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Brief Communication

# Direct observation of female mating frequency using time-lapse photography

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**One basic condition of postmating sexual selection is that females mate more than once before fertilizing their ova. Knowledge of the frequency and extent of multiple mating in a given population or species is therefore important in order to fully understand the potential for sexual selection, in the form of sperm competition, sexual conflict and cryptic female choice. Surprisingly, there are only a handful of studies that have attempted to estimate the frequency of multiple mating in insects (including Drosophila) and none have made direct observations over extended periods of time. Here we use time-lapse photography to directly score matings in isolated pairs of *D. melanogaster* and show that multiple mating in the laboratory occurs at a high frequency but at comparable rates with wild caught females. We also find that the interval to remating rises approximately additively with each mating, indicating either an increase in female resistance or male reluctance to remate. These results suggest that the opportunity for postmating sexual selection in laboratory and natural environments are not dramatically different and that there may be a causal link between the rise in female mating resistance and the concomitant rise in the cost of mating. The method is easily executed and could be adapted to other insect models.**

Multiple mating by females is poorly understood[1] and yet has a profound influence on sexual selection, extending its reach to postmating processes of sperm competition,[2] sexual conflict[3] and cryptic female choice,[4] which are important in shaping the behavior, morphology and physiology of internally and externally fertilizing animals.[5-7] Reliable and accurate information on female mating frequency is therefore a vital component to complete our understanding of the strength of postcopulatory sexual selection currently operating in a given study population. Surprisingly, for many taxa, including well studied model organisms such as fruit flies, there is almost no direct observational data on the number of sexual partners that females have.

Direct observations of female mating frequency in nature are of course logistically difficult and sometimes severely underestimate true values. For instance, reliance on field observations led Lack[8] in 1968 to suggest that 93% of all passerine birds were monogamous, whereas with the advent of DNA fingerprinting we now know that 86% of all bird species are actually promiscuous to some extent.[9] Obtaining data on the female mating frequency of insects in the wild is perhaps even more challenging, and although mating frequency data is accessible for Lepidoptera[10] (due to the persistence of spermatophore remnants), most of what we know about insect mating frequency comes from indirect measures of laboratory reared broods,[11,12] where paternity can be assigned to each offspring according to markers (phenotypic, allozyme or molecular sequences) or where some proportion of the potential sires have been sterilized using radiation or chemicals (the sterile male technique).[13] Obtaining female mating frequency of drosophilids is no exception, where there is a long tradition of using phenotypic markers (typically eye-markers) in sperm competition assays.[2,12,14,15] However, one serious drawback of using phenotypic markers or male sterilization methods is that usually only two classes of males are employed, so repeated mating by females with males belonging to the same class cannot be detected. Experiments using multiple markers are rare[16] or not possible (e.g., with the sterile male technique) and they still suffer from the same problem of being unable to distinguish repeated matings with males of the same class. Direct observations of matings in the laboratory are also difficult for extended periods of time. Usually a window of time is chosen in which to scan for mating pairs, or females are only exposed to males intermittently. These methods will therefore either underestimate or constrain the mating frequency of females that are continuously exposed to males, and can therefore only give an estimate of the proportion of a population that remates, rather than of mating frequency per se.

Molecular markers offer considerable advantages over other indirect methods, because if many polymorphic loci are included

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in an analysis then the ability to assign paternity unambiguously to each offspring is greatly enhanced. This data can then be used to infer both the female mating rate and the differential success of each male's sperm. However, these high-resolution techniques also require a high technical effort (at significant expense) because DNA sequences must be obtained from each offspring for each locus studied. To our knowledge this approach has been used only twice previously to estimate the mating frequency of wild-caught female *Drosophila melanogaster*; both indicated that multiple mating by females is common. First, Harshman and Clark[17] analyzed allele frequencies of two highly polymorphic microsatellite markers from broods of females caught at the Ravenswood Winery, Sonoma, CA. Using a maximum-likelihood method the mating rate was estimated to be 1.82 males per female. However, due to computational limitations, the maximum number of possible sires per brood in the model was limited to four. A reanalysis of the Ravenswood data using a Bayesian approach,[18] without this restriction, gave a revised estimate of 2.44 matings per female, with a 95% confidence interval of 1.64–3.32 matings per female. A second study examining seven microsatellite loci from offspring produced by females caught in Vienna, Austria,[19] concluded that females had mated with 4–6 different males. However, in both of these studies only a small number of broods were examined (19 from Ravenswood and four from Vienna), probably reflecting the effort required in these kinds of methods.

Here we describe an experiment where we directly recorded the number of times individual females mate in a laboratory situation using time-lapse digital photography. The method used has several benefits: (1) it provides data on actual mating frequency; (2) there is no need for rearing and scoring of offspring; (3) there is no need for backcrossing phenotypic markers into the focal population or for sterilizing males; (4) the start-up/running costs are considerably lower than for molecular methods; and (5) digital images compare favourably with film where the costs of developing hundreds of prints large enough to confirm mating pairs would be prohibitively expensive.

All flies were from a large outbred base population $LH_M$.[20] We began by mating individual females once to males from our base population in individual test-tubes. All copulations were observed and any pairs that had not mated after 60 min were discarded. Each male was then removed under light $CO_2$ anaesthesia and a second novel male introduced. The tubes were then placed on a white tray (to improve visual contrast) in two rows within a rearing cabinet set to 25°C temperature, 65% humidity and continuous light. The test-tubes contained 10 ml of light coloured cornmeal-agar-sugar food that had been tilted soon after pouring to obtain a slanted surface which was oriented upwards. These tubes were plugged with an obliquely cut foam closure, also facing upwards. The light coloured food facilitated locating mating pairs in the photographs and the slanted food and closure ensured there was no "dead-space" in which flies could hide from view of the camera. A digital SLR (Nikon D70) was positioned overhead using a tripod and connected via a USB cable to a PC computer running Nikon Camera Control Pro 2 software (Nikon Corporation, USA). The camera was pre-focused on the array of test-tubes with a 50 mm lens. The aperture of the lens was set to f10 to ensure sufficient depth of field and the exposure time was set to 1/125 s to avoid blurred images of flies (these settings will vary according to the amount of incident light available). The time-lapse tool in Camera Control Pro 2 was then used to capture an image every 5 minutes for 48 hours, resulting in 576 photographs (see Suppl. File 1 for an example). Copulation usually lasts around 19.5 min (s.d. = 3.87 min) in our population and so this 5-minute interval is likely to capture the start and finish of all successful copulations. Images were checked for putative mating pairs and scored as a mating if the pair appeared in copula in three or more consecutive frames: 14 matings occurred over three frames; 13 occurred over four frames; two occurred over five frames. An initial scan can be quickly performed to rule out a copulating pair if at least one individual fly is seen in each tube. Confirmation that a mating has occurred can be achieved by inspecting images at full magnification. We found that the mean mating frequency was 2.7 times (mode = 2) with a 95% confidence interval of 2.17–3.24 times over the two-day period of observation, a single female mated a total of five times (Fig. 1; n = 17). 94% of females had remated at least once, thereby creating the opportunity in the vast majority of females for postmating mechanisms of sexual selection to operate. The mating frequency point estimate and 95% confidence interval are remarkably similar to that estimated from wild caught females and represents the entire period of reproductive activity that this population experiences during normal culturing.[21] However, since this experiment used isolated pairs the mating frequency of females found here may be lower than would be obtained if males and females were not isolated, assuming either males gain by mating with multiple partners or the motivation to mate increases with the availability of novel partners.

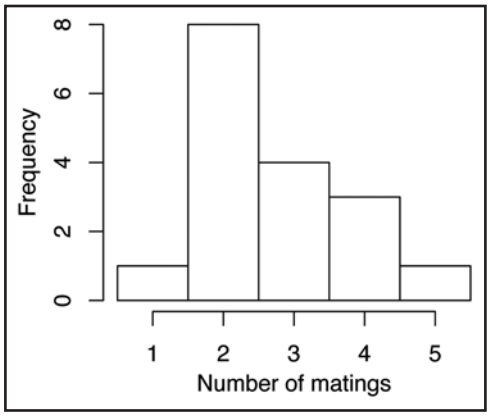


Figure 1. Frequency histogram of female mating frequency over a 48-hour period of continuous observation.

The sequence of photographs also allows us to examine how mating intervals are influenced by mating history. A linear mixed model including a random slope, mating interval duration as the dependent variable, number of matings as a fixed effect and individual female as a random effect indicates that there are significant differences in mating interval according to how many times a female has previously mated (Fig. 2), and that with each additional

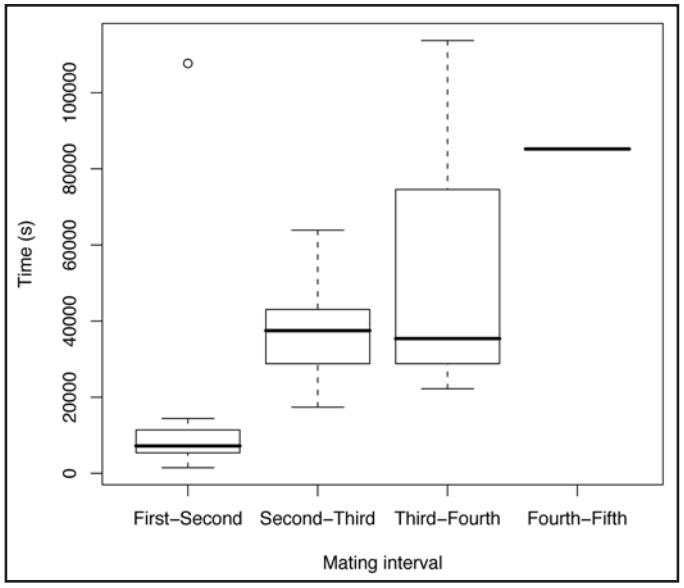


Figure 2. A box-and-whiskers plot of time intervals (s) between sequential matings showing a clear increase in mating interval with each additional mating.

mating the mating interval increases by a factor of 2.44 (coefficient $\log_{10}$ mating interval (±S.E.) = 0.893 ± 0.169, $t_4$ = 5.27, AIC = 79, p = 0.0002; p-values are based on an ANOVA comparison with a model in which the factor number of matings was removed). Including a polynomial term in this model, describing a non-linear relationship between mating interval and the number of previous matings, makes a small but significant improvement to the fit (coefficient $\log_{10}$ mating interval$^2$ = -0.528 ± 0.157, $t_7$ = -3.35, coefficient $\log_{10}$ mating interval = 4.028 ± 0.966, $t_7$ = 4.17, AIC = 75.67, p = 0.021), indicating that mating interval increased approximately additively between mating events (see Fig. 2). Thus, our data suggests that not only is the opportunity for postcopulatory processes of sexual selection in the laboratory environment comparable with that found in natural populations of *D. melanogaster*, but that female resistance to mating approximately doubles with each additional mating. This latter result is particularly interesting given that it is known that mating costs for females also rise (nonlinearly) with each additional mating,[22] suggesting either that part of these costs may derive from increases in the level of female behavioral resistance to male advances, or that female reluctance to remate is due in part to these accelerating costs. An alternative explanation is that the increasing mating interval is due to an increase in male (or female) reluctance to remate the same partner, a phenomenon known as the Coolidge effect.[23] Although this has been documented in several taxa,[23,24] and virgin flies have been found to prefer novel partners,[25] we know of no study that has shown this effect in Drosophila.

In conclusion, our study demonstrates that time lapse photography is an effective technique enabling one to estimate costs and benefits of repeated mating with much greater precision than previously possible. Further embellishments to the technique, such as use of infrared light during dark conditions and increased resolution for the observation of multiple individuals per vial, may see this technique's application to a wide range of studies investigating mating rates and other behavioral components involved in sexual selection.

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