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CONSPECIFIC SPERM PRECEDENCE IS AN EFFECTIVE BARRIER TO HYBRIDIZATION BETWEEN CLOSELY RELATED SPECIES

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Abstract.—Conspecific sperm precedence is widespread in animals, appears to evolve rapidly, and is thought to have the potential to prevent hybridization between closely related species. However, to date no study has tested the isolating potential of such a barrier in mixed populations of two taxa under conditions in which other potential barriers to gene flow are controlled for or are prevented from operating. We tested the isolating potential of conspecific sperm precedence in the ground crickets *Allonemobius fasciatus* and *A. socius* in population cage experiments in which the frequency of the two species was varied. Despite the observation of abundant interspecific matings, the proportions of hybrid progeny were low and differed statistically from the proportions expected in the absence of conspecific sperm precedence. The results demonstrate that conspecific sperm precedence can severely limit gene flow between closely related species, even when one species is less abundant than the other.

Key words.—*Allonemobius*, barrier to fertilization, conspecific sperm precedence, cricket, hybridization, reproductive isolation, speciation.

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Postmating but prezygotic barriers to fertilization have begun to attract wide attention among evolutionists, as a large number of recent studies suggest that such barriers arise quickly and may represent the primary isolating barrier between many species of animals (Nakano 1985; Katakura 1986; Katakura and Sobu 1986; Hewitt et al. 1989; Bella et al. 1992; Palumbi 1992, 1994; Howard and Gregory 1993; Gregory and Howard 1994; Wade et al. 1994, 1995; Price 1997). Until now, most studies of this phenomenon have consisted of simple, sperm-competition experiments involving closely related species. The usual finding is conspecific sperm precedence; and it has been inferred that this precedence may account for patterns of positive assortment seen in nature. However, clear experimental demonstrations of the isolating potential of conspecific sperm precedence in mixed populations are lacking. Here we report the results of such an experiment, which indicate that conspecific sperm precedence can serve as an effective barrier to gene flow between two closely related species.

The study organisms were the ground crickets *Allonemobius fasciatus* and *A. socius*, morphologically indistinguishable sister species that have been the subject of intensive study in our laboratory for the past decade. The former species occurs in the northeastern and north-central United States; the latter is found in the southeastern United States (Howard 1983, 1986; Howard and Furth 1986). They meet and hybridize along an extensive mosaic contact zone stretching from New Jersey at least as far west as Illinois (Howard and Waring 1991). One of the most interesting aspects of the hybrid zone is the lack of hybrids. In most "mixed" populations (populations containing individuals of both species), even those that have been studied for as many as 10 years, pure species individuals predominate (Howard and Waring 1991; Howard et al. 1993; Chu et al. 1995). Furthermore, the majority of individuals classified as "hybrid" in these pop-

ulations are not highly intermediate in genotype, rather they possess genotypes more characteristic of backcrosses (Howard and Waring 1991; Howard et al. 1993; Chu et al. 1995). Based on allozyme and RAPD genotypes, the proportion of individuals that could potentially be F₁ hybrids typically ranges from 0.01 to 0.07. Thus, the two species are strongly, but not completely, reproductively isolated, and F₁ hybrid formation appears to be a relatively rare event in mixed populations.

After extensive study (Howard and Furth 1986; Benedix and Howard 1991; Gregory and Howard 1993; Howard et al. 1993; Doherty and Howard 1996; Veech et al. 1996), the only factor we have been able to identify that isolates the two species is conspecific sperm precedence (Howard and Gregory 1993; Gregory and Howard 1994). Although females of both species paired exclusively with heterospecific males produce fertile hybrid offspring (Gregory and Howard 1993), when females of both species are mated to a conspecific male and a heterospecific male, heterospecific sperm rarely fertilize eggs (Howard and Gregory 1993; Gregory and Howard 1994). This is true regardless of the order of matings or whether the female is from an allopatric population or a mixed population.

Females of *A. fasciatus* and *A. socius* mate multiple times in nature (Gregory and Howard 1996), thus making it likely that their reproductive tracts will contain conspecific sperm (unless conspecifics are very rare). Multiple mating together with conspecific sperm precedence should lead to positive assortment, even between taxa that mate at random. But can conspecific sperm precedence acting alone explain the genetic isolation between *A. fasciatus* and *A. socius*? Because the operation of conspecific sperm precedence as a reproductive barrier should not depend on a field setting, the isolating potential of the barrier can be tested directly in the laboratory. We did so in three sets of population cage experiments.

MATERIALS AND METHODS

The first set of experiments consisted of four replicate cages, each containing 10 males and 10 females of both *A. fasciatus* and *A. socius* (40 crickets total). Each plastic cage was 53 × 38 × 23 cm and was topped with a piece of clear plexiglass, which allowed for observations of matings within each cage. The cages contained a sand-soil (2:1 mix) oviposition dish, two water vials, Purina Cat Chow®, and small square blocks of wood to provide shelter and stages for calling and mating. Individuals of one species were marked on the pronotum with a dot of white correction fluid, so that observers could identify the species of individual crickets. Individuals of *A. fasciatus* were marked in two cages; individuals of *A. socius* were marked in the other two cages. Crickets placed in the cages were 10–15 days posteclosion and originated from pure populations in New Jersey near or within the zone of overlap. Because *A. fasciatus* and *A. socius* were in equal frequency in these cages, females of each species were likely to mate often with conspecifics. Hence, conditions in this experiment were optimal for the operation of conspecific sperm precedence as a barrier to hybridization.

The second and third sets of experiments examined the effectiveness of the fertilization barrier when one of the species was in the minority. In four replicates, *A. fasciatus* was the minority species (four males and four females vs. 16 males and 16 females of *A. socius*). In another four replicates, *A. socius* was in the minority (four males and four females vs. 16 males and 16 females of *A. fasciatus*). Because females of the minority species were likely to mate infrequently with members of their own species, these experiments increased the possibility that conspecific sperm precedence could be overwhelmed by repeated matings to heterospecifics.

Crickets were allowed to mate and oviposit freely for three weeks. Each cage was observed several times a day and the types of matings taking place (conspecific or heterospecific) were noted. Previous laboratory studies have documented demographic differences in offspring production between *A. fasciatus* and *A. socius* females that were mated with conspecific or heterospecific males. Slight survivorship differences also occurred among the different classes of offspring (Gregory and Howard 1993). However, (1) heterospecific crosses in which multiple mating occurred produced many eggs and these eggs did not suffer increased embryonic mortality; (2) hybrids developed normally after hatching; and (3) hybrids survived and reproduced well (Gregory and Howard 1993). Hence, unless there were large deviations from random mating in the population cages, conspecific sperm precedence represents the only potentially strong barrier to gene flow between the two taxa.

After adults were removed from the cages, oviposition dishes and cotton from water jars (into which females often lay eggs) were treated as described by Gregory and Howard (1993). Offspring from each cage were reared to adulthood in groups composed of no more than 100 individuals in plastic cages measuring 40 × 28 × 22 cm. Within a week of reaching adulthood, a hind leg was removed from each individual and the identity of the individual (*A. fasciatus*, *A. socius*, or hybrid) was determined via genotyping at three loci coding for soluble enzymes: hexokinase, isocitrate dehydrogenase-1,

TABLE 1. Mating patterns in the mixed-species cages. In cages A1–A4, the two species were in equal abundance. In cages B1–B4, *A. socius* was in the majority (80% of the population). In cages C1–C4, *A. fasciatus* was in the majority (80% of the population).

Cage	<i>fas</i> male <i>fas</i> fem	<i>fas</i> male <i>soc</i> fem	<i>soc</i> male <i>soc</i> fem	<i>soc</i> male <i>fas</i> fem	χ^2	P-value
A1	10	10	15	10	1.67	> 0.50
A2	15	9	10	10	2.00	> 0.25
A3	16	4	12	8	8.00	< 0.05
A4	11	8	10	6	1.68	> 0.60
Total	52	31	47	34		
				Pooled	7.74	> 0.05
				Heterogeneity	5.89	> 0.75
B1	5	4	15	0	20.86	< 0.001
B2	3	3	24	5	3.21	> 0.30
B3	3	6	11	3	7.23	> 0.05
B4	0	3	11	3	0.74	> 0.80
Total	11	16	61	11		
				Pooled	14.08	< 0.005
				Heterogeneity	17.96	< 0.05
C1	18	4	1	3	0.44	> 0.90
C2	21	1	2	1	7.06	> 0.05
C3	14	3	3	5	4.75	> 0.10
C4	20	4	0	8	3.16	> 0.30
Total	73	12	6	17		
				Pooled	2.48	> 0.40
				Heterogeneity	12.92	> 0.10

and aspartate aminotransferase (formerly known as glutamic-oxalacetic transaminase). There is a fixed electrophoretic difference between the two species at hexokinase. At the other two loci, one or more alleles are specific to the gene pool of a single species (Howard 1986; Howard and Waring 1991). Individuals lacking alleles of *A. socius* were regarded as pure *A. fasciatus* and individuals without alleles of *A. fasciatus* were considered pure *A. socius*. Individuals harboring alleles of both species were considered to be hybrids.

We constructed a stochastic simulation model to examine whether the number of hybrids produced in our experiments differed from the number predicted in the absence of conspecific sperm precedence. The model was individually based. For each of the four possible crosses between *A. fasciatus* and *A. socius* females and males, the model was calibrated by observed patterns of mating (this study) and by the mean and variance of both the number of offspring produced in laboratory crosses and their survivorship to maturity (Gregory and Howard 1993). The demographic data in Gregory and Howard (1993) were collected under conditions in which conspecific sperm precedence could not operate, that is, from crosses in which a female was mated repeatedly to a single conspecific or heterospecific male. Thus, the data in Gregory and Howard (1993) allow us to predict the number of hybrid offspring expected in the absence of conspecific sperm precedence.

Simulations were performed on a cage-by-cage basis. This was done because mating frequencies differed significantly in the second set of population cages (cages B1–B4), and were heterogeneous (but not significantly different) in the other two sets of cages (Table 1). For each cage, the appropriate number of *A. fasciatus* and *A. socius* females mated with heterospecific males according to random mating fre-

TABLE 2. Species composition of the progeny of the 12 population cages.

Cage	Parental composition	N	Progeny		
			Proportion <i>A. socius</i>	Proportion <i>A. fasciatus</i>	Proportion hybrid
A1	Equal	247	0.652	0.287	0.061
A2	Equal	196	0.714	0.260	0.025
A3	Equal	157	0.790	0.197	0.013
A4	Equal	164	0.817	0.146	0.036
B1	0.80 <i>socius</i>	131	0.901	0.084	0.015
B2	0.80 <i>socius</i>	175	0.834	0.154	0.011
B3	0.80 <i>socius</i>	177	0.938	0.028	0.033
B4	0.80 <i>socius</i>	144	0.958	0.028	0.014
C1	0.80 <i>fas</i>	168	0.262	0.548	0.190
C2	0.80 <i>fas</i>	146	0.432	0.527	0.041
C3	0.80 <i>fas</i>	126	0.206	0.754	0.040
C4	0.80 <i>fas</i>	90	0.411	0.589	0.000

quencies, with the exception of cages A3, B1, B3, and C2. In these four cages, there were significant or nearly significant departures from random mating expectations (Table 1); hence, probabilities of mating with heterospecifics were set equal to the observed frequencies. The number and type of mature offspring produced by each female cricket in the absence of conspecific sperm precedence was determined by the probabilities that the female mated with conspecific or heterospecific males, and by stochastic sampling from the data in Gregory and Howard (1993) on the number and survivorship of nymphs produced in conspecific or heterospecific crosses. Finally, we calculated the proportion of *A. fasciatus*, *A. socius*, and hybrid offspring for each cage. We repeated this process 10,000 times. On a cage-by-cage basis, we used the resulting distribution of the proportion of hybrid offspring to determine the probability that the empirically observed proportion of hybrid offspring could have been obtained in the absence of conspecific sperm precedence. We used these probabilities to perform a one-sided statistical test, because our alternative hypothesis was that conspecific sperm precedence would cause the observed proportion of hybrids to be lower than the expected proportion of hybrids.

RESULTS

In the four cages in which *A. fasciatus* and *A. socius* occurred with equal abundance, conspecific mating occurred more frequently than would be expected under random mating, but overall the deviation did not achieve significance (Table 1). Clearly, a great deal of interspecific mating occurred and the potential for the production of hybrid offspring was high. When *A. socius* was in the majority, there were significant deviations from random mating expectations (Table 1). In general, there were more matings between *A. fasciatus* males and females than expected. This intriguing result suggests that females or males of *A. fasciatus* may more actively seek out conspecific mates when they are in the minority in a population. However, even in this group of cages, interspecific matings were frequent. In the absence of conspecific sperm precedence, one would expect the females to produce a large number of hybrid offspring. Finally, when *A. fasciatus* was in the majority, there were no significant

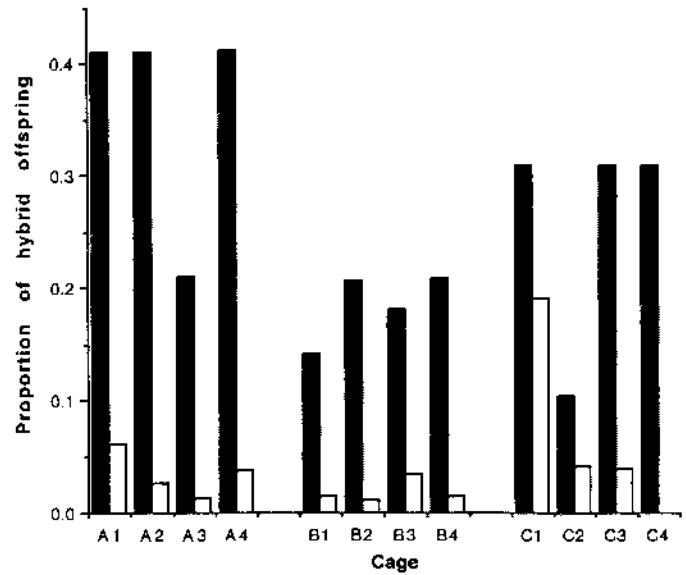


FIG. 1. Predicted (closed bar) and observed (open bar) proportions of hybrid offspring; cages are denoted as in Table 1. Predicted proportions of hybrid offspring were calculated from an empirically calibrated, stochastic simulation model in which there was no possibility of conspecific sperm precedence (see text for description). Statistical tests indicated that the predicted proportions of hybrids were significantly different from the observed proportions of hybrids in all cages ($P = 0.0317$ for cage C1; $P = 0.0089$ for cage C2; $P < 0.0001$ for the remainder of the cages).

deviations from random mating expectations (Table 1); again one would expect to find a large number of hybrids among the progeny of females.

The species composition of the progeny of each cage is summarized in Table 2. The results were quite clear. Despite abundant interspecific mating, hybrids were rare in the F_1 generation in 11 of the 12 population cages—ranging from 0 to 6%. These levels are consistent with the 1–7% of F_1 hybrids found in natural populations. We pooled data from the three experiments and found that the frequency of the parental species did not affect the number of hybrid offspring (ANOVA, $F_{2,9} = 0.74$, $P = 0.50$). Thus, the effectiveness of conspecific sperm precedence as a barrier to hybridization did not break down when *A. fasciatus* or *A. socius* were 20% of the crickets in a cage. The one exception to this pattern was cage C1, with *A. socius* in the minority: 19% of the progeny from this cage were hybrids. We have no ready explanations for this result other than to suggest: (1) that a particularly fecund *A. socius* female did not mate with any *A. socius* males and hence produced a large number of hybrid offspring; or (2) that there is individual variation in the effectiveness of conspecific sperm precedence. Another general pattern observed among the progeny was a greater abundance of *A. socius* than *A. fasciatus*. This result can be attributed to better survivorship of *A. socius* and greater fecundity of *A. socius* females (Gregory and Howard 1993).

The stochastic simulation model confirmed that the number of hybrids produced in the cages differed from the number predicted in the absence of conspecific sperm precedence. The proportions of hybrids predicted from the model were much higher than the levels observed in the cages (Fig. 1).

Even in cage C1, the only cage in which the proportion of hybrids was outside the range commonly found in the field, significantly fewer hybrids were produced than expected in the absence of conspecific sperm precedence ($P = 0.0317$). Overall, the results of these experiments provide strong evidence that conspecific sperm precedence can serve as an effective barrier to hybridization between *A. fasciatus* and *A. socius*.

DISCUSSION

The experiments described in this paper were designed to determine whether the conspecific sperm precedence that has previously been described between *A. fasciatus* and *A. socius* can account for the genetic isolation between the two taxa in areas of overlap. The need for such studies is clear. An increasing number of evolutionists report that conspecific sperm precedence exists between closely related terrestrial animals (Nakano 1985; Katakura 1986; Katakura and Sobu 1986; Hewitt et al. 1989; Bella et al. 1992; Howard and Gregory 1993; Gregory and Howard 1994; Wade et al. 1994; Price 1997), but we have little understanding of the isolating potential of this phenomenon. One would expect the level of isolation mediated by conspecific sperm precedence to vary depending on such factors as the number of times that females mate, the time interval between matings, the amount of fertilization that occurs between matings, the relative abundance of the two interacting species, and the strength of the precedence. A priori, we expected that conspecific sperm precedence would serve as a strong barrier to gene flow between *A. socius* and *A. fasciatus*. The fertilization advantage enjoyed by conspecific males is very strong (Howard and Gregory 1993; Gregory and Howard 1994; Howard et al., in press; Howard and Gregory, unpubl. data) and females mate multiply over short time intervals (Gregory and Howard 1996; Howard et al., in press; Gregory and Howard, unpubl. data). These are conditions that should enhance the isolating potential of conspecific sperm precedence.

In line with our expectations, relatively few hybrid offspring were produced in the three sets of population cages (Table 2), despite abundant interspecific matings (Table 1). A stochastic simulation model confirmed that this result could not be explained by hybrid embryo mortality or by differences in survivorship between pure species and hybrid individuals after hatching (Fig. 1). Thus, the isolation between the two species in the population cages, and presumably in nature as well, appears to be due to conspecific sperm precedence.

Of great interest is the finding that even when one species comprised only 20% of the individuals in a cage, a situation that should diminish the effectiveness of conspecific sperm precedence as a reproductive barrier, few hybrids were produced. Indeed, the proportion of hybrids produced in cages with one species in the minority did not differ significantly from the proportion of hybrids produced in cages with equal numbers of the two species (see Results). Two factors may explain this finding. First, conspecific sperm precedence may be strong enough that even a single conspecific mating assures fertilization by conspecific sperm, regardless of the number of heterospecific matings. This supposition is sup-

ported by preliminary evidence from controlled laboratory matings (Howard et al., in press; Howard and Gregory, unpubl. data). Second, when in a minority, females or males may become more discriminating and may more actively seek out mates of their own species. Evidence of this phenomenon was found when *A. fasciatus* was less abundant, in the second set of population cages (B cages; Table 1).

A cautionary note should be interjected at this point. A single cage, C1, was an outlier with regard to the proportion of hybrids among the progeny of the cage. In this cage, in which *A. socius* were in the minority, hybrids represented 19% of the progeny. Although the high proportion of hybrids in this cage was anomalous, the result may serve as a warning that conspecific sperm precedence can break down as a barrier to gene flow when one species becomes much less abundant than another.

Conspecific sperm precedence represents a very late-acting reproductive barrier. Thus, the question arises as to why earlier-acting barriers, such as a preference for conspecific male calling songs, have not arisen in *A. fasciatus* and *A. socius* from areas of sympatry. One potential explanation is that the strength of reinforcing selection may be weak. Conspecific sperm precedence will establish a selection pressure for the evolution of pre-mating barriers to gene exchange only if participation in heterospecific matings diminishes the reproductive success of females and males. At least for females of *A. fasciatus* and *A. socius*, this requirement does not appear to be met. In mating with a heterospecific, a female gains a nuptial feeding by chewing on the tibial spurs of the male and by eating the spermatophore transferred by the male (Mays 1971; Gregory and Howard, pers. obs.). Moreover, because a female mates repeatedly (Gregory and Howard 1996), she is likely (except when her species is rare) to mate with one or more conspecifics who will fertilize most of her eggs. The energy expended in mate searching and the increased risk of predation due to proximity to a calling male (Cade 1975) are the only identifiable costs to a female for engaging in a heterospecific mating. These costs are likely to be small in these abundant insects. The cost of a heterospecific mating appears to be higher for males, who provide nutrients to females with little probability of fertilizing eggs. There are a number of potential explanations for the failure of males to respond to the selection pressure generated by this cost, including: lack of variation for discriminatory ability (Barton and Hewitt 1981), the swamping effect of gene flow from populations not in contact with the second species (Bigelow 1965; Littlejohn 1981; Howard 1986), and/or recency of contact between the two taxa in areas of sympatry. Alternatively, females of the two species may be so similar in morphology, behavior, and pheromones that discrimination by males is not possible.

Barriers to fertilization appear to evolve rapidly. The most direct evidence for this comes from studies of sperm or pollen competition between closely related species, which almost invariably report conspecific sperm or pollen precedence (Nakano 1985; Katakura 1986; Katakura and Sobu 1986; Hewitt et al. 1989; Bella et al. 1992; Arnold et al. 1993; Wade et al. 1994; Rieseberg et al. 1995; Price 1997). Extremely rapid evolution also is indicated by molecular studies of the male and female reproductive tracts in *Drosophila*

(Choudhary et al. 1992; Civetta and Singh 1995) and by studies of fertilization proteins, particularly in broadcast spawning marine invertebrates (Lee and Vacquier 1992; Palumbi 1992; Shaw et al. 1993; Vacquier and Lee 1993; Lee et al. 1995; Swanson and Vacquier 1995; Metz and Palumbi 1996; Vacquier et al. 1997). The rapid, divergent evolution of proteins and traits related to fertilization, and the onset of conspecific sperm precedence have at least four potential explanations that are explored in detail in Howard et al. (in press): (1) response to pathogens; (2) sexual selection by male-male competition; (3) female choice; and (4) avoidance of polyspermy (fertilization of an egg by more than one sperm). The present study was not designed to distinguish among these hypotheses. However, the results of this study demonstrate that whatever the cause of its evolution, conspecific sperm precedence can severely limit gene exchange between closely related species, even when one species is less abundant than another. Thus, the divergence of proteins and traits related to fertilization may indeed serve as a major engine of speciation (Rice 1996, in press).

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