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A POSTINSEMINATION BARRIER TO FERTILIZATION ISOLATES TWO CLOSELY RELATED GROUND CRICKETS

PAMELA G. GREGORY AND DANIEL J. HOWARD
Department of Biology, New Mexico State University, Las Cruces, New Mexico 88003

Abstract.—Postinsemination barriers to fertilization generally have been ignored by biologists interested in the origin and nature of reproductive isolation among closely related terrestrial animals. Yet evidence presented in this paper indicates that such a barrier bears primary responsibility for the reproductive isolation between the ground crickets *Allonemobius fasciatus* and *Allonemobius socius*. Postinsemination barriers to fertilization may isolate many other terrestrial animals as well, but the design of most laboratory hybridization experiments precludes the detection of these barriers.

Key words.—Fertilization barrier, hybrid zone, reproductive isolation, speciation, sperm competition.

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Evolutionary biologists conduct laboratory hybridization studies to address many issues in the contentious area of speciation, from the role of hybridization in the direct formation of new species (Bullini and Nascetti 1990) to the genetic basis of reproductive barriers between species (Coyne 1985; Orr 1989). Laboratory studies have been particularly important in assessing the fitness of hybrids and the role of postmating barriers in isolating closely related taxa (e.g., Katakura and Nakano 1979; Barton 1980; Chapco 1991; Francescoli and Costa 1992). Frequently, laboratory matings lead to the formation of viable, fertile hybrids, a finding that has led to the widespread belief among evolutionists that many closely related species are isolated primarily by behavioral and ecological differences. Rarely considered is the possibility that closely related taxa are isolated by concentration-dependent barriers to fertilization, barriers that can be breached by the multiple matings typical of laboratory hybridization studies.

Allonemobius fasciatus and *Allonemobius socius* are small ground-dwelling crickets in the subfamily Nemobiinae that inhabit short grassland areas of eastern North America. *Allonemobius fasciatus* occurs from southeastern Canada to the northeastern and north-central United States, whereas populations of *A. socius* are found in the southeastern and south-central United States (Alexander and Thomas 1959; Howard 1983; Howard and Furth 1986). *Allonemobius fasciatus* and *A. socius* meet and hybridize, to a limited extent, in an extensive contact zone that stretches from New Jersey at least as far west as Illinois (Howard and Waring 1991). Within mixed populations (populations containing members of both species), pure species individuals usually predominate and individuals classified as hybrid typically possess genotypes characteristic of backcrosses (Howard and Waring 1991; Howard 1986). Thus, reproductive isolation between the two species in areas of contact appears quite strong, although not complete.

Up to now, the trait(s) responsible for this isolation have proven elusive to identify. With regard to traits that would isolate prior to mating, we cannot detect microhabitat or phenological differences between the taxa (Howard et al. 1993). Slight calling-song differences exist, but females from some mixed populations do not exhibit preferential movement toward conspecific songs, at least in a laboratory setting (Doherty and Howard, unpubl. data). Recent work on postzygotic barriers suggests that they are weak as well. Individuals with genotypes indicative of mixed ancestry are quite viable in the field (Howard et al. 1993). Moreover, laboratory hybridization results in the formation of many viable, fertile offspring (Gregory and Howard 1993).

Here we describe studies of sperm competition recently completed in our laboratory. These studies identify a significant barrier to gene exchange between *A. fasciatus* and *A. socius*—a postinsemination barrier to fertilization. The results of this work were previously summarized

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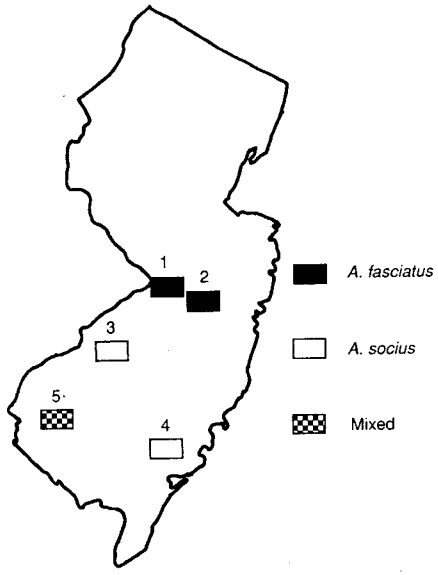

FIG. 1. Map of New Jersey showing the location and species composition of collecting sites of crickets used in sperm competition experiments. 1, Yardville; 2, Buddtown; 3, Woodbury; 4, Laureldale; 5, Pointers.

in a paper evaluating the interaction between postinsemination signaling systems and reinforcement (Howard and Gregory 1993).

MATERIALS AND METHODS

We collected females and males in July of 1991 as middle instar nymphs from one of five sites in southern New Jersey. *Allonemobius fasciatus* occurred alone at two sites and *Allonemobius socius* occurred alone at two sites (fig. 1). At the fifth site, both species as well as hybrids occurred. After reaching adulthood in the laboratory, two virgin males were mated with a virgin female in one of the following sequences: (1) conspecific followed by conspecific, (2) conspecific followed by heterospecific, (3) heterospecific followed by conspecific, or (4) heterospecific followed by heterospecific. Males mated to the same female were distinctive at one or more polymorphic protein loci. Genotypes were identified prior to mating via protein electrophoresis of a hind leg. Except for the matings among individuals from the mixed population, males came from different populations than females. By mating males and females from different populations in all matings involving individuals from "pure" populations, we controlled for the interpopulation aspect of heterospecific crosses.

Matings took place 24 h apart at 28°C. All crickets were 10–15 d posteclosion when mated. Each male was allowed one spermatophore transfer. For each pair, we recorded the time from the beginning of copulation to spermatophore removal. We tested whether spermatophore attachment lasted longer in conspecific than in heterospecific matings via a *t*-test [StatView 512+ (Abacus Concepts, Inc.)]. After the second mating, we moved females to an oviposition jar containing 50 g of lightly packed sand/soil (2:1) mixture. One week later, the female was transferred to a second oviposition jar. At the end of the second week, each female was frozen at −80°C along with her two mating partners. Oviposition jars were treated as described in Gregory and Howard (1993). After 5 mo at 4°C, eggs were allowed to hatch and the offspring were reared to a size adequate to ensure successful genotyping via protein electrophoresis. By comparing the genotypes of the offspring, the mother, and the two potential male parents we established which male fathered each of the progeny. Deviations from the expectation of equal offspring production by first and second males were tested via a *G*-test (Sokal and Rohlf 1981).

Sperm transfer and motility were evaluated in females mated singly to a conspecific or to a heterospecific male. After a single spermatophore transfer, the spermatheca of each female was dissected out and opened on a microscope slide in two drops of Ringer's solution. Sperm presence and motility were assessed under a light microscope by an observer who was unaware of the species identity of the male and female mating partners. Spermatozoa were considered motile if even a single sperm cell demonstrated noticeable movement.

RESULTS

When a female was mated with two conspecifics, both males fertilized eggs (table 1). However, the second male fertilized significantly more eggs than the first male in the second week of oviposition in cross class 1 and in the first week of oviposition in cross class 2, whereas the first male fertilized significantly more eggs than the second male during the first week of oviposition in cross class 4.

The pattern of paternity changed considerably when one of the mating partners was conspecific and the other was heterospecific. Regardless of the order of matings and regardless of whether the female was from a pure population or a mixed

TABLE 1. Patterns of fertilization when females were allowed to mate once with two males.

Cross class	Species designation of female and two males: F[1], M1[2], M2[3]	Site of origin[6]: F, M1, M2	Fraction of crosses producing offspring	Mean proportion of 1st male offspring 1st week (N[7])	Mean proportion of 2nd male offspring 1st week (N)	G	Mean proportion of 1st male offspring 2nd week (N)	Mean proportion of 2nd male offspring 2nd week (N)	G
				Conspecific males					
1	fas[4], fas, fas	2, 1, 1	17/17	0.468 (16)	0.532 (16)	0.01 NS[8]	0.213 (14)	0.787 (14)	15.93**
2	fas, fas, fas	5, 5, 5	4/4	0.415 (4)	0.585 (4)	5.79*[9]	0.443 (3)	0.557 (3)	0.16 NS
3	soc[5], soc, soc	4, 3, 3	9/9	0.537 (10)	0.463 (10)	0.58 NS	0.504 (8)	0.496 (8)	0.00 NS
4	soc, soc, soc	5, 5, 5	2/2	0.735 (2)	0.265 (2)	8.65*	0.500 (2)	0.500 (2)	0.20 NS
				Conspecific/heterospecific male					
5	fas, fas, soc	2, 1, 4	9/9	0.968 (5)	0.032 (5)	89.24**[10]	1.000 (3)	0.000 (3)	29.11**
6	fas, fas, soc	5, 5, 5	3/4	0.970 (3)	0.030 (3)	34.07**	1.000 (2)	0.000 (2)	29.11**
7	soc, soc, fas	4, 3, 2	5/5	0.983 (3)	0.017 (3)	73.00**	1.000 (1)	0.000 (1)	6.24*
8	soc, soc, fas	5, 5, 5	4/4	1.000 (4)	0.000 (4)	110.90**	1.000 (4)	0.000 (4)	42.97**
				Heterospecific/conspecific male					
9	fas, soc, fas	2, 4, 1	11/14	0.000 (6)	1.000 (6)	105.35**	0.000 (6)	1.000 (6)	42.97**
10	fas, soc, fas	5, 5, 5	4/4	0.000 (5)	1.000 (5)	119.21**	0.000 (5)	1.000 (5)	48.52**
11	soc, fas, soc	4, 2, 3	8/8	0.100 (4)	0.900 (4)	60.40**	0.000 (4)	1.000 (4)	52.67**
12	soc, fas, soc	5, 5, 5	3/3	0.083 (3)	0.917 (3)	48.81**	0.000 (3)	1.000 (3)	23.56**
				Heterospecific males					
13	fas, soc, soc	2, 4, 4	2/10	0.640 (1)	0.360 (1)	0.83 NS	0.000 (1)	1.000 (1)	1.39 NS
14	soc, fas, fas	4, 2, 2	7/7	0.368 (6)	0.631 (6)	12.7**	0.050 (5)	0.950 (5)	30.18**

F[1], female; M1[2], first male; M2[3], second male; fas[4], *Allonemobius fasciatus*; soc[5], *Allonemobius socius*; origin[6], see figure 1; N[7], number of families; NS[8], not significant; *[9], $P < 0.025$; **[10], $P < 0.001$.

population (the sites of origin of females and their mating partners are shown in the third column of table 1), conspecific males fathered the great majority of offspring (table 1). In the case of *Allonemobius socius* females, the heterospecific male fathered offspring in 6 of 14 trials, but the proportion of offspring sired by the heterospecific male did not exceed 18% in any trial. Conspecific sperm precedence was even stronger in *Allonemobius fasciatus* females. The heterospecific male fathered offspring in only 2 of 19 trials, never accounting for more than 12% of the offspring of a female. Eggs fertilized by heterospecific males were all laid in the first week, suggesting that the fertilization advantage enjoyed by conspecific sperm increases with longer storage.

When both mating partners were heterospecific, the pattern of paternity indicated sperm mixing, although the second male fathered more offspring when the female was *A. socius* (table 1). The unexpected finding in this series of crosses was the low number of offspring produced by *A. fasciatus* females. Only 2 of the 10 crosses of *A. socius* males to *A. fasciatus* females produced offspring (table 1, mean = 1.77 offspring). In contrast, *A. fasciatus* females mated to two conspecifics all produced offspring ($N = 17$ females; mean = 33.88 offspring). Matings to two heterospecific males were more successful when the female was *A. socius*. All the females gave rise to young and the mean number of offspring was 69% of the number yielded by matings to two conspecific males.

The fertilization advantage of conspecific males could not be attributed to lack of sperm transfer in heterospecific matings. The mean time from the beginning of copulation to spermatophore removal by the female did not differ significantly between conspecific and heterospecific matings (25.57 min versus 26.72 min, t-test, $P = 0.6629$). Moreover, dissections of five *A. fasciatus* and four *A. socius* females subsequent to a single heterospecific mating revealed the presence of numerous sperm in all spermathecae.

However, conspecific sperm precedence may be explained by sperm motility differences in the female reproductive tract. Motile sperm were clearly visible under a light microscope in all spermathecae dissected from *A. socius* ($N = 7$) and *A. fasciatus* ($N = 6$) females mated to a single conspecific. In contrast, when females were mated once to a heterospecific, only one of five and one of four spermathecae dissected from *A. fasciatus* and *A. socius* females, respectively, contained visibly motile sperm.

Discussion

The sperm competition experiments described here demonstrate that a barrier to fertilization exists between *Allonemobius fasciatus* and *Allonemobius socius*. In matings involving a heterospecific male and a conspecific male, the conspecific male fathered the vast majority of offspring regardless of the order of mating (table 1). Moreover, *A. fasciatus* females mated to two heterospecific males produced very few offspring (table 1). The lack of offspring in this last set of crosses was surprising because in an earlier hybridization study most heterospecific crosses involving an *A. fasciatus* female yielded offspring (52 of 62), and the mean number generated (32.76) was 58% of the number produced by conspecific crosses (Gregory and Howard 1993). The major difference in methods between the two studies was in the number of matings permitted. In the present study, females were mated once to each of two males, whereas in the earlier study males were allowed to inseminate females repeatedly over 4 d. The difference in outcomes suggests that the barrier to fertilization detected in this study is concentration dependent (at least in the case of *A. fasciatus* females) and can be overcome by multiple matings. In contrast to the results with *A. fasciatus* females, heterospecific matings to *A. socius* females provided results similar to those of the earlier study. All the females gave rise to young, and the mean number of offspring was 69% of the number yielded by matings to two conspecific males.

Available evidence suggests that the barrier to fertilization between *A. fasciatus* and *A. socius* cannot be attributed to insemination difficulties. The genitalia of the two species are very similar (Fulton 1931; Gregory pers. obs.), and heterospecific males experienced no apparent difficulty transferring spermatophores to females. Moreover, the mean time from the beginning of copulation to spermatophore removal by the female did not differ between conspecific and heterospecific matings, and heterospecific sperm clearly entered the female sperm storage organ (see Results).

The deficiency of offspring fathered by heterospecific males also cannot be attributed to hybrid embryo mortality or to differential survival of hatchlings. In earlier work, we demonstrated that

percent egg hatch did not differ between conspecific and heterospecific matings and that hybrids survived as well as nonhybrids in the laboratory (Gregory and Howard 1993). Instead, differences in offspring production between conspecific and heterospecific pairings could be ascribed to differences in the number of eggs laid (Gregory and Howard 1993).

Thus, the lack of hybrid offspring produced by a female mated to a heterospecific and a conspecific male was almost certainly caused by sperm-transport difficulties or by a divergence in gamete recognition systems between *A. fasciatus* and *A. socius*. The lack of motile sperm in the spermathecae of most females inseminated by heterospecific males (see Results) provides support for the idea that heterospecific sperm have difficulty moving into the vicinity of an unfertilized egg.

A barrier to fertilization that results in conspecific sperm precedence should lead to positive assortment even between taxa that mate at random if females mate multiply. Electrophoretic analysis of the offspring of field-collected adult females of *Allonemobius* demonstrates that females typically mate with more than one male in nature (Gregory and Howard unpubl. data). Thus, conspecific sperm precedence may account for the low numbers of hybrids found in most mixed populations of *A. fasciatus* and *A. socius*. This possibility is enhanced by the finding that conspecific sperm precedence was as strong in individuals from mixed populations as in individuals from pure populations (table 1).

Strong conspecific sperm precedence would serve most effectively as a reproductive barrier when two species occur with equal abundance and females mate many times. Under such circumstances, a female is likely to mate at least once with a conspecific and therefore lay eggs fertilized by a conspecific. As the abundance of a species decreases, so too does the chance of a female encountering and mating with a conspecific male. Thus, at some threshold of relative abundance, conspecific sperm precedence, by itself, will not serve as an effective barrier to reproduction between two taxa. The threshold level will depend on the strength of the conspecific sperm precedence and the number of times a female mates. Arnold et al. (1993) make similar arguments with regard to interspecific pollen competition and reproductive isolation in plants. Of course, when a strong barrier to fertilization is combined with some positive assortative mating, two taxa may remain isolated even when one of them is quite rare.

One of the questions that arises from this work is how important are barriers to fertilization in isolating other closely related animals? In marine animals that do not interact behaviorally prior to external gamete release, gamete recognition loci appear to evolve quite rapidly and differences at such loci are often critical in isolating closely related taxa (Lessios and Cunningham 1990; Lee and Vacquier 1992; Palumbi 1992; Howard and Gregory 1993). The situation is less clear for other types of marine animals and for terrestrial animals. Very few laboratory hybridization studies have reported evidence of barriers to fertilization among closely related terrestrial animals. However, the repeated matings typical of laboratory hybridization studies (e.g., Bicudo and Prioli 1978; Chapco 1991; Carracedo and Casares 1987; Goulielmos and Alahiotis 1989) will overcome concentration-dependent barriers to fertilization. Thus, the vast majority of such studies will not provide information about the presence or absence of fertilization barriers. Intriguingly, three other recent investigations designed to detect the presence of barriers to fertilization between closely related terrestrial animals have reported evidence of such barriers (Hewitt et al. 1989; Bella et al. 1992; Wade et al. 1994). But because the number of such investigations is small and none have yet incorporated field experiments, understanding the general significance of fertilization barriers in isolating closely related terrestrial animals will come about only with further study.

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