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Repeated mating in a lek-mating insect, *Drosophila melanogaster.*

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Introduction

Females of many diverse animal species mate multiple times in nature (Andersson, 1994; Johnson and Burley, 1997). Often, such "multiple mating" is performed with different male partners, as in the fruitfly *Drosophila melanogaster* (*e.g.*, Minamori and Morihira, 1969; Fuerst *et al*., 1973). In general, copulation is assumed to be costly for many reasons (Hunter *et al*., 1993). Thus, explaining the frequent occurrence of multiple mating, particularly when new copulations do not result in or are not necessary for fertilization of eggs, is an important area of research in evolutionary and behavioral biology. A number of hypotheses have been proposed to explain the occurrence of multiple mating, and there is general empirical support for these (reviewed in Petrie *et al*., 1992).

Sometimes, females remate with the same male partner ("repeated mating"; Hunter *et al.*, 1993). It is unclear how widespread repeated mating is, since reports from only a limited number of species exist (Petrie, 1992; Petrie *et al.*, 1992; Hunter *et al.*, 1993; Choe, 1995; Lens *et al.*, 1997; Brown, 1997; Andrade and Mason, 2000). Although the groups of hypothesized explanations of multiple mating and repeated mating overlap, they are different (Hunter *et al.*, 1993). We will not review these hypotheses in any detail here, but it is sufficient for this report to state that in general there is little empirical evidence in support of any of these hypotheses. Additional diverse species exhibiting repeated mating will be useful for further testing of these hypotheses. Here we report repeated mating in *D. melanogaster*.

Materials and Methods

General courtship behavior: The courtship and mating behavior of single pairs of *D. melanogaster* under laboratory conditions is well-characterized (Bastock and Manning, 1955; Hall, 1994; Greenspan and Ferveur, 2000). Typically, healthy (food and a water source), young (3-5 days old), virgin *D. melanogaster* males and females will begin the courtship ritual within 5-60 seconds post-introduction, copulate within 5-10 minutes, and will copulate for 12-20 minutes.

Fly strains used: John Carlson (Yale University) provided the standard laboratory *D. melanogaster* "Oregon-R" strain, Trudy Mackay (North Carolina State University) provided *D. melanogaster* iso-female strain "M99-419" (collected in 1999 in North Carolina), Gary Karpen (The Salk Institute, La Jolla, CA) and the Bloomington *Drosophila* Stock Center (Indiana University) provided the *yellow*$^{-}$ and *yellow*$^{+}$ body color mutants, and Michael Turelli (UC-Davis) provided the *D. simulans* "Watsonville" strain.

Remating assays: We assayed remating in two distinct ways which yielded quantitatively different outcomes (see Results). In the first, we utilized the "Copulatron", a multiple-mating chamber described in Drapeau and Long (2000). Very briefly, the Copulatron contains 49 cylinder-shaped

chambers about _" deep and the diameter of a U.S. quarter, about 1". Each chamber has a small side-chamber the diameter of a pencil eraser into which fly food is pipetted, allowing flies to survive in the chambers for a long time. In the second method for assaying remating, males and females were placed into 8-dram food vials (cornmeal/molasses food) for a day, and then males were transferred into female vials without anesthesia, by "tapping". This is a commonly-used setup for a general mating assay. In both treatments, virgin flies were collected at < 8 hours of age and stored in same-sex food vials for four days in groups of ~ 5-10. At 4 days old, flies were lightly and briefly aspirated and placed either into the top or the bottom of Copulatron chambers (separated by dark acetate) or into vials, one fly per vial. Flies were allowed to recover from CO_2 handling for ~ 24 hours. After that, acetate was removed from the Copulatron, and males from male vials were transferred without anesthesia into female vials by tapping. Courtships and copulations were then observed by eye and data recorded by hand in real time for 5 hours. Despite a reported "short-term copulation effect" (*i.e.*, females are not receptive for up to 24 hours after copulating) (Manning, 1967), it has been reported multiple independent times that fair proportion of *D. melanogaster* females will multiply mate within a 6 hour period (Scott and Richmond, 1985; Scott, 1987; Van Vianen and Biljsma, 1993). The sibling species *D. simulans* was used for an identical experiment to that with *D. melanogaster*, in the Copulatron only.

Viable offspring output: After repeated mating assays, flies were lightly anesthetized in the Copulatron with CO_2 (see Drapeau and Long, 2000) and placed in glass food vials with standard banana/corn syrup food (Rose Lab recipe). We kept track of individual females so that we could look for an association between the number of times a female mated and her lifetime adult offspring output. Adult offspring output was scored on Day 1 and Day 2, and then females were left in vials for 3 days at a time for the remainder of their lives. The total offspring number from each of those 3-day vials was scored. For every vial, ample time was allowed for every adult to hatch before counting.

Individual Recognition: We wished to determine whether in this species the female "knows" that she is mating with the same male. The "individual recognition" experiment involved "switching" the males after a first copulation had ended to determine if repeated mating was dependent on if the male had been changed ("experimental" treatment) or if the same male was reintroduced ("control" treatment). Initially, M99-419 males and females were placed into 8-dram food vials (cornmeal/molasses food) for a day. Males were left in small groups, and females were isolated singly. Female vials were numbered sequentially. On the day of assay, males were transferred into female vials by mouth aspiration and copulations were observed. Immediately after copulation ended, the male and female in each vial were separated without anesthesia by mouth-pipetting males out of female vials. We then waited 2 hours. Then, in roughly half the cases, the same male was put back in the vial by either "tapping" transfer or mouth pipetting, and in the other half, males were switched among vials in a haphazard fashion. We also randomly chose 18 vials (~ 10%), using a random-number table, to be controls in which we did not remove males at all. After the transferring of males, we watched vials to determine if remating occurred within 5-6 total hours of observation. The total sample size of the experiment was approximately 180 pairs of flies. Unfortunately, experimental design issues prevented us from sufficiently answering the question at hand. We observed no remating within 5-6 hours in any treatment in our experiment with strain M99-419. This result is most likely due to the fact that in order to "switch" male partners for females (see Methods), we transferred males from vials to vial by mouth pipetting, and otherwise handling the vials. This was done twice – once to remove the male and once to reintroduce him into a vial. Despite our best attempts to be gentle, this most likely disturbed the males enough to discourage active male courting after an initial copulation. It may have also disturbed females such that they were in some cases unreceptive despite male courtship.

Results

How often does repeated mating occur in 5 hours?: We confined single pairs of 5-day old virgin *D. melanogaster* flies in small mating chambers, either in the Copulatron or in food vials, for approximately 5 hours and observed the number of copulations that occurred between each pair. We performed two experiments with iso-female strain M99-419 on two consecutive days with flies collected from the same set of vials. On each day, an experiment in the Copulatron and an experiment in vials was performed. Some summary results from these four experiments are presented in Table 1. The most notable result is that the frequency of repeated mating was greatly reduced in both experiments when flies were assayed in vials compared to the Copulatron. This is most likely due to the fact that the flies in the vial treatment were treated more roughly than the flies in the Copulatron, thus disturbing them. Mean durations of Copulation #1 and Copulation #2, and the time between Copulation #1 and #2, are extremely similar between the two Copulatron treatments and the two vial treatments. Similarly, within the Copulatron treatment where sample sizes were sufficient for statistical analysis, the durations of Copulation #1 and Copulation #2 were not different from each other within either the first (t = 0.8996, d.f. = 12, P = 0.3863) or second (t = -1.003, d.f. = 13, P = 0.3344) experiment. The above general result, that remating often occurs within 5 hours of confinement in the Copulatron, was qualitatively confirmed in a completely independent experi-ment performed in the Copulatron over a month later with the same strain, M99-419. In this experiment, 7/31, or 22.6%, of females remated within 5 hours. The combined data from the two previous Copulatron trials showed that 28/81, or 34.6%, of females remated. Recourting and initial time-at-remating

Table 1. Behavioral aspects of laboratory repeated mating in *Drosophila melanogaster.* "D1" and "D2" are experiments 1 and 2 performed on days 1 and 2, respectively. "Tron" = Copulatron treatment and "Vial" = vial treatment (see Methods).

Frequency Distributions of Copulations

	0	1	2	3	N
D1 Tron	1	22	14	1	38
D1 Vial	8	30	2	0	40
D2 Tron	2	28	13	0	43
D2 Vial	8	29	3	0	40

Time Until Copulation 1 (minutes)

	Mean	SD	N	SE
D1 Tron	3.45	3.29	37	0.54
D1 Vial	15.31	14.88	32	2.63
D2 Tron	17.28	7.00	41	1.09
D2 Vial	20.86	14.84	32	2.62

Duration of First Copulation (minutes)

	Mean	SD	N	SE
D1 Tron	13.81	7.46	35	1.26
D1 Vial	15.00	3.49	32	0.62
D2 Tron	13.52	2.63	41	0.41
D2 Vial	14.89	4.31	32	0.76

Time Between Copulations 1 and 2 (minutes)

	Mean	SD	N	SE
D1 Tron	200.73	49.24	15	12.71
D1 Vial	222.16	31.03	2	21.94
D2 Tron	211.96	43.20	13	11.98
D2 Vial	110.57	44.27	3	25.56

Duration of Second Copulation (Minutes)

	Mean	SD	N	SE
D1 Tron	14.57	2.88	15	0.74
D1 Vial	14.34	3.45	2	2.44
D2 Tron	13.53	4.26	13	1.18
D2 Vial	17.03	6.70	3	3.87

occurred at qualitatively similar times in the third and in the first two trials. Anecdotally, we note that the initial observation of remating in the Copulatron was made on F_1 completely outbred male flies in both *yellow* mutant and wild-type genetic backgrounds, and highly inbred "Oregon-R" wild-type females. As many as 9 copulations in ~ 5-6 hours were observed between the same pair of flies. More common were 2-3 copulations in this time span, however. Contrastingly, chambers containing Oregon-R males with outbred *yellow* or *yellow*$^{-}$*; yellow*$^{+}$ flies rarely remated. In total, the data suggest that there may be genetic variation, at least among laboratory strains, for this behavior.

Does same-pair remating increase female offspring output?: Repeated mating may increase total lifetime offspring production. We hypothesized that there could be such a fitness benefit to females who remated with the same male in our experiments. We found no evidence for this hypothesis (Two-way ANOVA analysis, details not shown). In two replicate experiments, we found no differences in lifetime adult offspring production between females from strain M99-419 that mated a single time in our experiments, and females that mated twice (Mean ± SD: Expt. 1: copulated twice: 46.4 ± 40.8 (N = 10), copulated once: 27.7 ± 29.3 (N = 22); Expt. 2: copulated twice: 27.5 ± 32.1 (N = 13), copulated once: 26.0 ± 27.8 (N = 28)). Additionally, Wilcoxin tests on each day comparing females that mated with their male partner once versus twice yielded no significant values for any day in either treatment. In all treatments there were numerous females that had no adult offspring whatsoever (these data are included in the averages above). Hence, there did not appear to be a "material benefit" to mating twice, at least within 5 hours. This may be because repeated matings have to be spaced (until sperm is depleted) to receive a benefit (see Pyle and Gromko, 1978).

Does a sibling species remate in the lab?: We wished to determine if *D. simulans*, a sibling species of *D. melanogaster*, similarly remated within 5 hours under laboratory conditions. We found no evidence that *D. simulans* pairs of the "Watsonville" strain remate within 5 hours; indeed, we observed not a single case in 23 total pairs. We noted that 10/23 pairs mated within 30 minutes, 13/23 in 1 hour, 14/23 in 1.5 hours, 15/23 in 2 hours, 16/23 in 2.5 hours and 3 hours, and 18/23 in 4 hours and 5 hours. Male courtship after initial copulation was relatively rare, but was observed as early as 2:20:58 elapsed time. The observed remating difference between species may be because *D. simulans* genuinely behaves different in this respect from *D. melanogaster*. It is not known what the natural mating system of *D. simulans* is (*i.e.*, Do they mate in leks?). It may equally be explained by the fact the premating ritual of *D. simulans* and its sibling species *D. mauritiana* and *D. sechellia* is distinctly different from *D. melanogaster*. It is known that unlike *D. melanogaster*, which when virgin and healthy will begin their courtship ritual within seconds or at most ~ 5 minutes, males and females of the other species often, but not always, remain uninterested in each other for ~ 30 minutes, and then suddenly begin courtship and mating (M.D.D. and A.D.L., in writing; M.D.D. unpublished observations). That this is true is shown in the following *D. simulans* data: 10/23 copulations occurred in the first hour, but 8/23 copulations occurred between hours 1 and 5. (This can be compared to, for instance, one replicate of *D. melanogaster* strain M99-419: out of 31 pairs, 22 copulated in the first 1.5 minutes, and 26 copulated within the first hour. There were 7 rematings within 5 hours.) It is worth noting that the actual duration of both courtship (once it is initiated) and copulation are qualitatively similar among species. It is possible that 5 hours is not sufficient for remating to occur in *D. simulans* (we used 5 hours to allow a direct comparison to *D. melanogaster*).

Discussion

Mating terminology: Hunter *et al.* (1993) coined the term "repeated mating" to mean, specifically, repeated mating between the same male and female pair. Hunter *et al.* (1993) state that the

term “multiple mating” should be used to describe mating between a single individual and multiple, different, partners of the opposite sex. Because the hypotheses explaining the behaviors, and the behavioral, social, life-history, and genetic consequences of the behaviors, are generally different, the authors of this paper again note that different terms should be used in the literature. In the past, “repeated mating” has been used to describe what is really “multiple mating” as defined here, for instance, in *D. melanogaster* (Gromko and Pyle, 1978; Pyle and Gromko, 1978, 1981) and in other *Drosophila* species (Dobzhansky and Pavlovsky, 1967; Richmond and Ehrman, 1974). Additionally, the terms “remating” and “female remating”, often used, cease to be useful in the context of these two distinct female behaviors.

Mating behavior experimental design: We showed that the frequency of repeated mating is greatly affected by the experimental method used for setting up mating assays. In the less-disturbing Copulatron, repeated mating occurred at about 20-30%. Repeated mating nearly never occurred using a more disturbing method of “tapping” or “banging” flies from one vial into another. This suggests that for looking at behaviors that could possibly be sensitive to the treatment of the insect (most of them), methods should be used which minimize the disturbances. We designed the Copulatron (Drapeau and Long, 2000) for just this reason, and this behavioral report is a justification for this.

Hypotheses explaining same-pair remating: Although discussing theories of repeated mating is outside the scope of this short report, we would like to note that this observation of repeated mating by *D. melanogaster* in the laboratory may be useful for testing this general body of theory in the future. In particular, the observation by Taylor and Kekic (1988) that *D. melanogaster* mating occurs in leks on relatively undisturbed brandy barrels in huts on orchards Belgrade, Yugoslavia suggests that this species could act as a model for understanding this complicated mating behavior from both evolutionary and genetic standpoints.

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