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Courtship and remating in field populations of *Drosophila*

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Abstract. Data on courtship and remating in field populations of *Drosophila simulans* and *D. melanogaster* were gathered to determine whether the timing of female remating was related to the number of sperm females retain in storage. Several types of female rejection behaviour commonly terminated courtships, and did so after brief courtships. Efficacious rejections by females are necessary to any hypothesis that relies upon female influences on the timing of remating. To examine the sperm load of remating females, mating pairs were collected and separated as they initiated copulation, well before sperm transfer had occurred. When subsequently cultured in the laboratory, females from these interrupted matings produced significantly fewer progeny than randomly collected non-mating females. The difference in progeny production between remating and control females was significantly greater for *D. melanogaster* than for *D. simulans*, but in both species females carried fewer than average number of sperm at the time of remating. Correspondence (and the lack of it) between these field observations and those obtained in different laboratory-based experimental set-ups is discussed.

Drosophila melanogaster has been used extensively in laboratory studies of courtship behaviour, sexual selection and reproduction. The typical laboratory experiment differs from the field environment in ways that could affect the behavioural results of the experiment. For instance, many laboratory studies of courtship behaviour involve virgin females exclusively; in field settings males encounter and court previously mated females much more often than virgins (Bouletreau 1978; Partridge et al. 1987). Male mating success and intensity of courtship are both likely to be affected by the male's rate of encounter with unreceptive females. Furthermore, the laboratory environment is usually designed to produce healthy flies and to maximize reproductive output. In contrast, feeding and oviposition sites may not be abundant at some locations or seasons, and the reproductive potential of flies in such environments is substantially lower than in the laboratory (Bouletreau 1978). Environmental variation of this sort is known to produce both direct and indirect effects on the frequency of mating (Harshman et al. 1988; Marks et al. 1988). Another essentially unavoidable attribute of a laboratory experiment, the use of chambers in which to grow and observe the flies, is known to affect several types of behaviour. Spontaneous activity (Ewing 1963), courtship duration (Ewing & Ewing 1984) and copulation duration (Bower 1988) depend on chamber size. The walls of the observation chamber are physical barriers to movement that limit the effectiveness of female decamping (i.e. walking, jumping or flying away from a courting male) as a rejection behaviour (Ewing & Ewing 1984), possibly producing subsequent effects on the frequency of female remating (Manning 1967).

Females caught in nature and found to be carrying sperm from two or more males provide unambiguous evidence that females remate in nature while still carrying sperm from a previous mating (Milkman & Zeitler 1974; Gromko et al. 1980). However, the frequency and timing of remating with respect to the number of sperm remaining in storage remain unresolved issues. Manning (1967) first showed that mating reduces female receptivity to subsequent courtship. He showed that female receptivity is reduced in a short-term way by the physical act of copulation (which he called the copulation effect), and that a longer-term effect on female receptivity is correlated with the number of sperm transferred to the female (the sperm effect). However, in the laboratory the expression of the sperm effect depends markedly upon experimental design (Newport & Gromko 1984). In designs where males and females are confined together for 24–48 h, females remate more frequently and do so

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at random with respect to the number of sperm remaining in storage. In contrast, designs in which males and females are placed together for a discrete period each day produce a much lower frequency of female remating, with females remating only as their supply of stored sperm decreases (Newport & Gromko 1984). Results from studies of sperm loads of wild-caught females suggest that the number of sperm remaining in storage is a factor influencing female receptivity to remating in the field (Stalker 1976), but more direct evidence is necessary to settle this issue.

If females are waiting to remate until many sperm from a previous mating have been used, then (1) unreceptive females in field populations must be able to reject male courtship effectively, presumably producing short courtship durations, and (2) when females do remate, the number of sperm they carry in storage must be small in comparison with females not remating. Here we report courtship and mating data for field populations of *D. simulans* and *D. melanogaster*. The courtship observations demonstrate that the average courtship is short and that female rejection behaviour is efficacious. The mating data provide a test of the relationship between the number of sperm in storage (as estimated by progeny production) and female receptivity to remating. The remating data and the courtship data reflect two different aspects of a single phenomenon: the number of stored sperm is at least correlated with, and possibly functionally related to, the probability of female remating.

STUDY SITE

We carried out all observations and experiments on flies attracted to oranges and lemons beneath citrus trees in Tempe, AZ. We broke open and added new fruits to the site at approximately weekly intervals. The predominant species present were *D. simulans* and *D. melanogaster*. Flies were present in large numbers, which simplified observations; it was not difficult to find pairs involved in courtship. Numbers were not so great as to make it difficult to observe individuals, however.

COURTSHIPS IN THE FIELD

Materials and Methods

In March and April of 1989, April of 1990 and April of 1991 we gathered data on courtship durations and outcomes. We conducted observations of courting pairs between 0600 and 1000 hours and between 1600 and 1900 hours. To begin, an observer identified a single male or female that was not actively courting and then watched that individual until it became involved in a courtship. All the elements of courtship described for males in the laboratory (Bastock & Manning 1955) were observed, including orientation, wing vibration, tapping, licking, following and attempted copulation. The duration of the courtship was timed with a stopwatch and the behaviour that terminated the courtship was recorded as follows: (1) the female extruded her ovipositor and the male turned away; (2) the female kicked or preened and the male turned away; (3) the female walked away from the male and the male did not follow; (4) the female jumped or flew away and the male was unable to follow; (5) the male walked away from female without obvious rejection behaviour from female; (6) the male switched courtship from one female to another; (7) the male was displaced by a second male; and (8) copulation. It should be noted that female rejection behaviour did not always terminate a male's courtship; only those behaviour patterns that terminated courtship were recorded. Immediately following courtship termination, the observer attempted to aspirate (or poot) both the male and the female that had been involved in the courtship and save them in a numbered vial. On many occasions we were able to catch only one member of the courting pair. Flies were returned to the laboratory for species identification. We determined species identity of males by inspection of the genitalia. Females were cultured individually and their species identity was made from their male progeny.

Results

The relative frequency of *D. melanogaster* differed among years (Table I), so the data on frequency of pairings were treated separately for each year. The best estimate of the relative frequency of the two species for males and females separately included cases where only one sex was captured; consequently, the sample size for individuals was larger than the sample size for pairs. We calculated the expected frequency of courting pairs under the hypothesis of random encounter of males and females. The observed counts among pairs in which species identity of both sexes were known fit expectations based on random encounter (Table II). Expected values for this analysis were small, which

Table I. Relative frequencies *D. melanogaster* and *D. simulans* observed in courtship, for males and females separately*

	D. melanogaster	*D. simulans*	Total
Males			
1989	9	55	64
	(0·141)	(0·859)	
1990	8	21	29
	(0·276)	(0·724)	
1991	11	13	24
	(0·458)	(0·542)	
Total	28	89	117
$\chi^2=9{\cdot}96$, $df=2$, $P<0{\cdot}01$			
Females			
1989	6	50	56
	(0·107)	(0·893)	
1990	10	13	23
	(0·435)	(0·565)	
1991	14	7	21
	(0·667)	(0·333)	
Total	30	70	100
$\chi^2=25{\cdot}3$, $df=2$, $P<0{\cdot}01$			

*Including courtships in which only one member of the pair was caught. The numbers shown in parentheses are proportions, used to calculate expected pairing frequencies in Table II.

might argue against the use of chi-squared, however, small expected values would inflate the statistic artificially. Clearly, the small expected values did not produce the appearance of statistical significance where there really was none.

Despite the difference in species composition among years, we were unable to identify any differences in behaviour among years. Thus data were pooled across years in all subsequent analyses. We identified five categories of species pairs for further analysis. In three categories both sexes were caught and identified (*D. simulans–D. simulans* pairs, *D. melanogaster–D. melanogaster* pairs and mixed-species pairs). In two categories only one member of the pair (either sex) was caught and identified (*D. simulans*–unknown pairs and *D. melanogaster*–unknown pairs).

Most courtships were short. This was apparent within each species category (Table III) and for the distribution pooled across all species categories (Fig. 1). The distributions of courtship durations were skewed right and were leptokurtic. The considerable difference between the median and the mean was symptomatic of the asymmetry of the distributions. The differences in courtship duration among the three species pairings in which both sexes were identified were not significant (Kruskal–Wallis $H=4$, $df=2$, $P=0{\cdot}13$).

Courtships were terminated more often by female-initiated behaviour than by male-initiated behaviour (Table IV). It should be noted that female decamping is the sum of females walking away and females jumping or flying away. Thus female decamping was the most common behaviour found to terminate courtships, with extrusion next. The contingency of terminal behaviour on species category (Table IV) was not significant ($\chi^2=27$, $df=24$, $P=0{\cdot}32$). This result was unaffected by the inclusion or exclusion of the low-frequency behaviour (kick-preen, male switched mate and male-displaced).

The relationship between courtship duration and terminal behaviour was analysed based on data pooled across all species categories (Table V). The differences in courtship duration among the seven categories of terminal behaviour were significant (Kruskal–Wallis $H=33{\cdot}8$, $df=6$, $P=0{\cdot}0001$). However, multiple comparisons among all seven categories lacked power, apparently as a result of the small sample sizes for behavioural categories kick-preen, male switched mate, and male displaced. Non-parametric multiple comparisons (Zar 1984) among the remaining four categories showed three overlapping groups (Table V). Courtships ending with the two aspects of female decamping (female walked away and female jumped or flew away) did not differ significantly from each other in duration, but they were both longer than courtships ending with extrusions.

Finally, it is noteworthy that none of the timed courtships ended in copulation. A low copulation rate was also reported for *D. melanogaster* in the field by Partridge and co-workers (Partridge et al. 1987). However, three times during our observations pairs other than the focal pair were observed to begin copulation. We obtained no courtship duration data on these pairs, but it was possible to time the duration of copulation and, as the pair disengaged, to aspirate them for species identification. One of these pairs was *D. simulans* and its copulation duration was 39 min 29 s. The other two were *D. melanogaster* with copulation durations of 27 min 0 s and 21 min 49 s. These values are all larger than those reported to be characteristic of *D. melanogaster* (17–20 min) and of *D. simulans* (16–18 min) in laboratory studies (Spieth 1952).

Table II. Frequencies of pairs in which both sexes were caught

	Pair		Expected		Observed count
	Male	Female	Frequency	Count	
1989	*D. simulans*	*D. simulans*	0·767	25·3	28
	D simulans	*D. melanogaster*	0·092	3·0	2
	D. melanogaster	*D. simulans*	0·126	4·2	3
	D. melanogaster	*D. melanogaster*	0·015	0·5	0
	$\chi^2=1·464$, $df=2$, $P=$ NS				
1990	*D. simulans*	*D. simulans*	0·409	5·7	5
	D simulans	*D. melanogaster*	0·315	4·4	4
	D. melanogaster	*D. simulans*	0·156	2·2	4
	D. melanogaster	*D. melanogaster*	0·120	1·7	1
	$\chi^2=1·883$, $df=2$, $P=$ NS				
1991	*D. simulans*	*D. simulans*	0·181	3·1	2
	D simulans	*D. melanogaster*	0·361	6·1	6
	D. melanogaster	*D. simulans*	0·153	2·6	2
	D. melanogaster	*D. melanogaster*	0·305	5·2	7
	$\chi^2=1·154$, $df=2$, $P=$ NS				

Table III. Courtship durations (s)

Pair	*N*	Median	Mean	SE	Skewness	Kurtosis
Both *D. simulans*	35	6·0	20·9	4·8	1·86	3·03
D. simulans–unknown	68	14·5	45·6	10·3	3·12	9·47
Mixed-species	21	6·0	15·4	4·5	1·86	3·01
D. melanogaster–unknown	21	6·3	11·1	2·3	1·01	−0·10
Both *D. melanogaster*	8	20·2	28·0	7·7	0·38	−1·55
Pooled	153	11·0	30·1	4·9	4·48	22·51

MATINGS IN THE FIELD

Materials and Methods

Observations were carried out from 7–11 April 1990 between 0700 and 1030 hours and between 1530 and 1930 hours each day. These intervals spanned the time of peak mating activity. Several observers each continuously scanned from six to 12 specific pieces of fruit searching for *Drosophila* pairs that were initiating copulation. Because the frequency of courtships leading to copulation was small, as described in the previous section, we adopted a two-fold search strategy. The first aspect of the search strategy involved copulating pairs. During the first 30 min of each observation period, we aspirated and set aside in a vial (and later released) any pairs seen in copula. In this way the fruits each observer was scanning were cleared of pairs that had begun copulating before the observer's arrival. Subsequently, any pairs seen in copula must have initiated copulation between scans (i.e. within 30 s of sighting); these pairs were saved for study. We aspirated these copulating pairs from the fruit: separated males from females by gently blowing the pair in and out of the aspirator (with the aspirator in the vial); and saved the male and female in two separate, numbered vials. This experimental group will be called 'first seen in copula'.

The second aspect of the search strategy was to see pairs initiate copulation. Observers directed their attention to any males attempting copulation. Most attempted copulations ended abruptly, with the male walking away from the female after she had extruded her ovipositor or after she had herself decamped, as described in the previous section of this paper. In contrast, a receptive female responded to the male's attempt to copulate by spreading her wings, a brief but obvious behavioural signal (Markow & Hanson 1981; Tompkins et al. 1982).

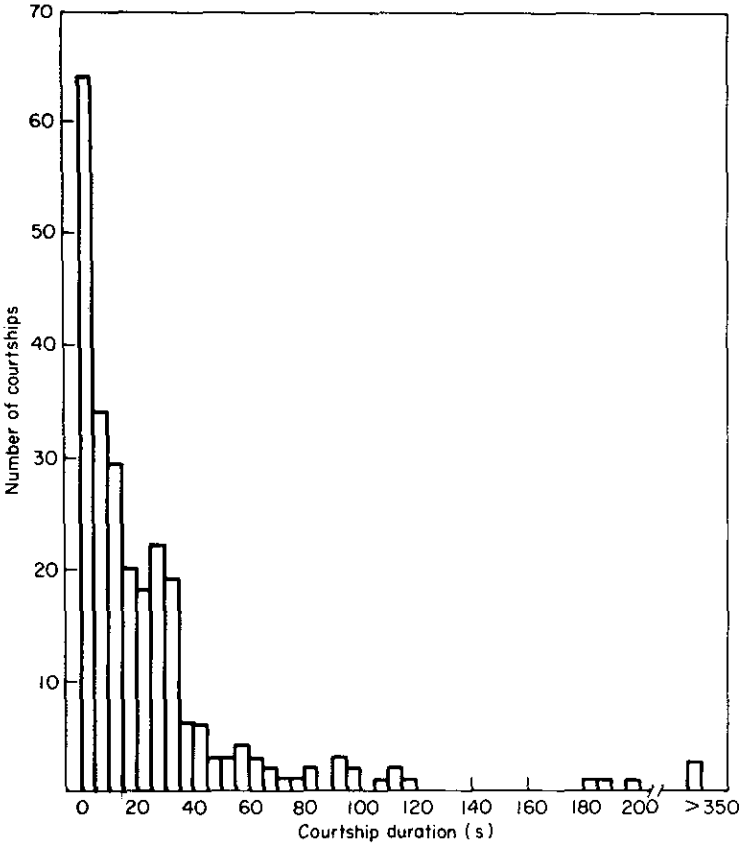

Figure 1. Distribution of courtship durations for all species categories.

Table IV. Behaviour that terminated courtship

	D. simulans and *D. simulans*	*D. simulans* and unknown	Mixed species	*D. melanogaster* and unknown	*D. melanogaster* and *D. melanogaster*	Total
Extruded ovipositor	15	17	7	8	6	53
Female jumped or flew	5	19	2	7	1	34
Female walked away	6	11	5	3	0	25
Male walked away	6	12	6	1	0	25
Male displaced	1	3	1	1	1	7
Female kicked or preened	2	2	0	1	0	5
Male switched females	0	4	0	0	0	4
Total	35	68	21	21	8	153

The genitalic union was established first and quickly, but it took the pair approximately 2–20 s for the male to settle down onto the female and for movement of the female's wings and the male's forelegs to cease ('settled pair'). Pairs that we observed to interact from attempted copulation to 'settled pair' were then aspirated, separated and saved separately as with the 'first seen in copula' group. This second experimental group will be called 'first seen initiating copulation'.

In the laboratory, *D. melanogaster* males transfer sperm about 10 min after the initiation of

Table V. Terminal behaviour and courtship durations, pooled across all species categories*

Terminal behaviour	*N*	Courtship duration (s)			Multiple comparisons
		Median	Mean	SE	
Female kicked or preened	5	3·8	4·0	0·9	—
Extruded ovipositor	53	5	12·8	2·7	A
Male walked away	25	5	17·3	4·9	A, B
Male displaced	7	14	27·5	10·7	—
Female walked away	25	16	57·4	18·7	B, C
Female jumped or flew	34	24·8	40·7	10·6	C
Male switched females	4	28·8	117·2	96·2	—

*Courtships terminated by the seven different behaviour patterns differed significantly in duration (Kruskal–Wallis $H=33·8$, $P=0·0001$). Multiple comparisons among the four behaviour patterns with appreciable sample sizes were carried out. Behaviour patterns followed by the same letter were not significantly different in non-parametric multiple comparisons.

copulation, although sperm transfer has been observed to occur as early as 4 min following the initiation of copulation (Gilbert et al. 1981b; Gromko et al. 1984). We interrupted pairs 'in copula' within 1 min of the initiation of copulation and 'initiating' pairs within a few seconds. In both cases, interruption was well before sperm transfer. Thus any progeny produced by females in the experimental groups must have been due to sperm received from a prior mate. Furthermore, ejaculatory components transferred before sperm have been shown to have no effect on the number of previously stored sperm (Gromko et al. 1984).

We aspirated control females from the fruit throughout the course of the observations. These females were a random sample of females not in copula. The number of control females was roughly double the number of experimentals.

Immediately after collection, we returned all control and experimental flies to the laboratory. We transferred individual females to fresh food vials daily for 10 days and then every other day for 10 more days. We determined species identity as described previously and counted all the progeny emerging. The primary purpose of this experiment was to compare the total number of progeny per female in experimental and control groups.

Results

The mean number of progeny produced by females 'first seen in copula' did not differ from the number produced by females 'first seen initiating copulation' for *D. simulans* ($t=0·662$, $df=39$, $P=0·51$) or for *D. melanogaster* ($t=0·739$, $df=6$, $P=0·49$, Table VI). The equivalence of the two aspects of the search strategy was thus verified; we pooled the two groups of experimental females for all subsequent analyses.

For both species, 'progeny per female' were normally distributed within the control group. However, within the experimental group, the data were not normally distributed, primarily because of the large number of females that did not produce progeny. When zero values were excluded (Table VII), the data were approximately normally distributed, allowing the use of parametric statistics. The exclusion of females producing no progeny also allowed the most rigorous test of our hypothesis, which was that previously mated females wait to remate until their supply of stored sperm from previous matings is reduced. By excluding females producing no progeny from the analysis we insured that we were considering only previously mated females.

We performed a two-way ANOVA on females that produced at least one offspring, with species and experimental treatment as main effects (Table VIII). The difference between control and experimental groups was highly significant, with experimental group females producing fewer progeny than controls. However, the interaction between species and experimental group was also significant. The pattern of differences among the means of the four groups (Fig. 2) shows that the interaction results

Table VI. Number of progeny produced by control and experimental females*

Group	Number of progeny			
	Median	Mean	SE	*N*
D. simulans				
Experimental				
Initiating copulation	25	110·2	33·2	16
In copula	83	140·2	30·0	25
Pooled	45	128·8	22·3	41
Control	268·5	263·4	11·9	60
D. melanogaster				
Experimental				
Initiating copulation	5	73·0	53·0	6
In copula	1·5	1·5	1·5	2
Pooled	3·5	55·1	40·5	8
Control	299·5	275·1	41·5	10

*Females producing zero progeny included.

Table VII. Distribution of females producing zero versus one or more progeny

	Females producing		
	One or more	Zero	Total
D. simulans			
Control	60	0	60
Experimental	26	15	41
Total	86	15	101
D. melanogaster			
Control	9	1	10
Experimental	5	3	8
Total	14	4	18

because the difference between control and experimental groups was much larger for *D. melanogaster* than for *D. simulans*. The experimental treatment effect was of different magnitude in the two species; it was none the less significant within each species separately (*D. simulans*: $t=2{\cdot}447$, $df=84$, $P=0{\cdot}0165$; *D. melanogaster*: $t=3{\cdot}506$, $df=12$, $P=0{\cdot}0043$). In both species, females delayed remating until many sperm from the previous mating were used, with females of *D. melanogaster* using more of the stored sperm before remating than did females of *D. simulans*.

DISCUSSION

Most courtships involving *D. simulans* and *D. melanogaster* in a natural field environment were of short duration, with the median value ranging from 6–20 s (Table III, Fig. 1). In contrast, males in vials or small chambers in the laboratory often court females in a series of bouts, accumulating long periods of courtship with a single female. For instance, average courtship durations of 2–3 min (Gromko 1987) and up to 10 min (Cobb et al. 1987) have been reported for *D. melanogaster*.

In the field, a variety of female behaviour patterns commonly ended courtship, including ovipositor extrusion and female decamping (walking, jumping or flying away). We inferred that patterns of female rejection behaviour were effective in terminating courtship. However some behavioural categories, particularly ovipositor extrusion, produced variable results. Some males turned immediately away from an extruding female, but other males persisted in courting and attempting copulation with extruding females, a result that has also been reported in laboratory studies (Bastock & Manning 1955; Connolly & Cook 1973; Ewing & Ewing 1987). Courtships that ended with female decamping were of significantly longer duration than those that ended with ovipositor extrusion. Females that decamped may have been ones for which ovipositor extrusion had previously failed to be effective in terminating male courtship. In contrast, we never observed a male that was able to follow a female that jumped or flew away.

Very few courtships ended because of interactions among males. Female rejection terminated courtship approximately 20 times more often than male–

Table VIII. Two-way ANOVA on female productivity*

Source	*df*	Sum of squares	*F*	*P*
Species	1	14 432·8	1·29	0·258
Treatment	1	210 610·0	18·86	0·001
Species × treatment	1	67 477·9	6·04	0·016
Error	96	1 072 210·8		

*Females producing zero progeny excluded.

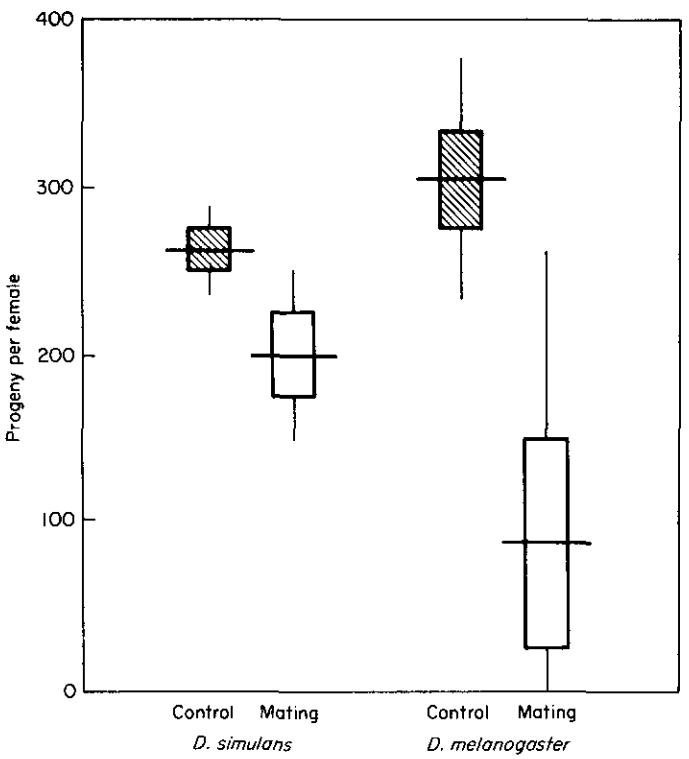

Figure 2. Mean number of progeny per female, excluding females that produced no progeny, for control (▨) and mating (□) groups for both species. The heavy horizontal line represents the mean, the rectangle represents the standard error and the vertical lines represent 95% confidence limits.

male interactions. The low frequency of competitive interactions among males leads us to suspect that female choice may play a larger role than male–male competition in these species, given that sexual selection is important.

In a separate experiment in which the search strategy was changed to improve chances of finding mating pairs, we aspirated and interrupted pairs as they initiated copulation. The relative numbers of progeny produced by these females reflected the relative number of sperm they had in their sperm storage organs from a previous mating. Remating females produced fewer progeny than control (non-mating) females in both species tested, although this difference was significantly larger for *D. melanogaster* than for *D. simulans* (Table VIII, Fig. 2). Thus, the reduction in female receptivity following mating observed by many in laboratory studies (Manning 1967; Gromko et al. 1984; Letsinger & Gromko 1985; Scott 1987; Chen et al. 1988) was plainly apparent in these natural populations. Although the sperm effect may be caused by some chemical component of the ejaculate and not the sperm themselves (Scott 1987), the result is

the same: females wait to remate until a substantial number of sperm from a previous mating have been used.

In interpreting the remating data, care should be taken to distinguish between female attractivenes and female receptivity. Recently mated females show both lowered attractiveness and lowered receptivity (Tompkins & Hall 1981; Jallon 1984). Given that males direct vigorous courtship towards mated females that respond with effective rejection, both in the laboratory and the field, it appears that males are attracted to females sooner after a previous mating than females regain receptivity to males. It is the lowered female receptivity (and not female attractiveness) that is correlated with the number of sperm remaining in female sperm storage organs.

The inference from progeny production to sperm storage is direct, although the relationship is not one-to-one. Efficiency of sperm use is low for females carrying a large number of sperm in storage (several sperm released for each egg fertilized), but the efficiency improves as the number in storage decreases (Gilbert 1981; Gilbert et al. 1981a). Consequently, for a given difference in progeny production for two groups of females, the corresponding difference in stored sperm is likely to be even larger. One factor possibly working against this inference would be a positive relationship between sperm storage capacity and female size. However, female size and number of sperm stored are uncorrelated in *D. melanogaster* (Pitnick 1991).

The data presented here suggest that the proportion of receptive females in nature is likely to be small. In this and in other studies in which random samples of wild females were dissected immediately upon collection (Bouletreau 1978) or were cultured in the laboratory (Spieth 1952; Table VII), between 85 and 96% of females were found to be carrying sperm from a previous mating. Both the courtship and remating data suggest that most non-virgin females are unreceptive at any one time. Some proportion of the virgin females are also likely to be unreceptive because they are immature until 2–3 days of age (Manning 1967; Connolly & Cook 1973).

The reproductive task presented to the male is one of finding a very small percentage of receptive females, which are a mix of virgin and previously mated females. Behavioural and chemical cues may be used by males to identify unreceptive females (Scott & Jackson 1990), as most courtships end within a few seconds; males spend very little courtship effort on individual females. The frequency of remating we observed is consistent with this distribution of courtship effort. Although a large proportion of females may remate soon after the first mating in a laboratory situation (Lefevre & Jonsson 1962; Bellen & Kiger 1987; Harshman et al. 1988), the data presented here suggest that females in the field are unlikely to remate until many sperm stored from a previous mating have been used.

Differences in behaviour in laboratory- and field-based observations were evident not just for the timing of female remating, but for courtship duration, effectiveness of female decamping and possibly for copulation duration as well. It would be too simple, however, to distrust all laboratory-based behavioural results, because some laboratory situations produce behaviour similar to those observed in the field. For instance, many aspects of the field observations of courtships reported in this paper, including short average courtships and effectiveness of female decamping, agree closely with laboratory data based on behaviour in large observation chambers (Ewing & Ewing 1984, 1987). Thus chamber size seems to be important to the correspondence between laboratory and field results. Similarly, the use of a divided chamber can reduce the agitating effects of aspirating flies into a chamber on the subsequent course and duration of courtship (Cobb et al. 1987). Chamber or apparatus effects are well known in behavioural research; the integration of field- and laboratory-based experiments is essential to the reduction or avoidance of such effects.

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