *M. trossulus* at the same three loci we used here carry high frequencies of *M. edulis* alleles at the *M7 lysin* locus (McCartney and Lima, 2007). The *M7 lysin* gene is expressed only in males and codes for a protein that dissolves the egg vitelline envelope prior to fertilization (Takagi *et al.*, 1994). Like other mollusc lysins, it shows a signature of positive selection, suggesting that it is involved in sperm-egg recognition (Riginos and McDonald, 2003; Springer and Crespi, 2007)

Reinforcement has often been invoked to explain gamete recognition and rapid evolution of the genes controlling it. Blue mussels offer a system in which the history of secondary contact between diverging populations is well established, but perfection of reproductive isolation has not been achieved. The present results, considered together with initial results from sperm lysins and egg proteins, suggest that the consequences for the evolution of gametic isolation may be complex. For example, alleles at gamete recogntion loci that introgress may be selected for in hybrid zones if their recipients can transmit some genes through hybrid offspring (Coyne and Orr, 2004). The high frequency of *M. edulis M7 lysin* alleles in otherwise "pure" *M. trossulus* individuals from Cobscook Bay (McCartney and Lima, 2007) suggests their selective maintenance over many generations of backcrossing. Introgression of novel compatibility alleles may

oppose selection against hybrid offspring to create complex dynamics in the evolution of gamete incompatibility in hybridizing populations.

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