

## CONSPECIFIC SPERM PRECEDENCE IN SISTER SPECIES OF *DROSOPHILA* WITH OVERLAPPING RANGES

AUDREY S. CHANG

Department of Ecology and Evolution, The University of Chicago, 1101 E. 57 Street, Chicago, Illinois 60637  
E-mail: audrey@ucdavis-alumni.com

**Abstract.**—Barriers to gene flow that act after mating but before fertilization are often overlooked in studies of reproductive isolation. Where species are sympatric, such “cryptic” isolating barriers may be important in maintaining species as distinct entities. *Drosophila yakuba* and its sister species *D. santomea* have overlapping ranges on the island of São Tomé, off the coast of West Africa. Previous studies have shown that the two species are strongly sexually isolated. However, the degree of sexual isolation observed in the laboratory cannot explain the low frequency (~1%) of hybrids observed in nature. This study identifies two “cryptic” isolating barriers that may further reduce gene flow between *D. yakuba* and *D. santomea* where they are sympatric. First, noncompetitive gametic isolation has evolved between *D. yakuba* and *D. santomea*: heterospecific matings between the two species produce significantly fewer offspring than do conspecific matings. Second, conspecific sperm precedence (CSP) occurs when *D. yakuba* females mate with conspecific and heterospecific males. However, CSP is asymmetrical: *D. santomea* females do not show patterns of sperm usage consistent with CSP. *Drosophila yakuba* and *D. santomea* females also differ with respect to remating propensity after first mating with conspecific males. These results suggest that noncompetitive and competitive gametic isolating barriers may contribute to reproductive isolation between *D. yakuba* and *D. santomea*.

**Key words.**—Conspecific sperm precedence, *Drosophila*, hybridization, reproductive isolation, speciation.

Received June 15, 2003. Accepted November 16, 2003.

For adherents to the Biological Species Concept (Mayr 1942), speciation is equivalent to the evolution of reproductive isolation between populations. Most studies of reproductive isolating barriers have focused on factors acting before mating (e.g., habitat isolation or mate discrimination) or after fertilization of the egg (e.g., hybrid sterility or inviability). “Cryptic” isolating barriers that act after copulation but before fertilization (i.e., forms of “gametic isolation”) are often neglected despite the possibility that they could form an important class of prezygotic isolating barriers in nature. Several such barriers have been found in insects, including both noncompetitive and competitive forms of gametic isolation. Noncompetitive forms of isolation include shortened duration of heterospecific copulation, decreased efficiency of heterospecific sperm transfer and use, and reduced storage of heterospecific sperm (Price et al. 2001). In contrast, conspecific sperm precedence (CSP) is a competitive phenomenon that occurs when sperm from two different species are present in a female. When CSP occurs, gene flow between two species decreases as the result of the differential fertilization success of conspecific versus heterospecific sperm in a single female (Howard 1999).

Studies have shown that competitive forms of gametic isolation prevent gene exchange in plants (Arnold et al. 1993; Rieseberg et al. 1995; Carney et al. 1996), marine invertebrates (Loeb 1915), and insects. Conspecific sperm precedence has been observed in beetles (Nakano 1985; Katakura 1986; Wade et al. 1994), grasshoppers, and crickets (Hewitt et al. 1989; Bella et al. 1992; Gregory and Howard 1994), and *Drosophila* (Price 1997). In *Drosophila*, single heterospecific matings often produce high numbers of hybrid offspring. As in many species of insects and birds (Gromko et al. 1984; Smith 1984), *intraspecific* multiple matings typically result in second male precedence (for double matings) or last male precedence (when more than two males are mated to a female). When CSP occurs, conspecific sperm fertilize

the majority of the eggs when females are mated to conspecific and heterospecific males (Price 1997).

Despite extensive documentation of CSP, in two respects it is still not well understood. First, it is difficult to judge whether and how much CSP contributes to reproductive isolation between closely related species in nature. Although the evolution of reproductive isolating barriers has been studied exhaustively in *Drosophila* (Coyne and Orr 1989, 1997), CSP has been seen only between allopatric species of the *D. simulans* group (Price 1997) and allopatric subspecies of *D. pseudoobscura* (Dixon et al. 2003). The relative importance of any reproductive barrier in impeding gene flow—including CSP—can only be evaluated by studying taxa that actually hybridize in nature (e.g., Howard et al.’s [1998] study of CSP in sympatric crickets).

Second, the rate at which CSP and other forms of gametic isolation evolve is unclear. In the ground crickets *Allonemobius fasciatus* and *A. socius*, CSP appears to be the only isolating barrier to gene exchange separating the sister species (Gregory and Howard 1994). However, in most other studies of gametic isolation (e.g., Hewitt et al. 1989; Bella et al. 1992; Wade et al. 1994; Price 1997; Dixon et al. 2003), some premating and/or postmating reproductive isolating barriers have already evolved between species by the time one finds CSP. Thus, only quantitative comparisons of the strength of reproductive isolating barriers between species pairs can determine the relative contribution of gametic isolation to speciation.

Here I examine the possibility of noncompetitive gametic isolation and CSP in crosses between *Drosophila yakuba* and its sister species, *D. santomea* (Lachaise et al. 2000). These species are unique within the well-studied *D. melanogaster* subgroup because they have overlapping ranges, forming a narrow hybrid zone on the island of São Tomé, approximately 320 km off the coast of West Africa. *Drosophila santomea* is endemic to São Tomé, whereas *D. yakuba* also occurs

throughout continental West Africa. Phylogenetic and historical evidence suggest that the two species diverged about 450,000 years ago and that the range overlap is due to recent secondary contact (Cariou et al. 2001). The species are morphologically distinct (Lachaise et al. 2000): *D. santomea* completely lacks the abdominal pigmentation characteristic of the other eight species, including *D. yakuba*, in the *D. melanogaster* subgroup. *Drosophila yakuba* and *D. santomea* are strongly sexually isolated in laboratory tests (Coyne et al. 2002) and hybrid F<sub>1</sub> males are sterile (Lachaise et al. 2000), although hybrid females are partially to fully fertile (Coyne et al., unpubl. data).

I show that two forms of cryptic isolating barriers exist between *D. yakuba* and *D. santomea*. First, noncompetitive gametic isolation occurs in single heterospecific matings between *D. yakuba* and *D. santomea*. Second, CSP occurs between these species: conspecific sperm enjoy a fertilization advantage over heterospecific sperm in the females of only one species. This CSP is asymmetrical: it appears in only one of the two reciprocal hybridizations. I also estimate the relative strength of gametic isolation between *D. yakuba* and *D. santomea* for comparison against estimates of sexual isolation and postzygotic isolation between these species.

## MATERIALS AND METHODS

### *Drosophila* Stocks

All flies were reared in uncrowded cultures at 24°C with a 12-h light-dark cycle on standard cornmeal-yeast-agar medium. *Drosophila yakuba* wild-type (yak<sup>+</sup>) males were taken from the Tai 18 isofemale stock collected in 1983 in the Tai rainforest on the border between Liberia and the Ivory Coast. *Drosophila yakuba sepia* (yak<sup>se</sup>) males and females were derived from a stock originally collected in Central Gabon; *sepia* is a recessive eye color mutation identical to that on the *D. melanogaster* 3rd chromosome (Llopart et al. 2002). *Drosophila santomea* wild-type (san<sup>+</sup>) males were derived from the STO.4 isofemale line, originally collected on São Tomé in 1998. *Drosophila santomea red, copper* (san<sup>rc</sup>) males and females were derived from the STO.18 isofemale line; *red* is a recessive eye color mutation on chromosome 2 and *copper* is a recessive X-linked eye color mutation that is easily distinguished from the wild-type eye color. All stocks were checked by M. Noor, Louisiana State University, for *Wolbachia* using PCR and confirmed free of infection.

### Mating Trials

Males and females were collected as virgins under CO<sub>2</sub> anesthesia and kept individually in eight-dram, food-containing vials for three days. On day four, flies were transferred without anesthesia into fresh vials for matings. Mating observations began within the first hour of the 12-hour light cycle. For each mating, I recorded copulation latency (the time between the introduction of the male into the vial and the start of copulation) and copulation duration. Females who did not mate after 90 min of observation were discarded. Males were removed promptly after termination of copulation to prevent remating. On day eight, singly mated females were randomly assigned to one of the following treatments: no

TABLE 1. List of matings performed. All second matings occurred four days after the first mating.

Mating type	Female	First male	Second male
1	yak <sup>se</sup>	yak <sup>se</sup>	—
2	yak <sup>se</sup>	yak <sup>se</sup>	yak <sup>+</sup>
3	yak <sup>se</sup>	yak <sup>se</sup>	san <sup>+</sup>
4	yak <sup>se</sup>	yak <sup>+</sup>	—
5	yak <sup>se</sup>	yak <sup>+</sup>	yak <sup>se</sup>
6	yak <sup>se</sup>	yak <sup>+</sup>	san <sup>+</sup>
7	yak <sup>se</sup>	san <sup>+</sup>	—
8	yak <sup>se</sup>	san <sup>+</sup>	yak <sup>+</sup>
9	yak <sup>se</sup>	san <sup>+</sup>	yak <sup>se</sup>
10	san <sup>rc</sup>	san <sup>rc</sup>	—
11	san <sup>rc</sup>	san <sup>rc</sup>	san <sup>+</sup>
12	san <sup>rc</sup>	san <sup>rc</sup>	yak <sup>+</sup>
13	san <sup>rc</sup>	san <sup>+</sup>	—
14	san <sup>rc</sup>	yak <sup>+</sup>	san <sup>rc</sup>
15	san <sup>rc</sup>	yak <sup>+</sup>	san <sup>+</sup>

second mating, conspecific second mating, or heterospecific second mating. Progeny from singly mated females (i.e., those not allowed to remate) were reared to adulthood and counted. Second mating trials were conducted in fresh vials and lasted for eight hours, with observations made every five minutes. Copulations typically lasted for 20 min; thus, observations made every five minutes ensured that all matings that occurred would be recorded. Females who did not remate were discarded. Females from successful second matings were transferred to fresh food vials every three days until they stopped laying fertile eggs. Progeny from these doubly mated females were reared to adulthood and scored for paternity. Table 1 lists the matings conducted in this study.

### Scoring Progeny

$P_2$ , the proportion of progeny sired by the second male in double matings, is based on the number of offspring produced after the second mating (Boorman and Parker 1976). Thus, for females who were not allowed to remate, progeny production was calculated by subtracting the number of offspring produced during the four days after mating from the total lifetime number of offspring. This correction allows for a comparison of progeny production between females who remated to those who were not allowed to remate.

In double matings involving *D. yakuba sepia* females, paternity of both male and female progeny was determined by either the presence or absence of the *sepia* marker or by differences in pigmentation among the progeny (Lachaise et al. 2000). F<sub>1</sub> hybrid males with *D. yakuba* mothers have pigmented abdomens. F<sub>1</sub> hybrid females with *D. yakuba* mothers are intermediate in pigmentation between *D. yakuba* and *D. santomea* females and can be distinguished from the parental species.

In double matings involving *D. santomea red, copper* females, only the paternity of female progeny could be determined. F<sub>1</sub> hybrid females with *D. santomea* mothers are intermediate in pigmentation between *D. yakuba* and *D. santomea* females. In contrast, F<sub>1</sub> hybrid males with *D. santomea* mothers cannot be distinguished from pure-species *D. santomea* males, as pigmentation in *D. santomea* is largely X-

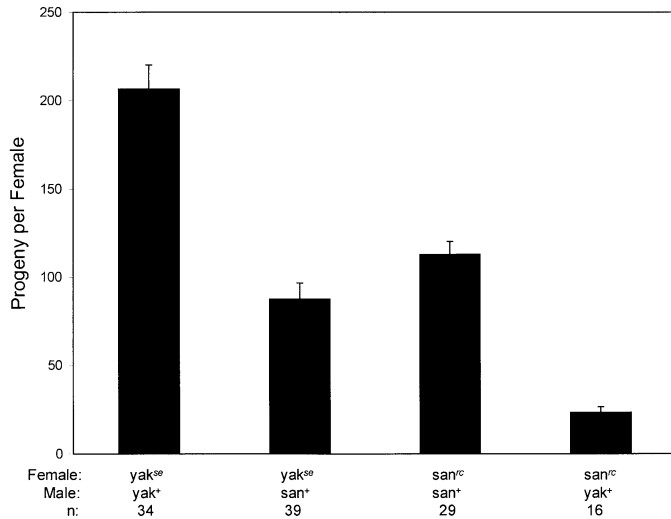


FIG. 1. Noncompetitive gametic isolation between *Drosophila yakuba* and *D. santomea*. Mean (SE) number of total offspring from conspecific and heterospecific single matings of *D. yakuba* and *D. santomea* females.

linked (Llopart et al. 2002). Moreover, the eye color mutations are uninformative in these matings: *copper* is a X-linked mutation and all male progeny show the *copper* phenotype; and though *red* is an autosomal mutation, it closely resembles the wild-type eye color in adult flies.

In two situations, I excluded females who did not produce any hybrid progeny. First, to minimize the possibility that low number of stored sperm increases the propensity of females to remate, I excluded females if the number of offspring produced four days after the first mating was more than 1.5 standard deviations below the mean of all females who remated. Second, to rule out trials in which sperm transfer most likely did not occur during second matings, I excluded females if the copulation duration of the second mating was less than the minimum amount of time for which there is evidence of sperm transfer. For example, the minimum amount of time for sperm transfer to occur in second matings of *D. yakuba sepia* females to *D. santomea* males was 7.8 min. The minimum amount of time for sperm transfer was determined by observation of hybrid progeny production in these matings.

#### Egg Hatchability

Experiments were performed to test for differences in hatchability between eggs from conspecific single matings and those from heterospecific single matings. *Drosophila yakuba sepia* females, *D. yakuba* Tai18 males, *D. santomea red*, *copper* females, and *D. santomea* STO.4 males were collected as virgins and kept in eight-dram corn food vials for three days. On day four, conspecific matings occurred en masse on corn media; all females mated successfully to conspecific males. Heterospecific matings were observed in fresh food vials and only eggs from successful heterospecific matings (i.e., the females were inseminated by heterospecific males) were collected. After mating, all females were transferred without anesthesia to medium containing blue food dye for egg laying. On days five and six, eggs were collected and

TABLE 2. Results of egg hatchability experiments. Egg hatchability was determined by counting the number of hatched versus unhatched eggs 24 h after the eggs were collected.

Female	Male	Hatched eggs	Unhatched eggs	% Hatched
yak <sup>se</sup>	yak <sup>+</sup>	438	2	99.5
yak <sup>se</sup>	san <sup>+</sup>	349	251	58.2
san <sup>rc</sup>	san <sup>+</sup>	562	38	93.7
san <sup>rc</sup>	yak <sup>+</sup>	121	139	46.5

transferred to black filter paper placed in corn food vials. Hatched and unhatched eggs were counted using a dissecting microscope after 24 h.

#### Larval Competition

Larval competition experiments were performed to distinguish sperm precedence from competitive inferiority or inviability of hybrid larvae (Gilchrist and Partridge 1997; Price et al. 2000). *Drosophila yakuba sepia* females, *D. yakuba* Tai 18 males, and *D. santomea* STO.4 males were collected as virgins and kept in eight-dram corn food vials for three days. On the fourth day, single matings between *D. yakuba sepia* females and either *D. yakuba sepia* males or *D. santomea* wild-type males occurred en masse on corn media. After three hours, flies were transferred to vials containing molasses-agar medium, allowing flies to acclimate to the novel medium. The dark-colored molasses-agar medium allows larvae to be easily recognized. After 20 to 24 h of acclimation, flies were transferred to bottles containing the same medium for egg laying. First-instar larvae were collected after 24 h from bottles containing the molasses-agar medium and transferred into corn food vials at a controlled density of 50 larvae per vial. I tested the viability of hybrid larvae at three relative frequencies: 10%, 50%, and 90% hybrid larvae. Larvae from these vials were allowed to develop into adults and their hybrid or pure-species origin determined by whether they carried the *sepia* marker.

### RESULTS

#### Noncompetitive Gametic Isolation

Figure 1 shows the mean offspring number for the four classes of single matings. Heterospecific matings produced fewer offspring than conspecific matings: *D. yakuba sepia* females mated to *D. santomea* wild-type males produced significantly fewer offspring than the same females mated to *D. yakuba* wild-type males (unpaired  $t_{71} = 7.432$ ,  $P < 0.0001$ ). Similarly, *D. santomea red*, *copper* females mated to *D. yakuba* wild-type males produced significantly fewer offspring than those mated to *D. santomea* wild-type males (unpaired  $t_{41} = 9.111$ ,  $P < 0.0001$ ). Because in these crosses, a female carries sperm from only one male, the reduced number of offspring from heterospecific single matings constitutes a noncompetitive form of gametic reproductive isolation.

Table 2 shows the hatchability of eggs from conspecific and heterospecific single matings. Eggs from matings between *D. yakuba sepia* females and *D. santomea* STO.4 males were significantly less likely to hatch than those from matings between *D. yakuba sepia* females and *D. yakuba* Tai 18 males

TABLE 3. Results of larval competition experiments. Hybrid larvae from matings between *Drosophila yakuba sepia* females and *D. santomea* wild-type males were competed against pure-species larvae from matings between (A) *D. yakuba sepia* females and *D. yakuba sepia* males and (B) *D. yakuba sepia* females and *D. yakuba* wild-type males at three fixed ratios (% hybrid). Number of larvae placed in vials and mean number (SE) of adults eclosed from vials;  $\chi^2$  tests were performed using number of adults eclosed pooled across trials.

Number of larvae placed in vials				Adults eclosed from vials		
Trials	<i>yakuba</i>	hybrid	% hybrid	<i>yakuba</i>	hybrid	$\chi^2$
(A) <i>yak<sup>se</sup></i> × <i>yak<sup>se</sup></i> vs. hybrids						
16	45	5	10	25.75 (1.02)	3.12 (0.38)	0.347
19	25	25	50	14.00 (0.76)	16.42 (0.72)	3.661
18	5	45	90	2.83 (0.29)	26.89 (1.02)	0.130
(B) <i>yak<sup>se</sup></i> × <i>yak<sup>+</sup></i> vs. hybrids						
15	45	5	10	28.87 (1.21)	2.87 (0.29)	0.493
16	25	25	50	17.78 (0.79)	16.56 (0.89)	0.182
15	5	45	90	3.07 (0.43)	28.27 (1.50)	0.024

( $P < 0.0001$ , Fisher's Exact test). Similarly, eggs from matings between *D. santomea red, copper* females and *D. yakuba* Tai18 males were less likely to hatch than those from matings between *D. santomea red, copper* females and *D. santomea* STO.4 males ( $P < 0.0001$ , Fisher's Exact test). Necrotized unhatched eggs (indicating early zygote death) were never observed, implying that unhatched eggs were unfertilized. These results suggest that decreased hatchability of eggs from heterospecific crosses, possibly through failure of fertilization, accounts for the differences in offspring production between conspecific and heterospecific single matings.

#### Comparison of Copulation Duration and Offspring Production between Singly and Doubly Mated Females

Any difference in the number of sperm stored between females who remated and those not allowed to remate should be reflected in the average number of offspring produced by a female during the four days after first mating ( $T_0$ ). (Note that for both *D. yakuba sepia* and *D. santomea red, copper* females,  $T_0$  is strongly correlated with the total number of offspring the female will produce during her lifetime [data not shown;  $n = 153$ ,  $r = 0.778$ ,  $P < 0.0001$  for *D. yakuba sepia*;  $n = 28$ ,  $r = 0.717$ ,  $P < 0.0001$  for *D. santomea red, copper*]. Thus,  $T_0$  provides a rough index for comparing lifetime offspring production.) To determine whether doubly mated females remated to compensate for inefficient sperm transfer or storage from the first mating, I compared copulation duration and  $T_0$  for singly versus doubly mated females. For matings involving *D. yakuba sepia* females, copulation duration of first matings do not differ between the singly (mean  $\pm$  SE = 44.7 min  $\pm$  1.1) and doubly mated females (42.8  $\pm$  1.1; unpaired  $t_{205} = 1.249$ ,  $P = 0.213$ ). Furthermore,  $T_0$  for singly mated females (52.8  $\pm$  1.8) is not significantly different from  $T_0$  for doubly mated females (54.7  $\pm$  2.0; unpaired  $t_{288} = 0.728$ ,  $P = 0.467$ ). These results suggest that *D. yakuba sepia* females remate even when first-mating males successfully transfer sperm; that is, the number of sperm transferred during first mating has no effect on remating.

For matings involving *D. santomea red, copper* females, the mean duration of copulation between singly (32.3  $\pm$  2.2) and doubly mated females (29.8  $\pm$  1.7) was not significantly different (unpaired  $t_{48} = 0.902$ ,  $P = 0.372$ ). However,  $T_0$  for

singly mated females (30.1  $\pm$  1.9) is significantly greater than  $T_0$  for doubly mated females (22.2  $\pm$  1.8; unpaired  $t_{54} = 2.992$ ,  $P = 0.0042$ ). These results suggest that *D. santomea red, copper* females remate because first-mating *D. santomea red, copper* males transfer few sperm. However, it is also possible that the low  $T_0$  is caused by the decreased viability of offspring homozygous for *red* and *copper*:  $T_0$  for *D. santomea red, copper* females mated singly to *D. santomea* wild-type males (37.8  $\pm$  2.9) is significantly greater than  $T_0$  for females mated to mutant males (29.3  $\pm$  2.0; unpaired  $t_{56} = 2.380$ ,  $P = 0.021$ ). Mean copulation duration does not differ between matings involving wild-type males (30.3  $\pm$  0.9) and mutant males (32.5  $\pm$  2.1; unpaired  $t_{50} = 0.973$ ,  $P = 0.335$ ).

#### Test for Viability Difference between Pure-Species and Hybrid Larvae

Table 3 shows the viabilities of pure-species versus hybrid larvae when reared together at different relative frequencies. I used  $\chi^2$  tests to compare the number of pure-species and hybrid adults eclosed to the number of pure-species and hybrid larvae placed in each vial, respectively. Hybrid larvae from crosses between *D. yakuba sepia* females and *D. santomea* wild-type males were not competitively inferior to pure-species larvae at any frequency class. Any difference observed in offspring number must therefore be due to differential sperm usage or egg mortality.

#### CSP in *Drosophila yakuba* Females

Figure 2 shows the results of all mating types performed in this experiment using *D. yakuba sepia* females. CSP is evident in double matings between *D. yakuba sepia* females and *D. santomea* males. In conspecific double matings involving *D. yakuba sepia* females mated first to *D. yakuba sepia* males and subsequently to *D. yakuba* wild-type males (mating type 2),  $P_2 = 0.93 \pm 0.02$ . Such intraspecific second male precedence is consistent with that found in other *Drosophila* species (Gromko et al. 1984; Turner and Anderson 1984). In contrast, in matings involving *D. yakuba sepia* females first mated to *D. yakuba sepia* males and then to *D. santomea* wild-type males (mating type 3),  $P_2 = 0.18 \pm 0.05$ . In *D. yakuba* females, then,  $P_2$  for heterospecific second males

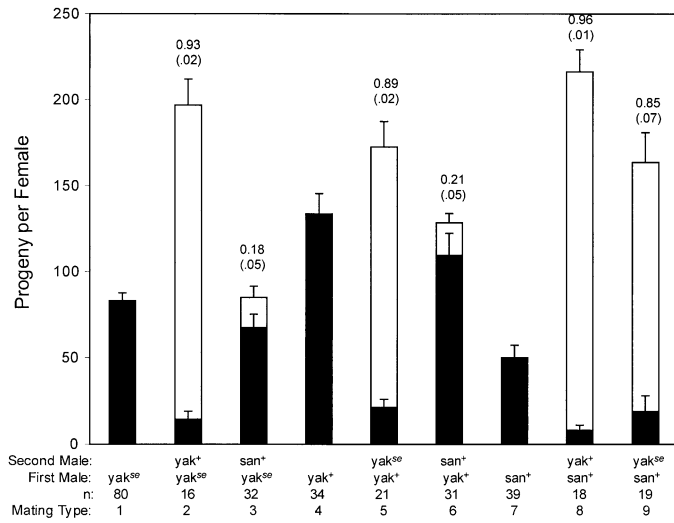


FIG. 2. Mean (SE) number of offspring per *Drosophila yakuba sepia* female sired by first (solid) and second (open) male. Number of offspring for single matings has been corrected by subtracting the number of offspring produced after the first four days. All double matings that occurred are included, regardless of evidence of sperm transfer (see text). Mean  $P_2$ -values (SE) are shown above bars.

is significantly lower than  $P_2$  for conspecific second males ( $P < 0.0001$ , Mann-Whitney  $U$ -test).

Many females who remated to *D. santomea* wild-type males did not produce any hybrid offspring. This may be due to either extreme CSP or failure of sperm transfer by *D. santomea* males. Despite having already controlled for this possibility (see Materials and Methods), I excluded  $P_2$ -values for those cases in which sperm transfer may not have occurred. When the  $P_2 = 0$  class is excluded, mean  $P_2$  increases to  $0.32 \pm 0.08$  for heterospecific second males. Even using the conservative criterion of excluding the  $P_2 = 0$  class,  $P_2$ -values for heterospecific second males are significantly smaller than those for conspecific second males ( $P < 0.0001$ , Mann-Whitney  $U$ -test). This suggests that doubly inseminated *D. yakuba sepia* females preferentially use conspecific sperm over heterospecific sperm or that heterospecific sperm are competitively inferior to conspecific sperm when both types are present in a single female.

To investigate the effect of mating order on sperm precedence, I compared matings in which *D. yakuba sepia* males mate after *D. yakuba* wild-type males (type 5;  $P_2 = 0.89 \pm 0.02$ ) to matings in which *D. yakuba sepia* males mate after *D. santomea* wild-type males (type 9;  $P_2 = 0.85 \pm 0.07$ ). Mean  $P_2$ -values do not differ significantly between these two mating types ( $P = 0.133$ , Mann-Whitney  $U$ -test). Similarly, I compared matings in which *D. yakuba* wild-type males mate after *D. yakuba sepia* males (type 2;  $P_2 = 0.93 \pm 0.02$ ) to matings in which *D. yakuba* wild-type males mate after *D. santomea* wild-type males (type 8;  $P_2 = 0.96 \pm 0.01$ ). Again, mean  $P_2$ -values do not differ significantly ( $P = 0.352$ , Mann-Whitney  $U$ -test). These results show that the species of the first male has little effect on  $P_2$ .

Differential transfer of sperm numbers in two successive matings (a noncompetitive form of isolation) may produce the same pattern of sperm usage as observed if CSP in double

matings (a competitive form of isolation) occurs in these species. Low  $P_2$  values are expected when *D. yakuba* females are mated conspecifically and then heterospecifically (e.g., mating type 3) simply because more conspecific than heterospecific sperm are present in the female's reproductive tract.

To distinguish between these possibilities, I estimated two values. First, I standardized the number of offspring from each single mating sired by a heterospecific male by the mean for all conspecific males:  $R_s = (\text{number of hybrid offspring from each } D. yakuba sepia \text{ female} \times D. santomea \text{ male single mating}) / (\text{mean number of offspring from } D. yakuba sepia \text{ female} \times D. yakuba sepia \text{ male single matings})$ . Similarly, I standardized the number of offspring sired by each heterospecific second male by the mean for all conspecific second males in double matings:  $R_d = (\text{number of offspring sired by the second male from each } D. yakuba sepia \times D. yakuba sepia \text{ male, then } D. santomea \text{ male mating}) / (\text{mean number of offspring sired by conspecific second males from } D. yakuba sepia \text{ female} \times D. yakuba sepia \text{ male, then } D. yakuba \text{ wild-type male matings})$ . If the number of offspring produced by *D. santomea* males reflects the number of sperm transferred by these males and if *D. santomea* males transfer, on average, less sperm than do *D. yakuba sepia* males, then  $R_s$  and  $R_d$  should not be significantly different from each other.

$R_s$  is significantly greater than  $R_d$  ( $n_1 = 39$ ,  $n_2 = 32$ ,  $P < 0.0001$ , Mann-Whitney  $U$ -test) for *D. yakuba sepia* females first mated to *D. yakuba sepia* males (mating types 2 and 3). When the  $P_2 = 0$  class is excluded,  $R_s$  still exceeds  $R_d$  ( $n_1 = 39$ ,  $n_2 = 18$ ,  $P = 0.0018$ , Mann-Whitney  $U$ -test). Thus, the low values of  $P_2$  for heterospecific second males are due to CSP and not to differential sperm transfer between the males of the two species.

The *sepia* mutant marker was used to score the paternity of progeny from double matings. It is conceivable (though unlikely) that the *sepia* mutation may affect sperm competition or usage in *D. yakuba*. To test for such a mutant effect, *D. yakuba sepia* females were first mated to *D. yakuba* wild-type males before remating to either *D. yakuba sepia* males or *D. santomea* wild-type males. All offspring were scored for paternity based solely on abdominal pigmentation differences between pure-species *D. yakuba* and hybrids (Lachaise et al. 2000.).

Heterospecific second matings, in which *D. santomea* males mate after *D. yakuba* wild-type males, result in a mean  $P_2 = 0.21 \pm 0.05$  (type 6, Fig. 2); exclusion of heterospecific second matings that produced no hybrid offspring gives a mean  $P_2 = 0.28 \pm 0.06$ . Thus,  $P_2$ -values for mating types 3 (yak<sup>se</sup> as first males) and 6 (yak<sup>+</sup> as first males) are not significantly different whether the  $P_2 = 0$  class is included ( $P = 0.2774$ , Mann-Whitney  $U$ -test) or excluded ( $P = 0.7928$ , Mann-Whitney  $U$ -test). Similarly,  $P_2$ -values for mating types 8 (yak<sup>+</sup> as second males) and 9 (yak<sup>se</sup> as second males) are not significantly different ( $P = 0.5434$ , Mann-Whitney  $U$ -test). Comparison of  $R_s$  and  $R_d$  for these matings shows that the pattern of sperm precedence is not due to differential sperm transfer in single matings between conspecific and heterospecific males ( $P = 0.0002$ ;  $P = 0.0394$  when  $P_2 = 0$  class excluded; Mann-Whitney  $U$ -test). Thus, the *sepia* marker has no detectable effect on sperm usage in

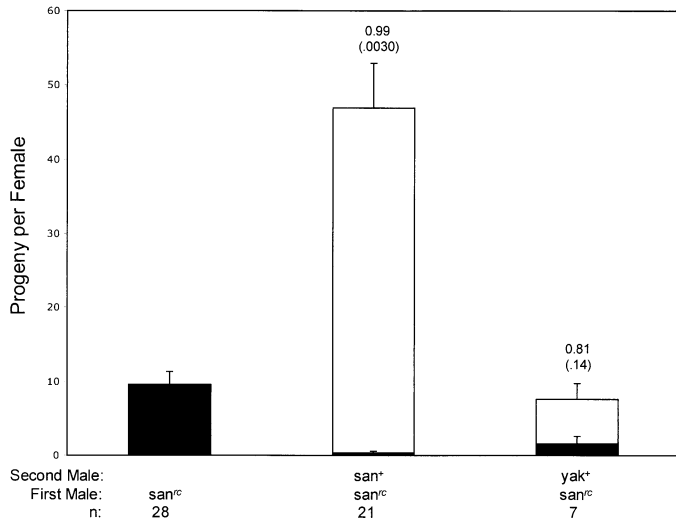


FIG. 3. Mean (SE) number of female offspring per *Drosophila santomea red, copper* female sired by first (solid) and second (open) male after single and double matings. Only female offspring were scored because all males are *copper* (*copper* is X-linked). Number of offspring for single matings has been corrected by subtracting the number of offspring produced after the first four days. Mean  $P_2$ -values (SE) are shown above bars.

double matings of *D. yakuba sepia* females, regardless of whether *D. yakuba* males are the first or second to mate.

In summary, *D. yakuba* females show patterns of sperm usage consistent with CSP. When *D. santomea* males mate after *D. yakuba* males, few hybrid offspring are produced. This is in dramatic contrast to results of conspecific double matings, in which the second males typically sire greater than 90% of the total progeny.

#### No Evidence of CSP in *Drosophila santomea* Females

Figure 3 gives the results of matings using *D. santomea red, copper* females. As in *D. yakuba* females, intraspecific second male precedence in *D. santomea* females is extremely strong, with  $P_2 = 0.99 \pm 0.003$ . However, in contrast to matings involving *D. yakuba* females, second male precedence is also evident in *heterospecific* second matings: in second matings of *D. santomea* females to *D. yakuba* wild-type males (mating type 12),  $P_2 = 0.81 \pm 0.14$ . The  $P_2$ -values for conspecific and heterospecific second matings are not significantly different ( $P = 0.396$ , Mann-Whitney  $U$ -test). These results should be interpreted with some caution, as few *D. santomea red, copper* females successfully mated with *D. yakuba* males after first mating with *D. santomea* wild-type males.

It was not possible to investigate the effect of mating order on patterns of sperm usage in *D. santomea* females. Single matings between *D. santomea* females and *D. yakuba* males occur infrequently, as sexual isolation between these species is very strong in this direction of the cross (Coyne et al. 2002). Moreover, *D. santomea* females rarely remate after first mating with *D. yakuba* males: only one of seven *D. santomea* female remated conspecifically after first mating to a *D. yakuba* male, which yielded a  $P_2 = 0.11$ .

Therefore, no evidence for CSP exists in *D. santomea* fe-

TABLE 4. Comparisons of remating propensity between *Drosophila yakuba sepia* (yak<sup>se</sup>) females and *D. santomea red, copper* (san<sup>rc</sup>) females to conspecific and heterospecific males.

Mating type	Female	First male	Second male	Total	Frequency of remating
2	yak <sup>se</sup>	yak <sup>se</sup>	yak <sup>+</sup>	194	0.0928
3	yak <sup>se</sup>	yak <sup>se</sup>	san <sup>+</sup>	194	0.206
5	yak <sup>se</sup>	yak <sup>+</sup>	yak <sup>se</sup>	278	0.0791
6	yak <sup>se</sup>	yak <sup>+</sup>	san <sup>+</sup>	262	0.137
8	yak <sup>se</sup>	san <sup>+</sup>	yak <sup>se</sup>	23	0.913
9	yak <sup>se</sup>	san <sup>+</sup>	yak <sup>+</sup>	22	1
11	san <sup>rc</sup>	san <sup>rc</sup>	san <sup>+</sup>	128	0.18
12	san <sup>rc</sup>	san <sup>rc</sup>	yak <sup>+</sup>	220	0.0454
14	san <sup>rc</sup>	yak <sup>+</sup>	san <sup>rc</sup>	7	0.143
15	san <sup>rc</sup>	yak <sup>+</sup>	san <sup>+</sup>	7	0

males. It is formally possible that this result is due to fewer number of sperm stored from first matings of *D. santomea red, copper* females who remated in these experiments produced fewer offspring during four days after their first mating (see above).

#### Index of Noncompetitive Gametic Isolation

The strength of gametic isolation can be estimated using a modification of Coyne and Orr's (1989, 1997) index of sexual isolation in no-choice mating experiments,  $I_s = 1 - (\text{number of heterospecific matings/number of conspecific matings})$ . Here, instead of considering the number of matings, I consider the relative mean number of eggs fertilized in heterospecific versus conspecific single matings. Thus, the index of noncompetitive gametic isolation is  $I_g = 1 - (\text{mean number of offspring produced by heterospecific single matings/mean number of offspring produced by conspecific single matings})$ . In matings involving *D. yakuba* females,  $I_g = 0.576$ , and for those involving *D. santomea* females,  $I_g = 0.795$ . Combining data from both directions of the cross, the total index of noncompetitive gametic isolation is  $I_g = 0.653$ . It is important to note that this index does not account for the additional isolation caused by CSP.

#### Remating Propensity

Table 4 gives the remating propensity between *D. yakuba sepia* females and *D. santomea red, copper* females. Comparison of remating frequencies show that *D. yakuba* females, once mated, are more likely to remate both conspecifically and heterospecifically than are *D. santomea* females (types 2, 3, 5, 6, 8, 9 vs. 11, 12, 14, 15;  $P = 0.0012$ , Fisher's Exact test). This difference in remating propensity between females of the two species cannot be explained by low numbers of sperm stored in the reproductive tracts of *D. yakuba* females: singly and doubly mated *D. yakuba* females produce comparable number of offspring (see above).

Surprisingly, *D. yakuba* females who first mate with conspecific males are twice as likely to remate heterospecifically as conspecifically (types 3 and 6 vs. types 2 and 5;  $P = 0.0002$ , Fisher's Exact test; all following  $P$ -values in this section are derived from Fisher's Exact tests of remating frequencies). In contrast, *D. santomea red, copper* females who first mated to *D. santomea red, copper* males remate far

more readily with *D. santomea* wild-type males than with *D. yakuba* males (mating type 11 vs. type 12,  $P < 0.0001$ ). This finding is consistent with the strong sexual isolation in single matings between *D. santomea* females and *D. yakuba* males (Coyne et al. 2002).

*Drosophila yakuba* females who mate first to *D. santomea* wild-type males are far more likely to remate than are females who mate first with conspecific males ( $P < 0.0001$  for type 2 vs. type 9, type 5 vs. type 8, and pooled for types 2 and 5 vs. 8 and 9). Among doubly mated *D. yakuba* females, those first mated conspecifically produce greater number of progeny before remating than those first mated heterospecifically (unpaired  $t_{135} = 6.246$ ,  $P < 0.0001$ ). This is consistent with the noncompetitive gametic isolation between *D. yakuba* and *D. santomea* shown above.

Heterospecific matings between *D. santomea* females and *D. yakuba* males are rare (approximately 20% of these matings are successful; see also Coyne et al. 2002); rematings occur even less frequently (14.3%). Thus, it is difficult to test if differences in remating behavior exist between *D. santomea* females who were first mated heterospecifically and those first mated conspecifically. However, the limited results suggest that there is no difference in remating propensity between *D. santomea* females who first mated conspecifically versus heterospecifically (types 11 and 12 vs. types 14 and 15,  $P > 0.9999$ ). *Drosophila santomea* females who first mate with *D. santomea red, copper* males are not more likely to remate with wild-type conspecific males than those who first mate with *D. yakuba* males (type 11 vs. 15,  $P = 0.6025$ ).

Thus, remating propensity appears to differ between *D. yakuba* and *D. santomea* females. Curiously, *D. yakuba* females who first mate conspecifically are more likely to remate with heterospecific males than with conspecific males. *Drosophila santomea* females who first mate conspecifically are more likely to remate with conspecific males than with heterospecific males. The reluctance of *D. santomea* females to mate "incorrectly" with heterospecific males affects not only first matings but also subsequent encounters with *D. yakuba* males.

## DISCUSSION

The present study identifies two "cryptic" reproductive isolating barriers that have evolved between *D. yakuba* and its sister species *D. santomea* in addition to the sexual isolation and hybrid male sterility in both directions of the cross previously documented (Lachaise et al. 2000; Coyne et al. 2002). First, noncompetitive gametic isolation occurs between these species. Heterospecific single matings produce fewer offspring than any conspecific single mating. Egg hatchability experiments show that eggs produced by heterospecifically mated females are less likely to hatch than those produced by conspecifically mated females. Second, conspecific sperm precedence, a competitive form of gametic isolation, occurs in one direction of hybridization.

### *Noncompetitive Gametic Isolation*

Heterospecific single matings between *D. yakuba* and *D. santomea* produce significantly fewer progeny, in both directions of the cross, than both types of conspecific mating

(Fig. 1). This deficit constitutes a noncompetitive form of "cryptic" reproductive isolation at the gamete level. Heterospecific sperm suffer some disadvantage within the reproductive tract of heterospecific females, resulting in decreased fertilization success. Females are penalized more severely than males for "incorrect" matings, as they will not be sexually receptive again for several days. Heterospecific single matings cause fitness losses for both sexes.

Other forms of noncompetitive gametic isolation have been documented in previous studies of isolating barriers in insects. In these studies, noncompetitive gametic isolation is typically the result of sperm transfer or storage problems in heterospecific females (Katakura 1986; Gregory and Howard 1994; Price et al. 2001). Interestingly, in *Drosophila*, heterospecific single matings often produced more offspring than conspecific single matings. For example, single matings between cosmopolitan and Zimbabwe "races" of *D. melanogaster* produced more offspring than single matings within both races (Dixon et al. 2003). In the *D. simulans* group, matings between *D. mauritiana* males and *D. simulans* females produced more hybrid offspring than matings between *D. mauritiana* males and *D. mauritiana* females (Price 1997). However, matings between *D. simulans* males and *D. mauritiana* females produced fewer hybrid offspring than matings between *D. simulans* males and *D. simulans* females. Thus, in contrast to *D. yakuba* and *D. santomea*, noncompetitive gametic isolation between *D. simulans* and *D. mauritiana* is asymmetric.

### *CSP in Drosophila yakuba Females*

In double conspecific matings of *D. yakuba* females, the second male sires the majority of the offspring. However, second male precedence does not occur when conspecific and then heterospecific males mate with *D. yakuba* females. Thus, in matings with *D. yakuba* females, first-mating *D. yakuba* males enjoy a fertilization advantage at the expense of second-mating *D. santomea* males.

Conspecific sperm precedence between *D. yakuba* and *D. santomea* is incomplete in two respects. For one, first-mating *D. yakuba* males do not have an advantage over first-mating *D. santomea* males when *D. yakuba* females are subsequently mated to *D. yakuba* males. In contrast, CSP occurs in *D. simulans* females in crosses with *D. mauritiana* males, regardless of mating order (Price 1997). For another, there is no evidence of CSP in *D. santomea* females. Conspecific sperm precedence is thus asymmetrical in crosses between these two sister species. Earlier studies on CSP between *D. simulans* and *D. mauritiana* were unable to examine CSP in both directions of hybridization, as *D. mauritiana* females rarely remate (Price 1997). Alipaz et al. (2001) documented asymmetric gametic isolation between Zimbabwe and cosmopolitan races of *D. melanogaster* and speculated that differences in gamete recognition may be responsible. In *Drosophila*, mismatches in the seminal receptacle length and sperm length can also generate asymmetries in sperm usage (Miller and Pitnick 2002). It is conceivable that differences in seminal receptacle and sperm length in *D. yakuba* and *D. santomea* contribute to the observed asymmetry in CSP. In any case, it is clear that the well-documented asymmetries

in sexual isolating barriers (Arnold 1976; Kaneshiro 1980; Shine et al. 2002) also extend to gametic isolating barriers.

Comparison of reproductive isolating barriers between different species pairs in the same group provides some insight to the relative rate of evolution of isolating barriers. Such information may indicate which barriers are important in impeding gene flow during the process of speciation (Coyne and Orr 1989, 1997). For example, the sympatric ground crickets *Allonemobius fasciatus* and *A. socius* appear to be isolated only by CSP, suggesting that CSP evolved faster than other forms of isolating barriers in this species pair (Gregory and Howard 1994). In *Drosophila*, gametic isolation has been studied only between a few taxa and no clear pattern of the relative rates of evolution of gametic isolating barriers emerges. The similarity in divergence times between *D. yakuba* and *D. santomea* (~450,000 years; Cariou et al. 2001) and between *D. simulans* and its two sister species (~260,000 to 410,000 years; Kliman et al. 2000) suggests that the evolution of CSP has been slower in the former species pair. In contrast, noncompetitive isolation appears to have evolved faster between *D. yakuba* and *D. santomea* than between *D. simulans* and *D. mauritiana*. The data currently available thus suggest that, in *Drosophila*, gametic isolation evolves at rates similar to other forms of isolation: gametic isolation is only found between taxa also isolated by behavioral isolation and hybrid sterility and/or inviability (Price 1997; Dixon et al. 2003; present study).

#### *Differences in Remating Propensity*

*Drosophila yakuba* and *D. santomea* females differ with respect to remating propensity after initially mating with males of their own species: *D. yakuba* females are more likely to remate heterospecifically than conspecifically. In contrast, *D. santomea* females more readily remate with conspecific males than with heterospecific males, paralleling their preference in single matings (Coyne et al. 2002).

Mate preference in single matings of *D. yakuba* females does not explain mate preference in second matings of *D. yakuba* females who first mated with conspecific males. Coyne et al. (2002) found that, in single matings, *D. yakuba* females are more likely to mate with *D. santomea* males than *D. santomea* females are with *D. yakuba* males. If mate preference in second matings were identical to that seen in first matings, one would expect *D. yakuba* females to remate more frequently to conspecific males. Furthermore, *D. yakuba* females who remate heterospecifically typically do not use a significant amount of sperm from the second matings. *Drosophila yakuba* females who mate twice with conspecific males invariably produce more pure-species progeny than those who first mate conspecifically and then heterospecifically (unpaired  $t_{98} = 6.961$ ,  $P < 0.0001$ ). Thus, relative to conspecific double matings, heterospecific second matings serve only to decrease the fitness of *D. yakuba* females who have already mated to conspecific males.

#### *Conclusions*

*Drosophila yakuba* and *D. santomea* have overlapping ranges on the island of São Tomé, forming a hybrid zone over an altitudinal gradient (*D. santomea* occurs at higher

altitudes). Interspecific crosses in the laboratory involving *D. santomea* females show stronger sexual isolation than those involving *D. yakuba* females (Coyne et al. 2002). Coyne et al.'s (2002) study noted that the level of sexual isolation observed in these lab crosses is insufficient to account for the very low frequency of  $F_1$  hybrids found in nature (~1%; Lachaise et al. 2000). If sexual isolation is not stronger in nature than in the laboratory, then other reproductive isolating barriers must operate to maintain these species as distinct entities in the face of hybridization. The findings reported here—noncompetitive gametic isolation and CSP—suggest that postmating, prezygotic barriers also contribute to the low frequency of hybrids. Differences in ecology (such as resource or habitat preference) probably play an additional role in isolating these species in nature, although ecologically based barriers are difficult to study in *Drosophila*.

#### ACKNOWLEDGMENTS

I thank S. Elwyn, S. Powers, and J. Cassidy for technical assistance. I also thank D. Howard and an anonymous reviewer for their suggestions. I am especially grateful to J. Coyne and D. Presgraves for many helpful discussions and comments on the manuscript. This work was supported by National Institute of Health grant no. GM58260 to J. A. Coyne.

#### LITERATURE CITED

- Alipaz, J. A., C.-I. Wu, and T. L. Karr. 2001. Gametic incompatibilities between races of *Drosophila melanogaster*. *Proc. R. Soc. Lond. B* 268:789–795.
- Arnold, M. L., J. L. Hamrick, and B. D. Bennett. 1993. Interspecific pollen competition and reproductive isolation in *Iris*. *J. Hered.* 84:13–16.
- Arnold, S. J. 1976. Sexual behavior, sexual interference and sexual defense in the salamanders *Ambystoma maculatum*, *Ambystoma tigrinum*, and *Plethodon jordani*. *Z. Tierpsychol.* 42:247–300.
- Bella, J. L., R. K. Butlin, C. Ferris, and G. M. Hewitt. 1992. Asymmetrical homogamy and unequal sex ratio from reciprocal mating-order crosses between *Chorthippus parallelus* subspecies. *Heredity* 68:345–352.
- Boorman, E., and G. A. Parker. 1976. Sperm (ejaculate) competition in *Drosophila melanogaster*, and the reproductive value of females to males in relation to female age and mating status. *Ecol. Entomol.* 1:145–155.
- Cariou, M.-L., J.-F. Silvain, V. Daubin, J.-L. DaLage, and D. Lachaise. 2001. Divergence between *Drosophila santomea* and allopatric and sympatric populations of *D. yakuba* using paralogous amylase lines and migration scenarios along the Cameroon volcanic line. *Mol. Ecol.* 10:649–660.
- Carney, S. E., S. A. Hodges, and M. L. Arnold. 1996. Effects of differential pollen-tube growth on hybridization in the Louisiana Irises. *Evolution* 50:1871–1878.
- Coyne, J. A., and H. A. Orr. 1989. Patterns of speciation in *Drosophila*. *Evolution* 43:362–381.
- . 1997. "Patterns of speciation in *Drosophila*" revisited. *Evolution* 51:295–303.
- Coyne, J. A., S. Y. Kim, A. S. Chang, D. Lachaise, and S. Elwyn. 2002. Sexual isolation between two sibling species with overlapping ranges: *Drosophila santomea* and *Drosophila yakuba*. *Evolution* 56:2424–2434.
- Dixon, S. M., J. A. Coyne, and M. A. F. Noor. 2003. The evolution of conspecific sperm precedence in *Drosophila*. *Mol. Ecol.* 12:1179–1184.
- Gilchrist, A. S., and L. Partridge. 1997. Heritability of pre-adult viability differences can explain apparent heritability of sperm

- displacement ability in *Drosophila melanogaster*. Proc R. Soc. Lond. B 264:1271–1275.
- Gregory, P. G., and D. J. Howard. 1994. A postinsemination barrier to fertilization isolates two closely related ground crickets. Evolution 48:705–710.
- Gromko, M. H., D. Gilbert, and R. C. Richmond. 1984. Sperm transfer and use in the multiple mating system of *Drosophila*. Pp. 371–426 in R. L. Smith, ed. Sperm competition and the evolution of animal mating systems. Academic Press, London.
- Hewitt, G. M., P. Mason, and R. A. Nichols. 1989. Sperm precedence and homogamy across a hybrid zone in the alpine grasshopper *Podisma pedestris*. Heredity 62:343–353.
- Howard, D. J. 1999. Conspecific sperm and pollen precedence and speciation. Annu. Rev. Ecol. Syst. 30:109–132.
- Howard, D. J., P. G. Gregory, J. Chu, and M. L. Cain. 1998. Conspecific sperm precedence is an effective barrier to hybridization between closely related species. Evolution 52:511–516.
- Kaneshiro, K. Y. 1980. Sexual isolation, speciation and the direction of evolution. Evolution 34:437–444.
- Katakura, H. 1986. A further study on the effect of interspecific mating on the fitness in a pair of sympatric phytophagous ladybirds. Kontyu 54:235–242.
- Kliman, R. M., P. Andolfatto, J. A. Coyne, F. Depaulis, M. Kreitman, A. J. Berry, M. McCarter, J. Wakeley, and J. Hey. 2000. The population genetics of the origin and divergence of the *Drosophila simulans* complex species. Genetics 156:1913–1931.
- Lachaise, D., M. Harry, M. Solignac, F. Lemeunier, V. Benassi, and M.-L. Cariou. 2000. Evolutionary novelties in islands: *Drosophila santomea*, a new *melanogaster* sister species from São Tomé. Proc. R. Soc. Lond. B 267:1487–1495.
- Llopart, A., S. Elwyn, D. Lachaise, and J. A. Coyne. 2002. Genetics of a difference in pigmentation between *Drosophila yakuba* and *Drosophila santomea*. Evolution 56:2262–2277.
- Loeb, J. 1915. On the nature of the conditions which determine or prevent the entrance of the spermatozoon into the egg. Am. Nat. 49:257–285.
- Mayr, E. 1942. Systematics and the origin of species. Columbia Univ. Press, New York.
- Miller, G. T., and S. Pitnick. 2002. Sperm-female coevolution in *Drosophila*. Science 298:1230–1233.
- Nakano, S. 1985. Effect of interspecific mating on female fitness in two closely related ladybirds (*Henosepilachna*). Kontyu 53:112–119.
- Price, C. S. C. 1997. Conspecific sperm precedence in *Drosophila*. Nature 388:663–666.
- Price, C. S. C., C. H. Kim, J. Posluszny, and J. A. Coyne. 2000. Mechanisms of conspecific sperm precedence in *Drosophila*. Evolution 54:2028–2037.
- Price, C. S. C., C. H. Kim, C. J. Gronlund, and J. A. Coyne. 2001. Cryptic reproductive isolation in the *Drosophila simulans* clade. Evolution 55:81–92.
- Rieseberg, L. H., A. M. Desrochers, and S. J. Youn. 1995. Interspecific pollen competition as a reproductive barrier between sympatric species of *Helianthus* (Asteraceae). Am. J. Bot. 82:515–519.
- Shine, R., R. N. Reed, S. Shetty, M. Lemaster, and R. T. Mason. 2002. Reproductive isolating mechanisms between two sympatric sibling species of sea snakes. Evolution 56:1655–1662.
- Smith, R. L. 1984. Sperm competition and the evolution of animal mating systems. Academic Press, London.
- Turner, M. E. and W. W. Anderson. 1984. Sperm predominance among *Drosophila pseudoobscura* karyotypes. Evolution 38:983–995.
- Wade, M. J., H. Patterson, N. W. Chang, and N. A. Johnson. 1994. Postcopulatory, prezygotic isolation in flour beetles. Heredity 72:163–167.

Corresponding Editor: M. Noor