

SPERM UTILIZATION IN ONCE- AND TWICE-MATED *DROSOPHILA PSEUDOOBSCURA* FEMALES

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Mating behavior in the fruit fly, *Drosophila*, has been studied extensively (Ehrman and Parsons 1976). It is known, for example, that females of many species in this genus copulate repeatedly both in the laboratory (Pruzan 1976; Richmond and Ehrman 1974; Fuerst et al. 1973; Bundgaard and Christiansen 1972) and in the field (Cobbs 1977; Stalker 1976; Milkman and Zeitler 1974; Anderson 1974). Information on the adaptive significance of rematings in females and on the utilization of sperm from successive ejaculates has come primarily from studies of *Drosophila melanogaster*.

Anderson (1974) suggested that repeated matings in *Drosophila* may replenish the female's store of sperm, resulting in higher fecundity. A single mating in *D. euronotus* is insufficient to fill a female's sperm storage organs (Stalker 1976), and both *D. pachea* and *D. mojavensis* must remate frequently in order to maintain sperm viability (W. B. Heed, personal communication). In these cases remating has obvious adaptive significance. This is not so for *D. melanogaster*, in which females receive sufficient sperm in one mating to fertilize their lifetime supply of ova (Lefevre and Jonsson 1962). With one exception (Pyle and Gromko 1978), to be discussed later, studies in this species reported no increase in productivity resulting from remating (see Fowler [1973] review).

Sperm competition, a term coined by Parker (1970) to denote the competition between sperm from successive ejaculates, can only occur if the female remates before her initial sperm supply is exhausted. In experiments by Manning (1962, 1967), *D. melanogaster* females normally remained unreceptive for 8–10 days after copulation. Return of receptivity was correlated with depletion of sperm supply. Gromko and Pyle (1978) reported a high degree of remating when most but not all of the stored supply of sperm had been utilized. Other studies have found considerable remating 48 h after the first mating when most of the sperm from the first mating was still present (Prout and Bundgaard 1977; Fuerst et al. 1973; Lefevre and Jonsson 1962). The discrepancy is probably the result of methodological differences (see Gromko and Pyle [1978] discussion).

One method of studying the fate of sperm in multiply inseminated females is to examine the genotypes of offspring following copulation with genetically different

types of males. From data obtained on *D. melanogaster* using this method, Nonidez (1920), Meyer and Meyer (1961), and Gromko and Pyle (1978) suggested that sperm from the second male mixed randomly with any residue of sperm remaining after the first mating—i.e., sperm mixing occurred. Dubinin (1928) and Lefevre and Jonsson (1962), on the other hand, found that newly deposited sperm eliminated those that had been previously stored—i.e., sperm displacement occurred. Kaufmann and Demerec (1942) attributed differences in frequency of the classes of offspring produced following multiple matings to differential viability of the developing embryos. Gugler et al. (1965) mated *D. melanogaster* females first to untreated males and second to males whose sperm had been radioactively labeled. They found that 20 min after remating, over half of the sperm in the ventral receptacle and the spermathecae were from the second male.

The adaptive significance of remating in *D. pseudoobscura* has not been investigated previously. It is known, however, that females of this species mate repeatedly (Levine et al., 1980; Pruzan 1976). When virgin females are presented with a choice of males of different genotypes, they often show a bias toward a particular type of male. On second mating after an initial copulation with the nonpreferred type, females show a bias toward copulating with males of the same genotype as their first mate (Pruzan et al. 1979; O'Hara et al. 1976; Pruzan 1976; Pruzan and Ehrman 1974).

This study investigated the following questions in *D. pseudoobscura*. (1) Do females remate before their sperm supply is exhausted? (2) Is there a difference in the number of progeny produced by singly mated versus remated females? (3) Is there a difference in the number of progeny resulting from double matings with males of the same genotype versus rematings with genetically different males? (4) What are the characteristics of sperm utilization with regard to sperm mixing versus sperm displacement?

METHOD

Two naturally occurring karyotypes of *Drosophila pseudoobscura*, Arrowhead (*AR*) and Standard, homozygous for autosomal recessive marker (*or*), were used. The flies were maintained in the laboratory in half-pint bottles containing food (formula 4-24, Carolina Biological Supply Co.) and fresh yeast. New cultures were initiated by serial transfer. Virgin flies were obtained by clearing bottles of all adults and separating by sex the newly emerged flies within 12 h. Flies were maintained and tested at 20° C, 60% RH (relative humidity) and 12:12 day-night cycle.

In order to distinguish between progeny of first and second matings, *ST;or/or* females bearing the visible marker orange eyes were used throughout. Because the *or* allele is recessive, progeny of *ST;or/or* females × *ST;or/or* males have orange eyes, whereas progeny of *ST;or/or* females × *AR;+/+* males, the latter homozygous for the wild allele of *or*, have normal, brick-red eyes.

Initial matings took place in an 8-dram (40-ml) vial containing food. One 3-day-old virgin *ST;or/or* female was placed together with two 3-day-old males and observed for 30 min. One group of females was given *ST;or/or* males and the other

TABLE 1
 PROCEDURE FOR OBTAINING ONCE- AND TWICE-MATED
Drosophila pseudoobscura FEMALES

FEMALE GENOTYPE	MALE GENOTYPE	
	First Mate	Second Mate
<i>ST;or/or</i>	<i>ST;or/or</i>	...
	<i>AR;+/+</i>	...
	<i>ST;or/or</i>	<i>ST;or/or</i>
	<i>ST;or/or</i>	<i>AR;+/+</i>
	<i>AR;+/+</i>	<i>ST;or/or</i>
	<i>AR;+/+</i>	<i>AR;+/+</i>

group was given *AR;+/+* males. No rematings were observed during this time. After 30 min all males were removed and the mated females were placed individually in vials containing medium and yeast for 3 days. Nonmated females were discarded.

Three days after the initial matings, all females were transferred to fresh food vials. A portion of the mated females were given the opportunity to remate through exposure to two 3-day-old males for 1 h. One-half of the females were placed with males of the same genotype as their first mate, while the other half were placed with males of the alternative genotype. This procedure resulted in six groups of mated females (table 1).

Females which refused to remate were given an additional opportunity to remate on day 6 after the original mating. All females were transferred to fresh food vials every 3 days for a total of seven transfers. Females were discarded 6 days after the seventh transfer.

Egg-containing vials were labeled and stored until the offspring eclosed. The progeny were then collected and counted daily. Paternity was assessed by eye color. Vials were discarded when no adults appeared for three consecutive days.

RESULTS

The mean number of progeny per female for once-mated (2 groups) and twice-mated (4 groups) *Drosophila pseudoobscura* *ST;or/or* are presented in table 1. Seventy-two percent of the females given an opportunity to remate did so within 6 days (transfer 3) after the initial mating. Forty-five percent of these females remated 3 days after their first mating (transfer 2), and 47% of the remaining females remated 3 days later (transfer 3). Females which did not survive through the seventh transfer were excluded from data analysis.

The number of progeny per transfer had significant heterogeneity of variance when transfer 1 was included, despite log transformation of the data (Bartlett's test, $M = 54.0$, $P < .05$). Further analyses excluded transfer 1, used untransformed data, and there was no heterogeneity of variance (Bartlett's test, $M = 17.8$, $P > .05$).

TABLE 2

MEAN NUMBER OF PROGENY PER *Drosophila pseudoobscura* FEMALE (\pm SD)

GROUP		TRANSFERS						
M_1	M_2	1	2	3	4	5	6	7
<i>ST;orlor</i>	...	41.56 (15.49)	55.44 (20.35)	59.78 (17.86)	45.78 (11.48)	50.22 (15.13)	33.11 (18.94)	28.33 (27.46)
<i>AR;+/+</i>	...	42.77 (14.14)	63.62 (17.77)	70.08 (29.09)	61.69 (28.54)	53.00 (22.98)	43.00 (23.81)	42.54 (20.67)
<i>ST;orlor</i>	<i>ST;orlor</i>	35.43 (55.60)	48.43 (24.31)	59.00 (20.52)	76.14 (26.66)	60.86 (18.22)	30.71 (30.49)	34.29 (22.19)
<i>ST;orlor</i>	<i>AR;+/+</i>	40.57 (36.95)	54.86 (22.18)	67.43 (20.76)	80.00 (33.97)	84.14 (33.66)	52.71 (28.54)	48.71 (33.66)
<i>AR;+/+</i>	<i>AR;+/+</i>	26.43 (36.47)	55.43 (25.19)	63.86 (25.24)	88.14 (44.69)	63.43 (32.18)	41.29 (29.08)	38.14 (35.64)
<i>AR;+/+</i>	<i>ST;orlor</i>	50.29 (38.24)	53.71 (26.37)	57.00 (29.94)	69.86 (32.65)	64.71 (16.52)	51.86 (31.97)	39.14 (39.70)

NOTE.—Females were transferred every 3 days in transfers 1–6 and after 6 days in transfer 7. M_1 and M_2 represent the genotypes of the first and second male mates, respectively.

Differences in mean number of progeny between once- and twice-mated females for transfers 2–7 were analyzed using 2×6 ANOVA (table 2). Twice-mated females had significantly more offspring per transfer ($\bar{X} = 57.6$) than once-mated females ($\bar{X} = 51.4$; $F = 4.19$, $P < .05$). Duncan's multiple comparison test for group X transfer interaction showed significant differences for transfers 4 and 5 ($P < .01$ and $P < .05$, respectively), where twice-mated females had more offspring than once-mated females (figure 1). All other transfer-by-transfer comparisons between once- and twice-mated females were not significant. The num-

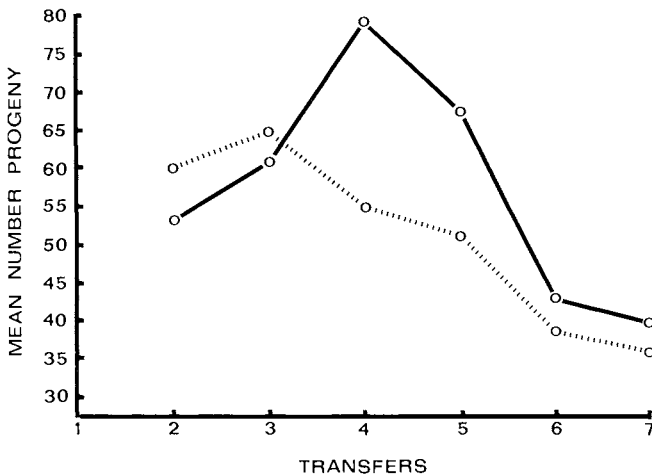


FIG. 1.—Mean number of progeny per transfers 2–7 produced by once- and twice-mated *Drosophila pseudoobscura* females. Dashed line = once-mated females; solid line = twice-mated females.

TABLE 3

ANALYSIS OF VARIANCE OF MEAN PROGENY PER TRANSFERS 2-7 OF ONCE-VERSUS TWICE-MATED *Drosophila pseudoobscura* FEMALES

Source	SS	df	F	P
Group (A)	2826.33	1	4.19	.04
Transfer (B)	36484.84	5	10.81	.0001
A × B	8518.87	5	2.52	.03

ber of offspring in both groups decreased significantly at transfer 6, i.e., 15 days after the initial mating.

A 4×6 ANOVA was used to test the difference in mean number of progeny per transfer among the four groups of twice-mated females. Table 3 shows that there were no differences among these groups of twice-mated females ($F = 1.45$, $P > .05$). Duncan's multiple comparison test on the transfer effect showed that a significant decrease in progeny occurred at day 15 (transfer 6) after the initial mating.

Sperm competition was determined in two groups of remated females: those which mated initially with *ST;or/or* then with *AR;+/+* males and those which mated initially with *AR;+/+* then with *ST;or/or* males. The number and paternity of offspring from each successive transfer are presented for representative individual females in figures 2 and 3. A heterogeneity of variance test (Sokal and Rohlf

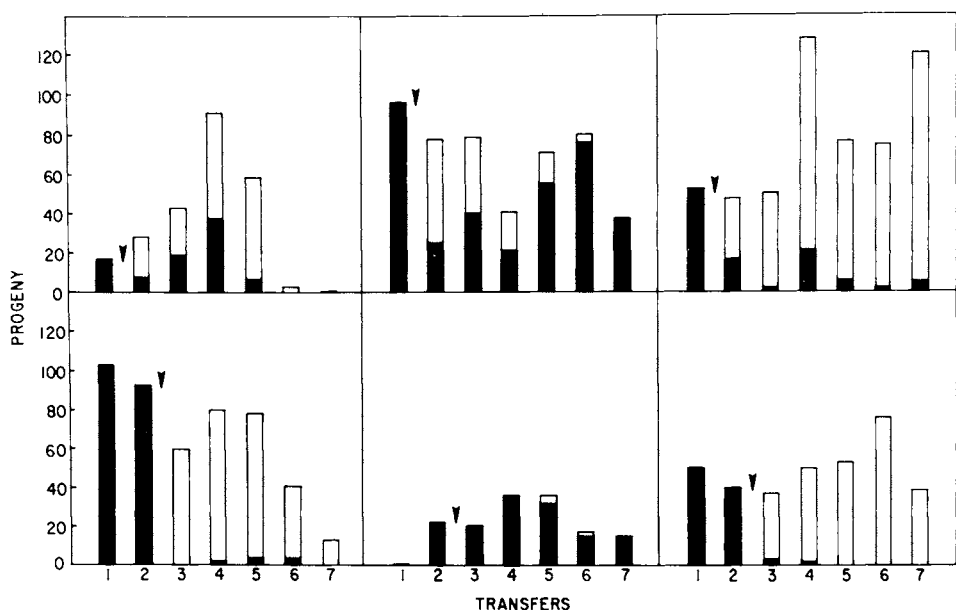


FIG. 2.—Sperm utilization in individual remated *Drosophila pseudoobscura* females. Females mated first with *AR;+/+* males and remated with *ST;or/or* males. Dark bars = progeny of the first male; light bars = progeny of the second male; arrow = day of remating.

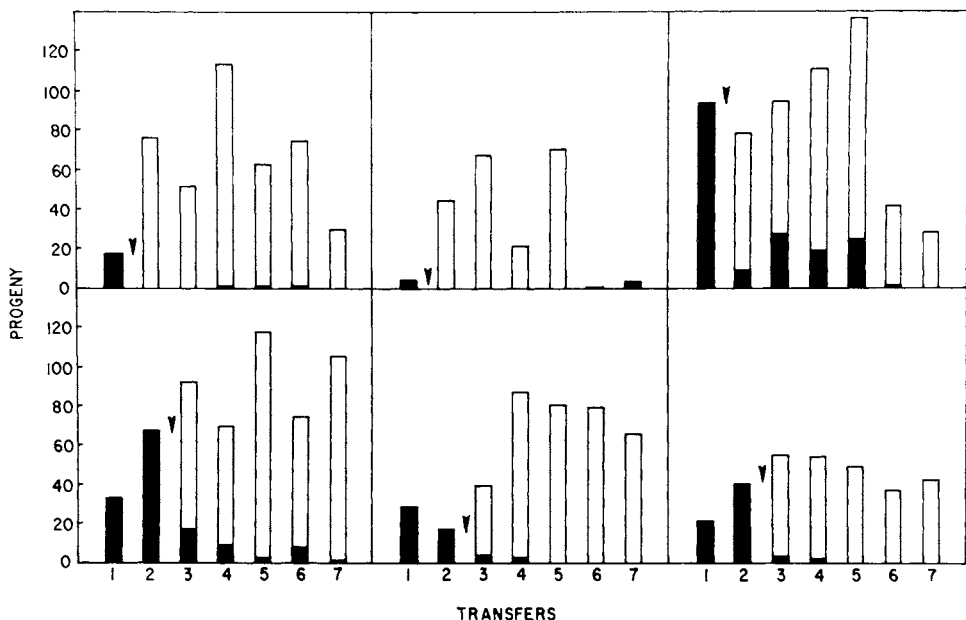


FIG. 3.—Sperm utilization in individual remated *Drosophila pseudoobscura* females. Females mated first with *ST; or/or* males and remated with *AR; +/+* males. Dark bars = progeny of the first male; light bars = progeny of the second male; arrow = day of remating.

1969) for sperm displacement ability of the two male karyotypes, measured as the proportion of offspring fathered by the second male, was statistically significant ($F = 6.71$, $P < .01$). The orange-eyed males were more variable than the *AR; +/+* males in their ability to displace the sperm of the prior male. The mean displacement by *ST; or/or* males (proportion = .68) was not significantly different from the mean displacement by *AR; +/+* males (proportion = .90; Sokal and Rohlf 1969, p. 374).

DISCUSSION

Multiple matings in *Drosophila pseudoobscura* were observed both in the laboratory and in the field. Initial field estimates of multiply inseminated females ranged from 2% (Crumpacker et al. 1974) to 55% (Cobbs 1977). Our laboratory result of 72% remating suggest that these field estimates may be low. Multiple mating can be distinguished from a single mating only if the males are of different genotypes with respect to the markers assayed. When this factor was taken into account the Cobbs (1977) estimate of multiple paternity increased to 79%. Recently Levine et al. (1980) estimated that 92% of *D. pseudoobscura* females collected in the Central Highlands of Mexico mated more than once.

Drosophila females studied here remated before their sperm supply was exhausted. As can be seen in figure 1, only a small proportion (about 10%) of the stored sperm was utilized in fertilizing the female's eggs by the time of the first opportunity to remate. It is possible that these females remate without regard to

the state of fullness of their sperm storage organs. The proportion of females remating at transfers 2 and 3 were approximately the same (45% and 47%, respectively). If remating were dependent on feedback from sperm storage organs, a higher percentage of remating would be expected at transfer 3, where approximately 30% of the sperm was utilized.

The mean number of offspring produced by remated females exceeded that of once-mated females (fig. 1 and table 3). The increased fitness of females may explain in part the biological significance of the repeated copulations reported in *D. pseudoobscura* (Levine et al., 1980). Recently Pyle and Gromko (1978) obtained similar results in *D. melanogaster*. Despite similarities in procedure, they found that most of the females of this species remated when their sperm supply was nearly exhausted. Remating sustained a high level of productivity longer than that found in single-mated females. Early rematings in our study stimulated females to produce progeny more rapidly without prolonging their productivity (fig. 1). Stone and Patterson (1954) reported similar results in species of the Virilis group. Rematings with conspecific males stimulated the females to lay eggs more rapidly.

When a female remates before her sperm supply is exhausted, sperm competition may prevail (Parker 1970). Evolution would favor males which, when mating with already mated females, displace as much as possible of the previously stored sperm. Evidence for sperm displacement in *D. melanogaster* abounds (Lefevre and Jonsson 1962; Minamori and Fukui 1970; Prout and Bundgaard 1977). In *D. pseudoobscura*, however, Dobzhansky and Pavlovsky (1967) reported sperm mixing, i.e., progeny of multiply mated females reflected the paternal karyotypes in approximately equal proportions. Our study showed sperm displacement. Both *ST;or/or* and *AR;+/+* males displaced the sperm of the first male, although the former were more variable than the latter. Numerous differences in procedure exist between the Dobzhansky and Pavlovsky (1967) study and the one reported here. Most notably, they placed the previously mated females into mass cultures with the new males for a period of 5 days. The females were then allowed to oviposit in individual vials for 5 days. Paternity was determined on the basis of chromosomal analysis of 10 randomly chosen larvae per remated female for the latter 5-day period only. Our results are based on classification of every offspring of each twice-mated female during the 24-day study.

Drosophila pseudoobscura ST;or/or males have lower fitness than wild-type males of this species. Results of female-choice experiments using young virgin females with *ST;or/or* and *AR* or *ST;or/or* and *CH* (Chiricahua) males indicate that *ST;or/or* males are at a mating disadvantage (Pruzan 1976). Yet *ST;or/or* males enjoy a mating advantage with females which were previously inseminated by these type males (Pruzan 1976; Pruzan et al. 1977). Comparison of mean progeny of twice-mated females (table 4) failed to provide an explanation for this phenomenon based on a hypothesized advantage to the females.

SUMMARY

We investigated the effect of single mating and remating on sperm utilization in *Drosophila pseudoobscura* females. Once-mated females produced progeny at a

TABLE 4

ANALYSIS OF VARIANCE OF MEAN PROGENY PER TRANSFERS 2-7 FOR
GROUPS OF TWICE-MATED *Drosophila pseudoobscura* FEMALES

Source	SS	df	F	P
Group (A)	3735.92	3	1.45	.23
Transfer (B)	30206.91	5	7.04	.0001
A × B	3615.40	15	.28	.99

high level for 15 days; thereafter the number dropped significantly. Seventy-two percent of the females remated within 6 days after the initial mating, when approximately 30% of the sperm was utilized. Thus, *D. pseudoobscura* females remate before sperm supply is exhausted. Remated females produced more progeny than once-mated females, regardless of the genotype of males involved in matings. The increase occurred shortly after remating and thereafter diminished to the same level as that of once-mated females. Sperm competition was evident when the two male mates were of different genotype. The mean sperm displacement ability of *ST;or/or* males was .67 and for *AR;+/+* males .90. The two means were not statistically significant. In each case, however, sperm displacement was evident.

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