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MECHANISMS OF CONSPECIFIC SPERM PRECEDENCE IN *DROSOPHILA*

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Abstract.—The postmating, prezygotic isolating mechanism known as conspecific sperm precedence (CSP) may play an important role in speciation, and understanding the mechanism of CSP is important in reconstructing its evolution. When a *Drosophila simulans* female mates with both a *D. simulans* male and a *D. mauritiana* male, the vast majority of her progeny are fathered by *D. simulans,* regardless of the order of mating. The dearth of hybrid progeny does not result from inviability of eggs fertilized by heterospecific sperm or from the relative inviability of heterospecific larvae. Instead, CSP apparently results from a prefertilization obstacle to heterospecific sperm. We identified two independent barriers to heterospecific fertilization, sperm displacement and incapacitation, whose action depends on the order of mating. When a *D. simulans* female mates first with a conspecific male, the seminal fluid from this mating incapacitates heterospecific sperm transferred two days later. This sperm incapacitation occurs with no change in the retention of stored sperm over time, but does not occur when the conspecific mating lasts for only 5 min. When the order of matings is reversed, the seminal fluid from the second mating physically displaces heterospecific sperm from storage, even if the conspecific copulation lasts only 5 min. Conspecific sperm are not susceptible to displacement by a second conspecific copulation, but are susceptible to interference by heterospecific sperm if the conspecific copulation is interrupted after 12 min. Curing the *D. mauritiana* males of their infection with the endosymbiont *Wolbachia* had no effect on CSP. Sperm displacement and incapacitation involve the same basic mechanisms seen in second-male sperm precedence within species, supporting the hypothesis that CSP is an evolutionary by-product of adaptations affecting sperm competition within species.

Key words.—Conspecific sperm precedence, *Drosophila,* homogamy, multiple mating, reproductive isolation, seminal fluid, sperm competition, *Wolbachia.*

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Some hybridizations between closely related insect species show an unexpected phenomenon: a female who copulates with both a conspecific and a heterospecific male will produce mostly conspecific progeny, regardless of the order of her matings (Howard et al. 1998b). This phenomenon, known as conspecific sperm precedence (CSP), differs from the results of multiple *intraspecific* matings, in which the last male to mate typically sires most of the offspring (Simmons and Siva-Jothy 1998). CSP may well be widespread in animals, and might also evolve quickly between related taxa if it is a by-product of rapidly evolving mechanisms for sperm competition within species (Rice 1996). By forming a barrier to gene flow between sympatric groups having incomplete sexual isolation (Howard et al. 1998a,b). CSP may be a major contributor to speciation.

At present, however, we know virtually nothing about either the ubiquity or the mechanism of CSP. The phenomenon can be documented only through controlled mating experiments that include some method to distinguish between hybrid and pure-species offspring. CSP has so far been demonstrated in ladybird beetles (*Epilachna*: Nakano 1985; Katakura 1986b), grasshoppers (*Podisma* and *Chorthippus*: Hewitt et al. 1989; Bella et al. 1992), flour beetles (*Tribolium*: Wade et al. 1994), ground crickets (*Allonemobius:* Gregory and Howard 1994), and *Drosophila* (Price 1997). In many ways, CSP resembles conspecific sperm-egg recognition in marine invertebrates (Palumbi 1998) and interspecific pollen competition in plants (Arnold et al. 1993; Rieseberg et al. 1995), because the dearth of hybrid offspring results from the fertilization advantage of conspecific over heterospecific gametes. However, because the biology of reproduction differs so greatly among plants, animals with external fertilization, and animals with internal fertilization, the mechanisms of CSP almost certainly vary among groups.

In sea urchins of the genus *Echinometra,* for example, heterospecific fertilization fails after sperm penetrate the egg's jelly coat and must then interact with the egg's vitelline layer (Metz et al. 1994). In *Rhododendron,* overgrowth or undergrowth of heterospecific pollen tubes yields few hybrid embryos (Williams and Rouse 1990). In the ground crickets *Allonemobius fasciatus* and *A. socius,* heterospecific sperm are less motile than conspecific sperm in the sperm-storage organs of the female, which probably affects their access to unfertilized eggs (Gregory and Howard 1994). A detailed understanding of such disparate mechanisms is necessary if we are to understand the evolutionary forces underlying CSP.

Here we report the results of experiments on the mechanisms of CSP among three sibling species of *Drosophila.* We determined previously (Price 1997) that *D. simulans* females, after double matings to one *D. simulans* and one *D. mauritiana* male, produce on average more than 85% pure-species offspring, regardless of the order of the matings. Far fewer hybrids are produced by these double matings than after single heterospecific inseminations, so reproductive isolation is caused by *competition* between ejaculates from different species. Furthermore, in experiments in which the fertile *D. simulans* male was replaced with a spermless (XO) *D. simulans* male, the transfer of conspecific seminal fluid greatly reduces the number of *D. mauritiana* sperm used for fertilization (Price 1997), so that much of CSP does not require the presence of conspecific sperm.

Any observation of CSP could in principle be due to two phenomena. First, heterospecific males may suffer from noncompetitive disadvantages in fertilization that operate irrespective of whether the female has also copulated with a conspecific male. Such disadvantages may result from trans-

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fer of few sperm during copulation, inviability of heterospecific sperm in the female reproductive tract, problems with storage or retention of heterospecific sperm, or problems with interspecific sperm-egg recognition. For example, in matings between the ladybird beetles *Epilachna pustulosa* and *E. vigintioctomaculata,* heterospecific sperm are inactivated in storage, and few hybrid progeny result from either single heterospecific matings or double heterospecific/conspecific matings (Katakura 1986a, 1997; Katakura and Sobu 1986).

Second, heterospecific sperm may suffer from competitive disadvantages revealed only when the female mates with both conspecific and heterospecific males. For example, stored heterospecific sperm may be uniquely susceptible to displacement or inactivation by a later-mating conspecific (Price et al. 2001). Alternatively, later-mating heterospecific males may be unable to counter defenses that protect stored conspecific sperm. For example, copulation with a conspecific male may alter the female's reproductive tract, interfering with the subsequent migration or storage of heterospecific sperm. Such interference would reduce the number of hybrids compared to that resulting from single heterospecific matings.

These two categories of mechanism are not mutually exclusive, and CSP may cause reproductive isolation by a combination of noncompetitive and competitive disadvantages of heterospecific sperm. Several obstacles limit fertilization after single inseminations in the *D. simulans* clade, including failures of heterospecific sperm transfer, storage, and retention (Price et al., 2001).

Here we focus on reproductive barriers observed only when heterospecific sperm compete with conspecific sperm. The existence of such barriers implies that males are not well adapted for sperm competition within the reproductive tract of heterospecific females, and suggests that CSP may be an incidental by-product of evolutionary divergence between species in traits affecting sperm competition. This hypothesis predicts that CSP will involve the same basic mechanisms as does second-male sperm precedence observed *within* species.

To identify the attributes giving conspecific males a competitive advantage over heterospecific males, we manipulated the conspecific males in various ways (making them sterile, interrupting their copulations, or curing their infection by the endosymbiotic bacterium *Wolbachia*) and examining the effects on the production of hybrid offspring. The ideal way to reveal the mechanisms of CSP would be to compare the patterns of sperm storage and use after single heterospecific inseminations to patterns seen after double inseminations by a heterospecific and conspecific male. Unfortunately, there is at present no way to discriminate between heterospecific and conspecific sperm within the reproductive tract of a single *Drosophila* female. (However, this is possible using two different genotypes of *D. melanogaster*; Price et al. 1999.) Instead, we took advantage of our discovery that the conspecific seminal fluid is largely responsible for CSP (Price 1997). This enables us to examine the mechanism of CSP using fertile heterospecific males and sterile conspecific males that transfer seminal fluid but no sperm.

Materials and Methods

Drosophila Stocks

Flies were reared in uncrowded cultures at 24°C with a 12-h light-dark cycle on standard cornmeal/yeast/agar medium. All females used in this study were taken from the *D. simulans* Florida City (FC) stock, an isofemale line collected in 1985 in Florida City, Florida and maintained in large numbers (Coyne 1989). Fertile *D. simulans* males were taken from the FC stock, and spermless *D. simulans* males were produced by crossing virgin FC females to males from a *D. simulans* attached-X, attached-XY stock provided by T. Yamamoto (Price 1997). Male offspring from this cross lack a Y chromosome (henceforth XO) and produce normal seminal fluids but no sperm (Price 1997). *Drosophila mauritiana* males were taken from the synthetic (SYN) stock, made by combining six isofemale lines collected on Mauritius by O. Kitagawa in 1981 (Coyne 1989, 1993) and maintained in large cultures.

Polymerase chain reaction (PCR) amplification of *Wolbachia* gene sequences by K. Dyer in the laboratory of M. Turelli (see Turelli and Hoffmann 1995) showed that flies from the *D. mauritiana* SYN stock are infected with *Wolbachia,* whereas flies from the *D. simulans* FC stock are not. To eliminate infection and control for the effects of antibiotic treatment, flies from both stocks were raised on food medium containing 1.0 mg/ml tetracycline (Werren and Jaenike 1995). These treated flies were used to establish stocks that were reared on normal food medium for three generations before being used for experiments. Repeat PCR assays after treatment confirmed that these stocks were free of *Wolbachia.*

Matings and Progeny

Table 1 lists the matings performed and the abbreviations used in the text to identify each type of mating. For all mating experiments, males and females were collected as virgins under CO_2 anesthesia and stored in 8-dram food vials. Each female's first mating took place on the fourth day after eclosion, and her second mating, if any, occurred two days later. All males were four days old at the time of mating.

All copulations were observed and timed. Mating observations began within 1 h after lights came on in the incubators and lasted from 45 min to 5 h, depending on the rapidity of mating. Each mating trial included one female and one or two males transferred without anesthesia into a fresh food vial. Males were removed from the vial immediately after copulation ended. If females failed to mate on the first day, they were discarded. Of the females who did mate successfully, a random subset was never given the opportunity to remate, and these females were used as single-mating controls (see below). Females who refused to remate were discarded. Females failing to produce any offspring after mating were excluded from analysis. For experiments involving interrupted matings with *D. simulans* males, the mating pair was gently separated with a small brush at a specific time after copulation began.

Females were stored individually in food vials for the two days between the first and second matings. They were then transferred to a fresh vial for mating, and thereafter transferred individually to fresh vials every three days until either

TABLE 1. List of matings performed and abbreviations given in text. All second matings occurred two days after the first mating. Time in parentheses is the interval after which matings were manually interrupted.

Mating type	Female	First male	Second male
s	*D. simulans* FC	*D. simulans* FC	—
sm	*D. simulans* FC	*D. simulans* FC	*D. mauritiana*
Xm	*D. simulans* FC	*D. simulans* XO	*D. mauritiana*
s_{12}	*D. simulans* FC	*D. simulans* FC (12 min)	—
s_{12}m	*D. simulans* FC	*D. simulans* FC (12 min)	*D. mauritiana*
s_5	*D. simulans* FC	*D. simulans* FC (5 min)	—
s_5m	*D. simulans* FC	*D. simulans* FC (5 min)	*D. mauritiana*
sX	*D. simulans* FC	*D. simulans* FC	*D. simulans* XO
m	*D. simulans* FC	*D. mauritiana*	—
ms	*D. simulans* FC	*D. mauritiana*	*D. simulans* FC
mX	*D. simulans* FC	*D. mauritiana*	*D. simulans* XO
ms_{12}	*D. simulans* FC	*D. mauritiana*	*D. simulans* FC (12 min)
ms_5	*D. simulans* FC	*D. mauritiana*	*D. simulans* FC (5 min)

they stopped laying fertile eggs or were used for a separate experiment (see below). Progeny were reared to adulthood at 24°C with a 12-h light-dark cycle on standard medium. All progeny were counted, and male *D. simulans* progeny distinguished from male *simulans*/*mauritiana* hybrid progeny by the shape of the genital arch (Coyne 1983; Price 1997).

Egg Hatchability

A subset of the mated females (including those mated singly and doubly) was set aside for examination of egg hatchability. On a given day, corresponding to two days after a single mating or immediately after a double mating, females were transferred individually without anesthesia to vials containing small plastic spoons filled with grape-juice-tinted medium. The females were allowed to lay eggs on these spoons for 24 h, then transferred without anesthesia to fresh spoons for another 24-h egg-laying period. Females were then discarded. Spoons were stored at 24°C for 28 h after removal of the female, at which time hatched and unhatched eggs were counted using a dissecting microscope. Brown unhatched eggs, which may indicate zygotes that died early in development, were observed only rarely. Nevertheless, we did not determine whether the unhatched eggs we observed were unfertilized or had been fertilized but died early in development.

Larval Competition

Hybrid eggs were produced by mass matings of *D. simulans* FC females to *D. mauritiana* SYN males, and conspecific eggs by mass matings of *D. simulans* FC females and males. These eggs were placed into food vials at a total density of 50 eggs per vial, using three different ratios of egg types (25 *D. simulans* and 25 hybrids; 45 *D. simulans* and five hybrids; five *D. simulans* and 45 hybrids). Eggs from these vials were allowed to develop into adults, the males of which were identified as hybrids or *D. simulans* by the shape of the genital arch.

Number and Location of Stored Sperm

For each mating type, five to 10 females were dissected per day at timed intervals after the end of their last copulation. Females were etherized and their reproductive tracts removed in a drop of phosphate-buffered saline (PBS). The female's uterus and three sperm-storage organs (the paired mushroom-shaped spermathecae and the long, tubular seminal receptacle) were each transferred to a separate drop of PBS to prevent mixing of sperm from different organs. The sperm from each organ were then removed with insect pins. Slides were dried at 60°C for 5–10 min, fixed in 3:1 methanol and glacial acetic acid for 5 min, rinsed three times in PBS, and treated with 0.5 μg/ml DAPI (4, 6-diamidino-2-phenylindole) in glycerol, which labels sperm heads so that they glow bright blue under an epifluorescent microscope. All sperm heads in each of the three storage organs and the uterus of every female were counted using this microscope.

Dissections of Male Genitalia

To determine whether males who mate second directly remove stored first-male sperm during copulation, a subset of the *D. simulans* XO males used for matings of type mX (first male: *D. mauritiana*, second male: *D. simulans* XO) were etherized immediately after the end of copulation. Their external genitalia (including genital arches, lateral plates, claspers, and penis) were removed in a drop of PBS and then dried, fixed, and labeled with DAPI as described above. The entire preparation was examined for sperm under an epifluorescent microscope. Because the conspecific XO males produce no sperm, any sperm observed must be *D. mauritiana* sperm from the first mating.

Sperm Extrusion by Females

A subset of females of mating type mX was set aside to examine the possibility that the second mating caused the extrusion of first-male sperm. These females were transferred individually without anesthesia, either immediately or 24 h after mating, into boxes formed by assembling six 18-mm^2 coverslips into a cube. Each cube contained a small drop of moist yeast paste to provide a site for oviposition. The boxes were placed in an airtight container with a wet paper towel and stored at 24°C for 24 h. Females were then discarded, the boxes disassembled, and the internal surface of each coverslip coated with 0.5 μg/ml DAPI. The entire area of each

TABLE 2. Doubly mated females can be considered a random subset of all singly mated females. Two-tailed *t*-tests revealed no significant differences (ns) between singly and doubly mated females for either the duration of the first copulation or the number of progeny produced in the first two days after mating.

Mating type	*n*	Mean (SE) duration of first copulation		Mean (SE) progeny in first two days	
s	164	30.34 (0.39)		34.11 (2.10)	
sm	44	30.05 (0.94)	ns	32.25 (3.82)	ns
m	184	11.60 (0.21)		28.25 (2.26)	
ms	52	11.10 (0.26)	ns	23.23 (4.28)	ns
mX	122	11.28 (0.22)	ns	24.78 (5.92)	ns

coverslip, including the yeast and any eggs, was examined for sperm under an epifluorescent microscope.

RESULTS

Comparing Singly and Doubly Mated Females

To make valid comparisons between single and double matings, it is essential that doubly mated females represent a random subset of all singly mated females. If, for example, the only females who remate are those having few sperm in storage from their first mating (Gromko et al. 1984), comparing the number of first-male sperm or offspring after single and double matings could reflect this remating bias rather than any effects of the second mating itself.

For singly mated females (mating types s and m), the number of progeny produced during the first two days is strongly correlated with the number of progeny produced over the remainder of the female's lifetime ($n = 149$, $r = 0.72$, $P < 0.0001$). This correlation allows us to use the number of progeny produced during the first two days as an index of lifetime productivity, and thus to compare the sperm load of control females with those who remate (Gilchrist and Partridge 1997). Two-tailed *t*-tests revealed no significant differences between singly and doubly mated females for either the duration of the first copulation or the number of progeny produced in the first two days after the initial mating (Table 2). Combining probabilities from all three independent comparisons (Sokal and Rohlf 1995) also revealed no significant differences between singly and doubly mated females (combined $P > 0.5$ for both copulation duration and progeny production). Doubly mated females can therefore be treated as a random subset of females who mate once. Any differences between control and doubly mated females in sperm storage or progeny production can thus be attributed to the effects of the second mating.

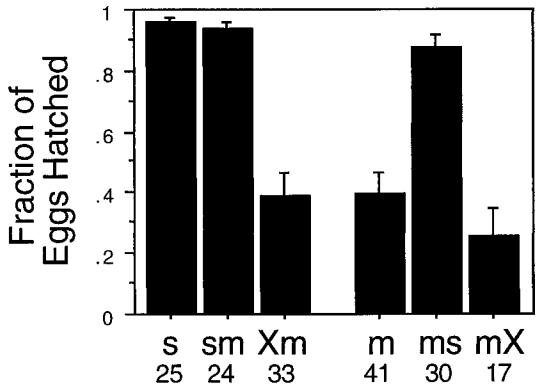

FIG. 1. Conspecific sperm precedence is not explained by low hybrid egg hatchability after double matings. Mean (+SE) fraction of eggs hatched after single and double matings of *Drosophila simulans* females. Hatchability was scored for all eggs laid two to three days after the first mating (0–48 h after the second mating, if any). Sample size (number of females) is given below each bar.

The Barrier to Hybridization Occurs before Fertilization

Figure 1 shows the hatchability of eggs laid two to three days after the only or first mating (in the latter case, this is 0–48 h after the second mating). There is no evidence of increased mortality among eggs laid by females mated to one heterospecific and one fully fertile conspecific male (sm vs. ms). Instead, egg hatchability is actually *higher* for mating types ms and sm than for mating type m. Thus, the reduced number of hybrid offspring in the former two crosses compared to the latter—the observed CSP—cannot be due to either failure of heterospecific sperm to fertilize or to successful fertilization but subsequently aborted development.

Females who received *D. simulans* sperm have a mean hatchability near or above 0.9 (mating types s, sm, and ms), whereas females receiving *D. mauritiana* but no *D. simulans* sperm have a mean hatchability below 0.5 (mating types Xm, m, and mX). In a separate group of females of mating type m, the mean hatchability of eggs laid during the 48 h immediately following mating was also less than 0.5 (data not shown; Price et al. 2001), and there is no significant drop in hatchability in the two days after a single heterospecific mating ($P > 0.3$, Mann-Whitney *U*-test). The low hatchability of *D. simulans* eggs after insemination by *D. mauritiana* males is probably a consequence of females releasing more eggs than they can fertilize with a small supply of *D. mauritiana* sperm, and is a distinct isolating mechanism not dependent on competition between con- and heterospecific inseminations (Price et al. 2001).

To determine whether the paucity of hybrid progeny after double matings could be attributed to a competitive inferiority of hybrids during development, we looked for differential mortality (over the period from egg through adult eclosion) under competitive rearing conditions similar to those found in broods produced by doubly mated females. Table 3 shows no significant differences between the frequencies of eggs placed into vials and the frequencies of adults eclosing from those vials ($P > 0.4$, chi-square tests). These results,

TABLE 3. Results of larval competition experiment. Number of eggs placed in vials and mean number (SE) of adult males eclosed from vials. Only males can be identified as hybrids versus *D. simulans* by the shape of the genital arch, so the total progeny size is roughly double the number of males.

	No. of eggs placed in vials			Adult males eclosed from vials			
n	*simulans*	hybrid	% *simulans*	*simulans*	hybrid	% *simulans*	X^2
15	25	25	50.0	8.40 (0.42)	8.33 (0.63)	50.9	0.004
5	45	5	90.0	16.60 (1.96)	1.40 (0.51)	91.9	0.494
5	5	45	10.0	2.00 (0.71)	14.80 (1.39)	10.5	0.338

in combination with the egg-hatchability data, indicate that *D. simulans* males interfere with *D. mauritiana* sperm at some time before those sperm fertilize *D. simulans* eggs.

Conspecific Sperm Precedence with and without Wolbachia

Infection with the endosymbiotic bacterium *Wolbachia* has been implicated in postzygotic isolation in *D. simulans* (Turelli and Hoffmann 1995). As noted above, flies from the *D. mauritiana* SYN stock are infected with the endosymbiont, whereas flies from the *D. simulans* FC stock are not. After treating both stocks with tetracycline, we repeated the matings used to detect CSP occurring in *D. simulans* females. We observed strong CSP using the tetracycline-treated stocks, with results virtually identical to those seen in nontreated stocks (Fig. 2). The competitive inferiority of *D. mauritiana* sperm within the reproductive tract of a *D. simulans* female cannot therefore be attributed to *Wolbachia* infection.

Sperm Displacement Versus Incapacitation

When a *D. simulans* female mates with both a *D. mauritiana* and a *D. simulans* male, she produces fewer hybrids than she would had she mated only once to a *D. mauritiana* male (Price 1997). The competitive disadvantage suffered by *D. mauritiana* males is greatest when the *D. simulans* male is fully fertile (Price 1997; Fig. 2), but is found also with double matings involving spermless conspecific males (Price 1997; Fig. 3A). Figure 3A shows that females of mating types Xm and mX produce on average only about one-third as many hybrids as m females. The reduction in the number of hybrid offspring caused by seminal fluid alone ranges from 40% to 80% of the effect of the normal ejaculate containing sperm and seminal fluid.

We compared the number of stored *D. mauritiana* sperm in m, mX, and Xm females to determine whether the decrease in hybrid progeny production after double matings could be caused by a decrease in sperm storage. Figure 3B shows the mean number of sperm stored in the entire reproductive tract of females 24–72 h after remating (and over the equivalent interval after the single mating). A visual comparison of Figures 3A and 3B reveals clearly that the nature of the conspecific male's interference with interspecific fertilization depends on mating order. If the *D. simulans* XO male mates first, hybrid formation is suppressed without any change in the number of heterospecific sperm initially stored: Females of mating type Xm have no fewer stored sperm than do females of mating type m (Fig. 3B; $P > 0.5$, one-tailed Mann-Whitney *U*-test). In contrast, if the *D. simulans* XO male

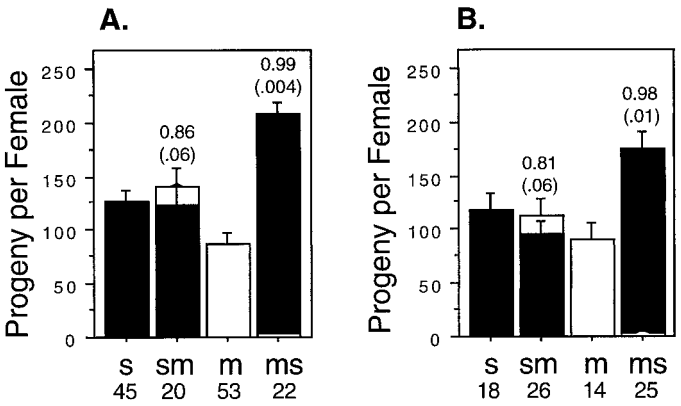

FIG. 2. Conspecific sperm precedence with and without *Wolbachia* infection. Mean (+SE) number of pure-species (solid) and hybrid (open) progeny per *Drosophila simulans* female after single and double matings. (A) Data reported in Price (1997). (B) Experiments repeated with tetracycline-treated stocks. Progeny produced within 48 h of the first (or only) mating are omitted. Sample size (number of females) is given below each bar. Fractions shown above the bars are mean (SE) proportion of pure-species progeny produced after remating.

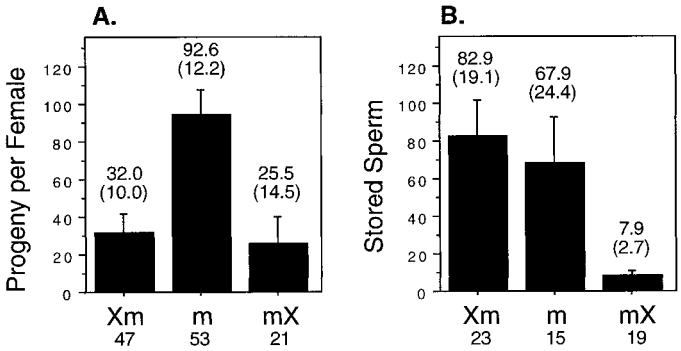

FIG. 3. The mechanism of conspecific sperm precedence depends on mating order. (A) Mean (+SE) number of progeny per *Drosophila simulans* female after single matings to *D. mauritiana* males (m) and double matings involving spermless *D. simulans* males (Xm and mX). Progeny produced within 48 h of the first (or only) mating are omitted. (B) Mean (+SE) number of sperm stored by females dissected 48–96 h after the first mating (24–72 h after the second mating, if any).

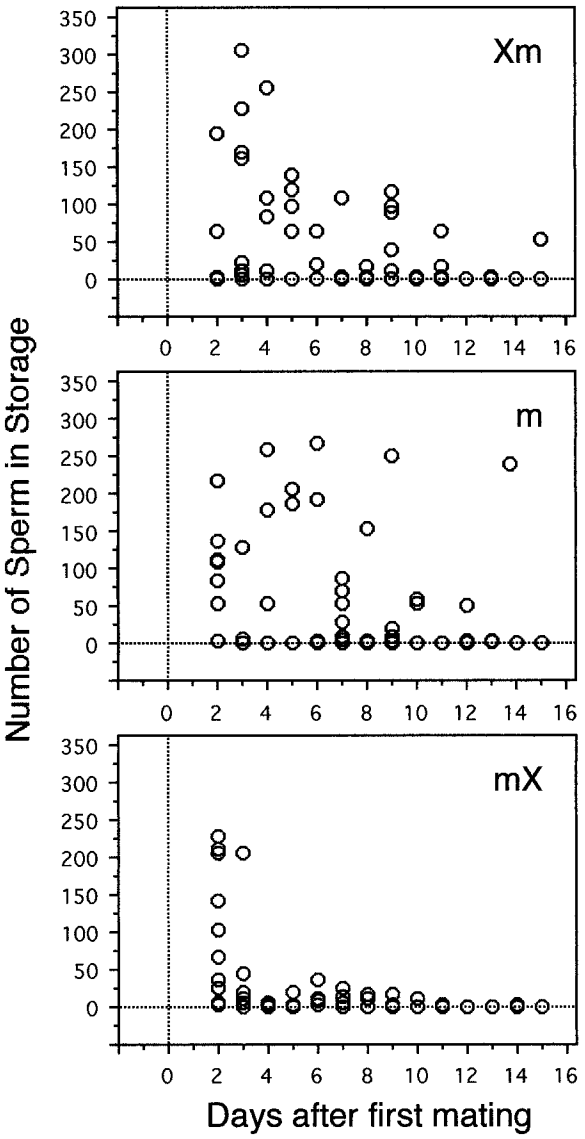

FIG. 4. The effect of sperm displacement and incapacitation on patterns of sperm storage over time. Number of sperm in the spermathecae and seminal receptacle combined, of females dissected on successive days after their second mating (and at the equivalent time for singly mated females). Five to 10 females were dissected each day.

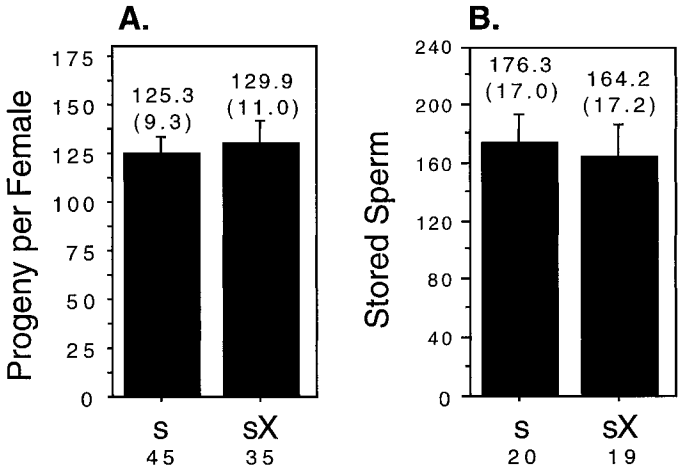

FIG. 5. Conspecific sperm are not susceptible to displacement by a later-mating spermless conspecific, with two days between matings. (A) Mean (+SE) number of progeny per *Drosophila simulans* female after single matings to *D. simulans* males (s) and double matings with a spermless *D. simulans* second male (sX). Progeny produced within 48 h of the first (or only) mating are omitted. (B) Mean (+SE) number of sperm stored by females dissected 48–96 h after the first mating (24–72 h after the second mating, if any).

mates second, previously stored heterospecific sperm are lost from storage: Females of mating type mX have significantly fewer stored sperm than do females of mating type m (Fig. 3B; $P < 0.005$, one-tailed Mann-Whitney *U*-test). In this latter cross, the reduction in number of hybrids is almost certainly due to the loss of heterospecific sperm.

Displacement of stored sperm apparently does not occur immediately after remating (Fig. 4), because mX females dissected 0–12 h after the second mating (day 2 in Fig. 4) have a mean of 102.8 (± 28.1 SE, $n = 10$) sperm in storage, compared to a mean of 101.9 (± 25.5, $n = 7$) in singly mated females. But mX females have consistently fewer stored sperm than do singly mated females from 24 h after remating (day 3 in Fig. 4) until they are depleted of stored sperm about 12 days later (Fig. 4; $P < 0.03$, one-tailed Kolmogorov-Smirnov test). In contrast, there is no significant difference between the Xm and m females in the distribution of sperm stored over time (Fig. 4; $P > 0.5$, one-tailed Kolmogorov-Smirnov test).

Heterospecific sperm are more susceptible to being displaced from storage than are conspecific sperm. *Drosophila simulans* females mated first to a *D. simulans* FC male and two days later to a *D. simulans* XO male (mating type sX) produce just as many offspring do as singly mated females (Fig. 5A; $P > 0.5$, one-tailed Mann-Whitney *U*-test), and they have no fewer sperm in storage than do singly mated females (Fig. 5B; $P > 0.5$, one-tailed Mann-Whitney *U*-test).

To estimate the efficiency of usage of stored sperm, we juxtaposed the ranked observations of sperm number 24–72 h after mating with the ranked observations of progeny produced after this time for females of mating types Xm, m, and mX (Fig. 6). The fewer sperm that females waste during fertilization, the more these distributions will overlap (Zimmering and Fowler 1968). There is indeed remarkable overlap of the distributions of sperm number and progeny number for both m and mX matings, suggesting that these females use nearly all of their stored sperm to produce progeny. Most of the mX females have very few stored sperm, but it appears that sperm not displaced from storage are used efficiently in fertilization. In contrast, a sizable fraction of the Xm females store many more sperm than are used to fertilize eggs, suggesting that heterospecific sperm are somehow incapacitated when their transfer is preceded by a conspecific mating.

Copulation Duration Determines the Seminal-Fluid Effect

To further examine the effect of a conspecific mating on a *D. simulans* female's use of heterospecific sperm, we interrupted conspecific copulations at two different intervals and examined the effect on the production of hybrid progeny.

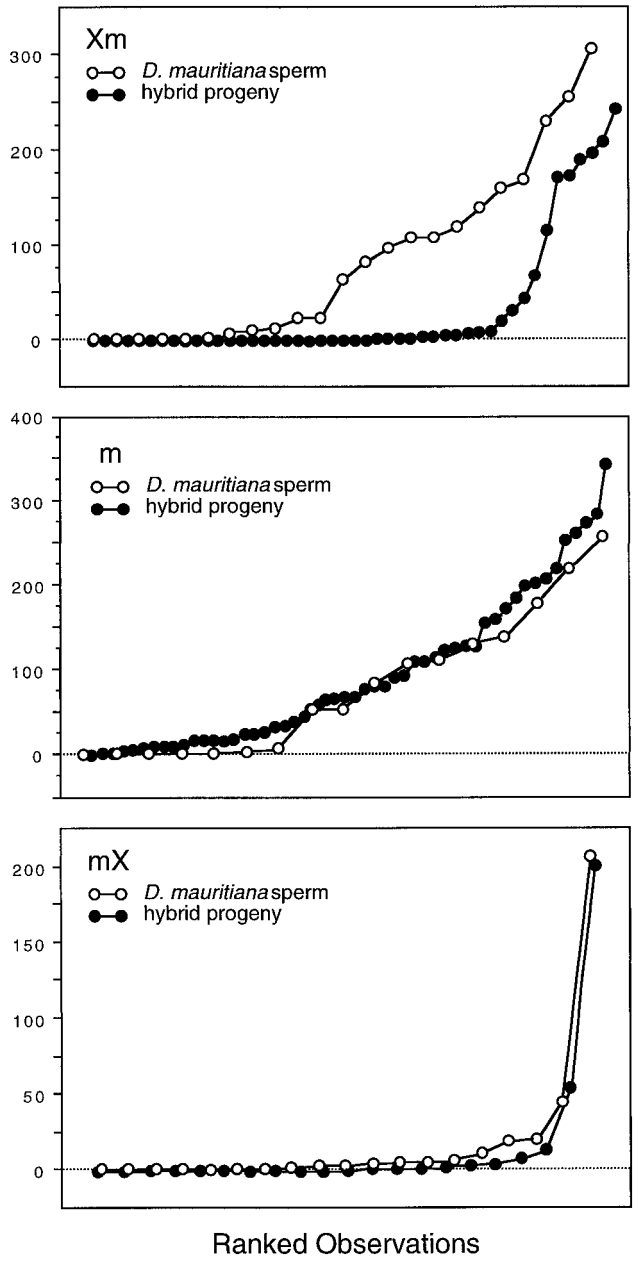

FIG. 6. Efficiency of heterospecific sperm use after single and double matings. Ranked distributions of total number of progeny produced after mating (solid circles) and number of sperm stored in the spermathecae and seminal receptacle 24–72 h after mating (open circles).

A preliminary study suggested that sperm transfer does not occur until *D. simulans* females and males have copulated for at least 10 min, and that transfer is usually complete by 14 min (Fig. 7). After 14 min, longer copulations do not result in more progeny (regression of progeny on copulation duration after 14 min: $n = 51$, $r = 0.11$, $P > 0.4$). However, the average conspecific copulation lasts about 30 min. It is thus possible that males continue to transfer either excess sperm or nonsperm components of the seminal fluid during the second half of copulation, perhaps including substances

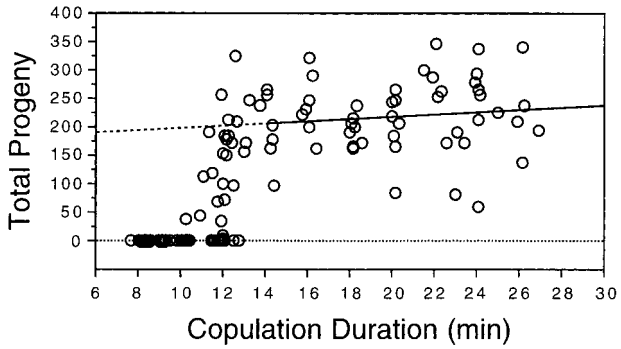

FIG. 7. Regression of progeny production on copulation duration for artificially interrupted copulations of *Drosophila simulans* females with *D. simulans* males. Linear regression line is calculated only for copulations longer than 14.0 min ($y = 179.6 + 1.9x$).

that influence sperm competition (see review in Wolfner 1997).

The component of *D. simulans* seminal fluid that incapacitates subsequently stored *D. mauritiana* sperm is not transferred during the first 5 min of copulation. Females for whom a 5-min conspecific mating was followed by a heterospecific mating produce just as many hybrids as singly mated females (Fig. 8A; s_5m compared to m; $P > 0.4$, one-tailed Mann-Whitney *U*-test). There is, however, a moderate but significant reduction in the number of hybrids produced if the heterospecific insemination is *followed* by a 5-min conspecific mating (Fig. 8A; ms_5 compared to m; $P < 0.01$, one-tailed Mann-Whitney *U*-test). Therefore, early in copulation, second-mating males may transfer some substance that causes physical displacement of stored heterospecific sperm.

To determine whether a conspecific copulation that lasts only as long as the average heterospecific copulation can still produce CSP, matings between *D. simulans* females and *D. simulans* males were interrupted after 12 min. Heterospecific matings that are long enough to result in sperm transfer last on average 11.60 ± 0.21 min (Table 2, mating type m). The data from Figure 7 suggest that conspecific copulations interrupted at 12 min are likely to produce a wide range of outcomes, leaving some females with no sperm, some with intermediate numbers of sperm, and some with a full sperm load. The s_{12} females produce a mean of 126 (± 22.3, $n = 14$) progeny, and the m females produce a mean of 93 (± 12.2, $n = 53$) progeny.

CSP breaks down when the conspecific mating lasts only 12 min (Fig. 8B). A closer examination of the data reveals that s_{12}m and ms_{12} females typically produce either pure-species progeny or hybrid progeny, but not both (Fig. 8C). Even females who produce just a few *D. simulans* progeny also produce very few hybrids. One interpretation of this finding is that even a small number of *D. simulans* sperm suffices to inhibit the formation of hybrids. We also find, however, that those females producing more than about 40 hybrids also rarely produce *D. simulans* progeny. In fact, double copulations in this trial produce fewer *D. simulans* progeny than do single copulations (Fig. 8B; $P < 0.001$, Mann-Whitney *U*-tests). This implies that, under these conditions, *D. simulans* sperm are susceptible to interference by *D. mauritiana* sperm in a *D. simulans* female. During the

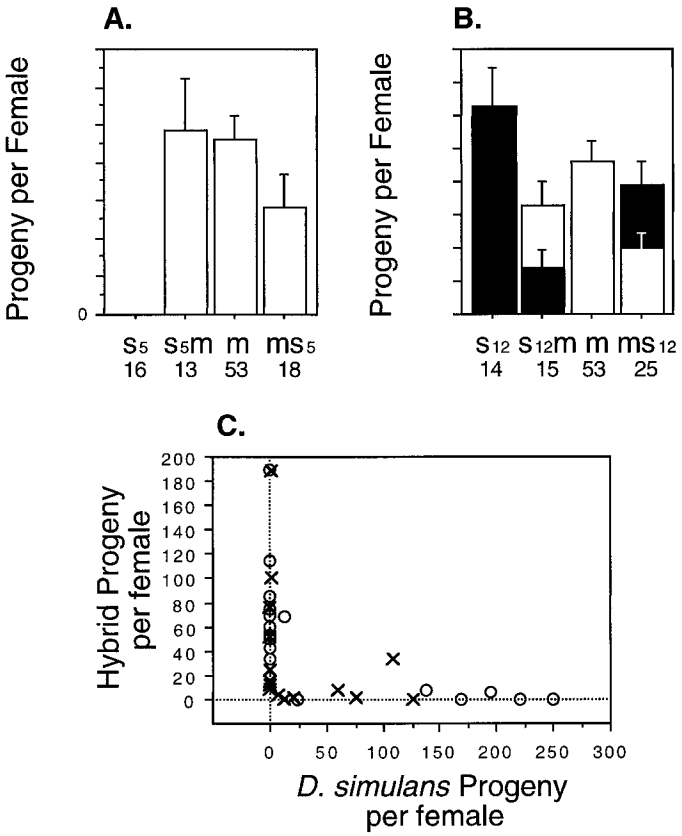

FIG. 8. Conspecific sperm precedence breaks down with interrupted conspecific copulations. Mean (+SE) number of pure-species (solid) and hybrid (open) progeny per *Drosophila simulans* female after single and double matings. (A) Conspecific copulations were interrupted after 5 min. (B) Conspecific copulations were interrupted after 12 min. (C) Relationship between number of hybrid progeny per female and number of pure-species progeny per female after $s_{12}m$ (X symbols) and ms_{12} matings (O symbols).

second half of normal, uninterrupted copulations, *D. simulans* males seem to transfer something that protects their sperm from such interference.

What Happens to the Displaced Sperm?

The observation that *D. mauritiana* sperm are lost from storage after a second mating to a *D. simulans* XO male (Fig. 3B) raises the question of what happens to the missing sperm. We dissected the genitalia of 10 *D. simulans* XO males immediately after mX matings to look for *D. mauritiana* sperm adhering to the penis, genital arches, lateral plates, or claspers. Eight of 10 dissected males had no visible sperm, and the remaining two each had fewer than 10 sperm on their external genitalia. These males apparently do not remove stored sperm during copulation, as expected because the male genitalia of these species show no obvious morphological adaptation for such a strategy.

To determine whether heterospecific sperm are expelled by mX females, we placed these females in boxes made of coverslips either immediately or 24 h after mating. If females release first-male sperm within 48 h of remating, this sperm should be found somewhere on the internal surface of the box. Four of six boxes had a few sperm, but none had more than 20, and all sperm were found on the surface of a laid egg. This finding is consistent with normal waste of sperm during early oviposition (Gilbert 1981), and it cannot explain the large loss of sperm shown in Figure 3B. Unfortunately, for now we do not know what happens to heterospecific sperm displaced from storage.

DISCUSSION

When a *D. simulans* female mates with just one *D. mauritiana* male, she produces a substantial number of hybrids. These females store a smaller fraction of transferred *D. mauritiana* sperm than of conspecific sperm, but those *D. mauritiana* sperm that *are* stored remain viable and are used efficiently for fertilization (Price et al. 2001). *Drosophila mauritiana* males encounter a major obstacle to heterospecific fertilization only when the *D. simulans* female also mates with a *D. simulans* male. Egg hatchability is high after such double matings (Fig. 1), and hybrid progeny do not suffer in competition with *D. simulans* progeny (Table 3). The main postmating, prezygotic impediment to gene flow between the species thus occurs when a *D. mauritiana* sperm must *compete* with a *D. simulans* ejaculate to fertilize a *D. simulans* egg. Seminal fluid from *D. simulans* males interferes in at least two independent ways with *D. mauritiana* sperm stored within a *D. simulans* female:

Incapacitation.—If the *D. simulans* male mates first, some component of his seminal fluid appears to inhibit the effectiveness of *D. mauritiana* sperm that are transferred and stored two days later (Fig. 3A, 6). This incapacitation occurs without any reduction in the number of *D. mauritiana* sperm stored (Fig. 3B) and without any effect on their retention in storage over time (Fig. 4). Furthermore, the component of seminal fluid that incapacitates subsequently stored sperm is transferred some time after the first 5 min of copulation (Fig. 8A).

Displacement.—If the *D. simulans* male mates after the *D. mauritiana* male, some component or components of his seminal fluid can physically displace *D. mauritiana* sperm from storage (Fig. 3B). At least some of these substances are transferred during the first 5 min of copulation, before sperm are conveyed to the female (Fig. 8A). *Drosophila simulans* males themselves must have a way to protect their own sperm from such an effect, because males are unable to displace conspecific sperm under the same conditions (Fig. 5). This observation somewhat resembles that seen in second-male sperm precedence in *D. melanogaster*, in which first-male sperm become susceptible to incapacitation by a later-mating male only after that sperm has been in storage for several days (Price et al. 1999).

We were unable to determine what happens to heterospecific sperm displaced by the seminal fluid of a later-mating conspecific male (mX matings). Examination of *D. simulans* male genitalia after mating showed no direct removal of previously stored sperm, in contrast to observations in some insect species (Waage 1979; Ono et al. 1989; Siva-Jothy and Tsubaki 1989; Helversen and Helversen 1991). We also found no evidence that the female expels first-male sperm after the end of the second copulation, a phenonemon seen

in rove beetles (Gack and Peschke 1994). Perhaps *D. simulans* females normally extrude *D. mauritiana* sperm when living on food medium, but fail to do so when ovipositing on small clumps of yeast within our glass chamber. It is also possible that, instead of being physically displaced from the storage organs, *D. mauritiana* sperm disintegrate after a conspecific copulation.

All of these conclusions about incapacitation and displacement of heterospecific sperm are based on experiments that either use spermless (XO) *D. simulans* males or that interrupt copulations with *D. simulans* males before sperm transfer. We demonstrated previously that, although the nonsperm components of the *D. simulans* seminal fluid have a dramatic effect on *D. mauritiana* sperm, this effect is not sufficient to completely explain the degree of CSP seen in two normal inseminations (Price 1997). Until it is possible to distinguish heterospecific from conspecific sperm stored within the reproductive tract of a doubly inseminated female, we will not know exactly how conspecific sperm themselves influence the storage and use of heterospecific sperm. In double conspecific fertilizations of *D. melanogaster* females, males who transfer sperm can physically displace and/or incapacitate previously stored sperm, whereas spermless second males can only incapacitate stored sperm without removing them (Price et al. 1999). Similarly, fertile *D. simulans* males may have methods of interfering with *D. mauritiana* sperm that are not available to spermless males—methods that we were unable to identify.

By interrupting *D. simulans* copulations after 12 min, before sperm transfer was complete, we found that even a few *D. simulans* sperm may prevent the production of hybrids (Fig. 8C). However, these interrupted matings also revealed that *D. simulans* sperm themselves can be susceptible to some form of interference by *D. mauritiana* males, a phenomenon not evident after conspecific copulations of normal length (Fig. 8B). This implies that, during the second half of copulation, *D. simulans* males may normally transfer a substance that protects their sperm from the negative effects of competition with another male. Because *D. mauritiana* sperm *do* outcompete previously stored *D. simulans* sperm for fertilizations within a *D. mauritiana* female (Price 1997), *D. mauritiana* males probably also have a mechanism to protect their sperm from competitive interference. But our results imply that this mechanism depends on the nature of the female reproductive tract, because *D. mauritiana* sperm do not enjoy such protection in the storage organs of a *D. simulans* female.

We have identified two independent mechanisms of CSP: displacement and incapacitation of heterospecific sperm. These are the same two mechanisms recently implicated in conspecific second-male sperm precedence in *D. melanogaster* (Price et al. 1999). Nevertheless, there are at least four differences between the details of CSP and conspecific second-male precedence. First, in conspecific double matings the second male always wins, whereas in CSP matings the conspecific male always wins, regardless of the order of mating. Second, males do not reduce the number of progeny of subsequently mating males of the same species, but severely reduce the number of hybrids if the second male is heterospecific. In this case, the first-mating males apparently incapacitate subsequently transferred *D. mauritiana* sperm (Fig. 3). Third, in CSP, conspecific seminal fluid lacking sperm reduces the number of hybrids when the second mating occurs within two days of the first (Price 1997; Fig. 3A). In double conspecific matings, on the other hand, seminal fluid reduces the number of first-male offspring only if sperm have been stored for a longer period of at least 7 days (Price et al. 1999). Finally, in conspecific matings, spermless males incapacitate but do not displace stored sperm (Price et al. 1999); in CSP matings, however, spermless males displace stored heterospecific sperm (Fig. 3B).

Our analysis suggests, then, that sperm competition within and between species involve the same basic phenomena (incapacitation and displacement), but that these phenomena operate in different ways and to different degrees in the two types of matings. In particular, heterospecific sperm competition seems to be an exaggerated version of phenomena involved in conspecific competition. This observation supports the hypothesis that CSP has evolved as a by-product of cryptic sexual selection within *D. simulans* and *D. mauritiana.* Such a scenario may also account for the heterospecific exaggeration of the ''insemination reaction'' in *Drosophila,* another phenomenon causing postmating isolation. As described by Dobzhansky (1951, p. 191):

> ''Some species of *Drosophila* show the so-called insemination reaction following copulation (Patterson 1946, 1947). A rapid secretion of a fluid into the cavity of the vagina takes place immediately after the copulation, causing a great swelling of the organ. This swelling persists for some hours after intraspecific copulations, whereupon the vagina returns to its normal condition. Insemination by a male of a foreign species gives a more violent reaction. The vagina remains swollen for days, and sometimes the secretion inside the vagina solidifies and obstructs the passage of eggs, making the female sterile.''

Such forms of postmating, prezygotic isolation are likely to be much more common than we realize. Their study may allow us to determine how normal evolution within species leads to abnormalities in crosses between species.

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