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Female Remating in *Drosophila ananassae*: Evidence for the Effect of Density on Female Remating Frequency

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Drosophila ananassae, *a cosmopolitan and domestic species, is largely circumtropical in distribution and belongs to the* ananassae *species complex of the* ananassae *subgroup of the* melanogaster *species group. In the present study, experiments were conducted to investigate the effect of density on female remating frequency by employing different wild-type and mutant strains of* D. ananassae. *Two experimental designs, i.e., 2-h daily observation and continuous confinement, were used. The results show that there is significant dependence of remating frequency on density in all strains tested under both experimental designs except in a wild-type strain (Bhutan), which shows no dependence of remating frequency on density under 2-h daily observation design. This finding provides evidence that density may increase the frequency of female remating in* D. ananassae.

KEY WORDS: *Drosophila ananassae*; female remating; density.

INTRODUCTION

Female remating is an important aspect of sexual behavior in *Drosophila* that has received considerable attention, partially because it is intimately associated with patterns of sperm usage and sexual selection (Parker, 1970; Smith, 1984). Parker (1970) used the term "sperm competition" to describe

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the selection pressures operating on males to produce adaptations which may include behavioral and physiological characteristics of the males as well as competitive properties of their sperm. This increases the possibility of his sperm, in the presence of another male, to be used by a female for insemination of her eggs. Female fitness is also influenced by multiple mating (Pyle and Gromko, 1978); females can be directly harmed by male adaptations for sperm competition (Chapman *et al*., 1995), so females should be under strong selection to mediate sperm competition. Remating is common in many species of *Drosophila* under both field and laboratory conditions (Lefevre and Jonsson, 1962; Anderson, 1974; Richmond and Ehrman, 1974; Craddock and Johnson, 1978; Loukas *et al*., 1981; Markow, 1985; Barbadilla *et al*., 1991; Aspi, 1992; Etges and Heed, 1992; Joly and Lachaise, 1993; McRobert *et al*., 1997; Price, 1997; Singh and Singh, 1997; Harshman and Clark, 1998). Females of some *Drosophila* species have been shown to gain direct benefits from remating (Markow and Ankney, 1984). Female remating frequency is dependent on the amount of sperm stored (Manning, 1962; Gromko *et al*., 1984; Gromko and Markow, 1993), components of the male seminal fluid (Chen *et al*., 1988; van Vianen and Bijlsma, 1993), levels of nutrition, and the egg-laying rate (Gromko and Gerhart, 1984; Harshman *et al*., 1988; Trevitt *et al*., 1988; Chapman *et al*., 1994; Chapman and Partridge, 1996). The frequency of remating is also influenced by density. Boorman and Parker (1976) suggested that at higher population densities, there is an increase in the incidence of courtship, resulting in a higher frequency of multiple mating in *D. melanogaster*. Marks *et al*. (1988) provided evidence for a positive correlation between population density and remating frequency in the field in the same species. However, increasing density can also decrease the frequency of remating in *D. melanogaster* (Gromko and Gerhart, 1984). Harshman *et al*. (1988) reported that the frequency of remating in *D. melanogaster* was unaffected by density for some combinations of the fly strains but was reduced at low relative densities for other combinations. In *D. pseudoobscura* and *D. persimilis*, increasing density increased the frequency of female remating (Richmond, 1976; Levine *et al*., 1980; Turner, 1986). In a natural population of *D. montana*, Aspi and Lankinen (1992) reported that low population densities at the end of the mating season probably force females to remate shortly after first mating.

D. ananassae has been extensively used for genetical studies, particularly population genetics, behavior genetics, and crossing-over by Singh and his co-workers (for references see reviews by Singh, 1996, 2000). The results of mating propensity tests in wild-type strains, mutant strains, and inversion karyotypes and the results of selection experiments have been used as evidence for genetic control of sexual behavior in *D. ananassae* (Singh and Chatterjee, 1986, 1987, 1988a,b; Chatterjee and Singh, 1987,

1988). We have conducted experiments on female and male remating frequency by employing several geographical strains of *D. ananassae* (Singh and Singh, 1997, 2000; Singh and Singh, 1999). The results have shown that (a) male remating occurs more frequently than female remating, (b) there is interstrain variation for remating time for both males and females, and (c) copulations are shorter in second matings compared to first matings. *D. ananassae* females show increases in productivity after remating (Singh and Singh, 2001). In *D. melanogaster*, results regarding the effect of density on female remating frequency obtained by different investigators vary. The effect of density on female remating frequency in *D. ananassae*, another cosmopolitan and domestic species, has not been tested. *D. melanogaster* and *D. ananassae* are members of the *melanogaster* species group but belong to different subgroups and also to different lineages as evidenced by taxonomic and cytogenetic studies (Lemeunier *et al*., 1986). In view of this, we conducted experiments to test the effect of density on female remating frequency in *D. ananassae* by employing 2-h daily observations and continuous confinement.

METHODS

To test the effect of density on female remating frequency in *Drosophila ananassae*, two experimental designs, i.e., (a) a 2-h daily observation design and (b) a continuous confinement design, were used (Gromko and Gerhart, 1984).

The 2-h Daily Observation Design

In this experiment the following stocks were used: Bhutan (wild type), DP (wild type) and *ca* (*claret* eye color—recessive mutation on chromosome II). Crosses 1–3 (Table I) were used in this design. In each cross, virgin females and males were collected and aged for 7 days in food vials. A single virgin female was placed in a fresh food vial (3-in. length $\times$ 1-in. diameter) with a single virgin male and the pair was observed for 60 min. When mating occurred, the pair was allowed to complete copulation and the male was discarded within 1 h of the completion of copulation. Once-mated females were left overnight in a group of six per vial. The next morning mated females and fresh virgin males were put into fresh food vials at each of the four densities. There were 12 vials with 2 pairs per vial, 4 vials with 6 pairs per vial, 2 vials with 12 pairs per vial, and 1 vial with 24 pairs (see Table II). No anesthesia was used. All transfers were accomplished by aspiration and tapping between vials with a funnel. The vials were observed continuously

Table I. Experimental Designs for Testing the Effect of Density on Female Remating Frequency in *Drosophila ananassae*

Cross	Females	First males	Second males
	(A) 2-h daily observation design		
1	Bhutan	Bhutan	Bhutan
2	*ca*	DP	*ca*
3	*ca*	*ca*	DP
	(B) Continuous confinement design		
4	*ca*	DP	*ca*
5	*ca*	*ca*	DP
6	*ct rb*	*y*	*ct rb*
7	*ct rb*	*ct rb*	*y*

for 2 h, the number of remated females in each vial was recorded, and remated females were aspirated out. After 2 h of observation, the females and males were separated by aspiration. Females were stored in groups of six per vial and the same density was maintained by adding new once-mated females. Males were discarded. The procedure was repeated on 6 consecutive mornings. The flies receiving a particular density treatment on 1 day always received the same density treatment on following days. Two replicates were run for wild-type (Bhutan) flies.

Table II. Results of the 2-h Daily Observation and Continuous Confinement Remating Experiments at Different Densities in *D. ananassae*

Cross	Number of remated females at different densities[a]			
	2/V	6/V	12/V	24/V
A. 2-h daily observation design				
1. Bhutan ♀ × Bhutan ♂ × Bhutan ♂				
Replicate I[b]	5	7	4	3
Replicate II[b]	6	2	4	5
2. *ca* ♀ × DP ♂ × *ca* ♂*	2	3	7	10
3. *ca* ♀ × *ca* ♂ × DP ♂*	3	5	9	11
B. Continuous confinement design				
4. *ca* ♀ × DP ♂ × *ca* ♂**	7	10	15	19
5. *ca* ♀ × *ca* ♂ × DP ♂**	10	12	17	22
6. *ct rb* ♀ × *y* ♂ × *ct rb* ♂**	6	10	13	18
7. *ct rb* ♀ × *ct rb* ♂ × *y* ♂***	5	7	11	20

[a] Twenty-four pairs were tested in each cross at each of the four densities.
[b] Variation between remated and not-remated females nonsignificant (χ^2 test from contingency table). Variation between remated and not-remated females: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (χ^2 test from contingency table).

The Continuous Confinement Design

In this experiment the following stocks were used: DP (wild type), *ca* (*claret* eye), *ct rb* (*cut* wing, *ruby* eye color—recessive mutation on the X chromosome), *y* (*yellow* body color—recessive mutation on the X chromosome). Crosses 4–7 (Table I) were used in this design. In each cross 7-day-old virgin females and males were pair-mated in individual food vials. Males were aspirated out within 1 h of the completion of copulation. All mated females were stored in a group of six females per vial. The next day the same density conditions were set up as in the 2-h daily observation design. These density treatment vials were left undisturbed at room temperature (approx. 24°C) for 5 days. At the end of that time the flies in all vials were etherized, and the females were put individually into fresh food vials. Males were discarded. The females were subcultured once in fresh food vials, 3 days later. A female was indentified as having remated by the presence of second male types among her progeny.

To test whether there is a significant difference in the number of remated females at different densities, the data were analyzed by calculating the chi-square values from an R × C contingency table and the correlation coefficient (r).

RESULTS

The results of the 2-h daily observation remating and continuous confinement remating experiments, conducted to test the effect of density on female remating frequency in *D. ananassae*, are presented in Table II. In the 2-h daily observation design there are three crosses in total. In cross 1 there are two replicates with wild-type females and males (Bhutan). The frequency of female remating at all four densities in cross 1 shows little variation, which is statistically not significant [correlation coefficient (r) between density and remating frequency: Replicate I, $r = -0.753$, df $= 2$, $P > 0.05$; Replicate II, $r = -0.102$, df $= 2$, $P > 0.05$]. In crosses 2 and 3, in which *ca* mutant females were tested with wild-type and mutant males (DP and *ca*), there is a considerable increase in the frequency of female remating at higher densities, which is statistically significant (correlation coefficient between density and remating frequency: cross 2, $r = 0.978$, df $= 2$, $P < 0.05$; cross 3, $r = 0.952$, df $= 2$, $P < 0.05$).

In continuous confinement, there are four crosses in total (Table II). In crosses 4 and 5 *ca* mutant females were tested with wild-type and mutant males (DP and *ca*) at different densities. In these crosses also there is a significant increase in the frequency of female remating at higher densities

(correlation coefficient between density and remating frequency: cross 4, $r = 0.974$, df = 2, $P < 0.05$; cross 5, $r = 0.989$, df = 2, $P < 0.05$). Female remating frequency was tested at four densities employing *ct rb* mutant females with *y* and *ct rb* mutant males in crosses 6 and 7. In both these crosses there are significant increases in the frequency of female remating at higher densities (correlation coefficient between density and remating frequency: cross 6, $r = 0.983$, df = 2, $P < 0.05$; cross 7, $r = 0.998$, df = 2, $P < 0.01$).

The contingency χ^2 test, to test the variation between remated and not-remated females, shows nonsignificant variation for wild-type females (cross 1, 2-h daily observation design; Table II). However, the χ^2 test for contingency for crosses 3 to 7 shows significant variations in both designs (Table II).

DISCUSSION

During the course of this study, experiments were conducted to investigate the effect of density on female remating frequency by employing different wild-type and mutant strains of *D. ananassae*. It is evident from the present results that all strains tested during the present study show a significant dependence of remating frequency on density under both the 2-h daily observation design and the continuous confinement design, except in a wild-type (Bhutan) strain which shows no dependence of remating frequency on density.

In female remating studies two experimental designs have been used by various investigators and the female remating frequency varies considerably in different species, i.e., the periodic confinement design and continuous confinement design (Pyle and Gromko, 1978; Loukas *et al*., 1981; Markow, 1985; Aspi, 1992; McRobert *et al*., 1997). The periodic confinement (brief duration of male–female interaction) is correlated with a low incidence of remating (Manning, 1962) and the continuous confinement is correlated with a high frequency of remating (Lefevre and Jonsson, 1962; Pyle and Gromko, 1978). During the present study, it has also been observed that the frequency of female remating is higher in the continuous confinement design compared to the periodic confinement design. Thus our results support the statement that a brief duration of male–female interaction is correlated with a low incidence of remating, and continuous confinement (longer duration of male–female interaction) is correlated with a high frequency of remating.

The relationship between density and frequency of multiple mating was examined using two experimental designs, i.e., periodic (2-h daily) and continuous confinement, in *D. melanogaster* by Gromko and Gerhart (1984). They found that increased density per se inhibits rater than facilitates remating. In the present study same experimental designs used by Gromko and

Gerhart (1984) were followed to examine the effect of density on remating frequency in *D. ananassae*. The results in the 2-h daily observation design with wild-type females were similar to those of Gromko and Gerhart (1984) who found that the frequency of female remating did not increase with a higher density. However, *ca* mutant females in the 2-h daily observation and *ca* and *ct rb* mutant females in the continuous confinement design show a significant increase in remating frequency at higher densities in *D. ananassae*.

Gromko *et al*. (1984) have reported that under the periodic confinement design the receptivity of females is sperm dependent. In periodic confinement designs, Gromko and Gerhart (1984) reported that the number of sperm in storage is not affected by density, hence female remating is also not affected by density. Further, for continuous confinement they have postulated that in all high-density vials, males were seldom uninterrupted while courting. However, in low-density vials males frequently directed sustained courtship at individual females without being displaced by other males. They also suggested that the decrease in female remating frequency at higher densities is due to increased effectiveness of female "decamping" in the continuous confinement design. However, Eckstrand and Seiger (1975) proposed that in a population of high density, the probability of a given fly's being a fast mater would be higher than in a less dense population because the incidence of proper cues for the manifestation of fast mating would be higher. Pearl (1932) has suggested that an increase in density could lead to more mating simply by increasing the opportunity for interaction between individuals in the population. Further, Harshman *et al*. (1988) proposed that there is genetic variation for density dependence in remating frequency, i.e., the effect of density on the frequency of remating depended on the fly strains used.

The present findings in *D. ananassae* support the statements by Pearl (1932), Eckstrand and Seiger (1975), and Harshman *et al*. (1988). If female remating has a genetic basis (see Sgro *et al*., 1998), individual females may vary in their receptivity to remate. van Viannen and Bijlsma (1993) suggested that female remating frequency is affected by the genotype of their first male and proposed that this could be due to differences in the amount or quality of seminal fluid transferred during copulation.

In *D. ananassae*, female remating with respect to productivity and sperm displacement has been studied by employing different mutant strains and a wild-type strain (Singh and Singh, 2001). The comparison of productivity between once-mated and remated females reveals that the productivity of remated females was significantly higher than that of once-mated females in all crosses. Thus female productivity is increased after remating in *D. ananassae* (Singh and Singh, 2001). Further, high $P2$ values (the proportion of second male progeny produced after remating) were found in all the crosses, which

provides evidence for sperm precedence of the second male to mate, suggesting the existence of sperm displacement in *D. ananassae* (Singh and Singh, 2001).

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