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Multiple paternity in wild-caught *Drosophila mojavensis*

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Abstract

Female remating frequency and sperm allocation patterns can strongly influence levels of sperm competition and reproductive success in natural populations. In the laboratory, *Drosophila mojavensis* males transfer very few sperm per copulation and females remate often, suggesting multiple paternity should be common in nature. Here, we examine female sperm loads, incidence of multiple paternity, and sperm utilization by genotyping progeny from 20 wild-caught females at four highly polymorphic microsatellite loci. Based on indirect paternity analyses of 814 flies, we found evidence for high levels of multiple paternity coupled with relatively low reproductive output, consistent with the high levels of female remating predicted in this sperm-limited species. Overall, we found little evidence for last - male sperm precedence though some temporal variation in sperm utilization was observed, consistent with laboratory findings.

Keywords: mating frequency, microsatellites, multiple paternity, natural populations, sperm utilization

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Introduction

Species of *Drosophila* exhibit remarkable diversity with respect to the number of sperm males transfer during a single copulation and the frequency with which females remate (Markow 1996; Singh *et al*. 2002). These two characters appear to be inversely correlated: species in which females receive few sperm are characterized by more frequent female remating than those species in which females receive thousands of sperm (Markow 2002). For example, males of *Drosophila pachea*, *Drosophila hydei*, *Drosophila mojavensis*, and *Drosophila buzzatii* have been estimated to transfer less than 150 sperm (Markow *et al*. 1990; Pitnick & Markow 1994a, b; Bundgaard & Barker 2000) while over 4000 and 25 000 sperm are transferred in *Drosophila melanogaster* and *Drosophila pseudoobscura*, respectively (Gilbert 1981; Snook *et al*. 1994). Females of *D. pachea*, *D. hydei*, and *D. mojavensis*, and *D. buzzatii* all remate within 24 h or less (Markow 1982, 1985; Pitnick *et al*. 1991; Bundgaard & Barker 2000), while *D. melanogaster* and *D. pseudoobscura* females usually wait several days before remating (Pyle & Gromko 1981; Markow 2002). Frequent female remating appears to have arisen first and led to a variety of pre- and postcopulatory evolutionary responses by males in different species (Markow 2002). In addition to the importance of mating system features to studies of sexual selection, the frequency and timing of remating and sperm utilization in relation to dispersal can have important implications for population genetic structure (Hurtado *et al*. 2004; Pfeiler *et al*. 2005).

The majority of studies of *Drosophila* remating and sperm utilization have been conducted in the laboratory; however, their implications for natural populations have yet to be systematically examined. Average sperm loads of wild-caught females have been measured with progeny counts for only a few *Drosophila* species, but support the species differences in reproductive output observed in the laboratory. For example, *D. melanogaster* females collected in the field produce an average of about 300 offspring (Stalker 1976a; Gromko & Markow 1993) and *D. pseudoobscura* females produce nearly 400 (Snook & Markow 2002). Conversely, smaller broods have been observed for two species that exhibit rapid remating in the laboratory, *D. euronotus* (~30 offspring per brood; Stalker 1976b) and *D. buzzatii* (175–240 offspring per brood; Bundgaard & Barker 2000). Similarly, only a few studies have used genetic markers to detect female remating in wild populations of *Drosophila*. Multiple paternity has been detected in all cases: *D. pseudoobscura* (Anderson 1974; Cobbs 1977), *D. euronotus* (Stalker 1976b), *D. melanogaster* (Harshman & Clark 1998; Imhof *et al*. 1998), *D. buzzatii* (Bundgaard *et al*. 2004), and *Drosophila simulans* (Schlötterer *et al*. 2005).

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Here we examine sperm loads and the frequency of multiple paternity in wild-caught females of *D. mojavensis*. Based upon mating system characteristics and ecology, we expect the progeny of wild-caught female *D. mojavensis* to reflect a large number of sires. First, as mentioned above, female *D. mojavensis* show a high remating rate in the laboratory. Because males transfer fewer sperm per copulation (68.9 ± 8.1 based on laboratory progeny counts; Markow *et al*. 1990) than a female could potentially use, singly mated females are essentially sperm limited (e.g. Wedell *et al*. 2002). Hence, frequent remating may be essential in assuring high female reproductive output (Markow 1982). Furthermore, males provide proteins in their seminal fluid that are utilized by female somatic tissues and developing oocytes (Markow & Ankney 1984). In nature, *D. mojavensis* feeds and breeds on necrotic cactus (Heed 1982) and adult flies may frequently experience nutritional stress (Brazner *et al*. 1984). When dietary resources are limited, nutrients derived from seminal fluid may play an important role in maximizing female fecundity (Markow *et al*. 1990). In the case of multiple matings, ejaculatory donations from previous males appear to benefit progeny sired by subsequent males (Markow 1988). If last-male sperm precedence were common in nature, then the potential for cuckoldry would be especially high in this system. However, in laboratory experiments, last-male sperm precedence disappears relatively quickly following insemination, and sperm utilization patterns are consistent with a model of equal mixing in eggs oviposited a few days after copulation (Markow 1988). Therefore, we expect that when multiple fathers are detected, they should contribute somewhat equally to the recovered broods. We tested these predictions by examining the sperm loads and the prevalence and pattern of multiple paternity in the progeny of 20 wild-caught inseminated *D. mojavensis* females using genetic variation at four highly polymorphic microsatellite loci.

Materials and methods

Sample collection

We collected a total of 52 female *Drosophila mojavensis* by a combination of bait trapping and direct aspiration off of active cactus rots in Organ Pipe National Monument, Arizona, USA. All samples were immediately aspirated into individual vials and transferred to new vials every 24 h for the first 5 days followed by every 48 h until they stopped laying eggs. Newly eclosed adult offspring were collected and snap frozen every 24 h.

Microsatellite genotyping

We used a modified squish preparation to extract DNA from all samples (Gloor *et al*. 1993). Multilocus genotypes were generated using three dinucleotide (A2131, M2192, and M496) and one trinucleotide (M3147) repeat microsatellite loci. All four markers were developed from genomic libraries of *D. mojavensis* and *D. arizonae* (Ross *et al*. 2003) and are unlinked (C. L. Ross & T. A. Markow, unpublished). We tagged one primer per locus with 6-FAM or HEX fluorescent dye (Applied Biosystems) and amplified all four loci using a single multiplexed polymerase chain reaction (PCR). The primer sequences were as follows: A2131 forward 5′-CAGAAATCGTTTCATTCATGC-3′, reverse 5′-CGCTTGGACAACTTTCAGC-3′; M2192 forward 5′-CCTTATCGCTGCTCGACTCC-3′, reverse 5′-AGGAAAACTTCAGCCAGACG-3′; M3147 forward 5′-CAAGATAGCCACAATCAAGTCG-3′, reverse 5′-TGTAACCCACTCGCTAAATGC-3′; M496 forward 5′-TCAACTGGAAGCTGTTAAATATCG-3′, reverse 5′-CATGCATCAGGCTTATCTCC-3′. Diluted PCR products were genotyped by the Genomics and Technology Core facility at the University of Arizona using an ABI 3100 Genetic Analyser. We used GENOTYPER version 1.1 (Applied Biosystems) to score all alleles.

Sperm loads in wild-caught females

Given the low number of sperm transferred during laboratory matings, we wanted to determine the average sperm load found in a natural population of *D. mojavensis*. We used total progeny counts to estimate the minimum number of sperm within an inseminated wild-caught female. Females not producing progeny were assumed to be virgins.

Indirect paternity analyses

To estimate the incidence of multiple paternity, we genotyped the mother and a sample of progeny from each of 20 broods. Initially, we genotyped approximately 20 progeny per brood (range 15–25). Broods were selected from across the distribution of brood sizes (see Fig. 1). Offspring were chosen from a number of oviposition vials to avoid potential bias introduced from nonrandom sperm usage across time. Subsequently we expanded our sampling in four broods in order to determine if we were systematically underestimating the number of fathers within each brood and to better examine potential temporal variation in sperm usage.

Direct information on the genotype of potential sires was absent from our data. Therefore, we performed likelihood-based tests of relatedness to estimate the number of fathers within each brood, as implemented in the program KINSHIP version 1.3.1 (Goodnight & Queller 1999). We tested the relatedness among offspring within a brood based on the probability that shared alleles are identical by paternal (R_p) or maternal (R_m) descent. To identify the number of

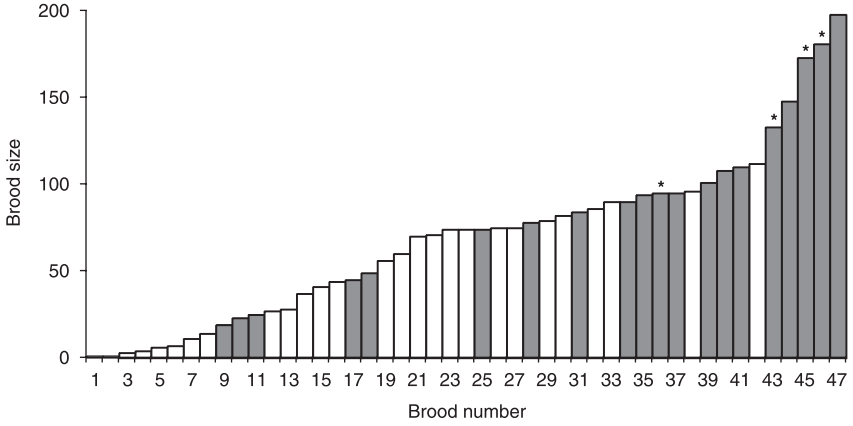

Fig. 1 The distribution of brood sizes for the 47 wild-caught *Drosophila mojavensis* females that successfully produced at least one offspring. Shaded bars represent broods included in paternity analyses. The four broods indicated with an * were sampled in greater detail to examine the impact of sample size on our estimates of multiple paternity and potential temporal variation in sperm usage.

sires per brood, we tested the primary hypothesis that offspring pairs shared both a mother and a father through direct descent ($R_p = 0.5$, $R_m = 0.5$). Since all individuals in a brood share the same mother, the null hypothesis-is that two individuals will only share maternal alleles ($R_p = 0$, $R_m = 0.5$). Offspring pairs were identified as half- or full-siblings based on the log-likelihood ratio (LOD base-10) of these two hypotheses (null/primary). Population frequencies of alleles at each locus ($N = 232$ individuals; C. L. Ross & T. A. Markow, unpublished) were used to account for background levels of allele sharing in the likelihood calculations. We determined statistical significance by simulating multilocus genotypes for 10 000 pairs of individuals conforming to the null and primary hypotheses and the specified population allele frequencies. These data were then used to determine the likelihood ratio needed to reject the null hypothesis at a 95% confidence level. Individuals were considered full-siblings if at least 80% of the pairwise LOD scores were significant at the 95% confidence level (Storz *et al*. 2001). In situations where individuals failed to meet this criterion, a unique father was inferred only if less than 20% of the individual's LOD scores were significant with all other full-sibling groups. All individuals not meeting either criterion were excluded.

Results

Microsatellite variation

The number of alleles sampled at each locus and estimates of observed and expected heterozygosities from an Organ Pipe National Monument population sample ($N = 232$ individuals; C. L. Ross & T. A. Markow, unpublished) are given in Table 1. Two loci, M3147 and M496, showed significantly reduced heterozygosity in this sample compared to Hardy–Weinberg expectations (C. L. Ross & T. A. Markow, unpublished).

Table 1 Variability of microsatellites

Locus	Alleles*	H_O†	H_E†
A2131	11	0.78	0.85
M2192	11	0.86	0.82
M3147	9	0.69	0.80
M496	16	0.68	0.79

*Number of alleles sampled in this study.
†Population-level heterozygosity (C. L. Ross & T. A. Markow, unpublished).

Sperm loads in wild-caught females

Of the 52 wild-caught females we examined, 47 produced a brood (90.4%). We collected a total of 3247 progeny from these 47 females, resulting in an average of 69.1 (± 7.0 SE) offspring per brood. The distribution of progeny numbers across these 47 broods is given in Fig. 1.

Incidence of multiple paternity

Overall, we genotyped a total of 814 flies (794 total progeny and 20 mothers; Fig. 1) at each of four microsatellite loci (see Table S1, Supplementary material). Using indirect paternity analysis (Goodnight & Queller 1999), we found that all 20 broods had been sired by at least two fathers and one had as many as six sires. Figure 2 gives the number of fathers represented in each brood and the proportion of offspring sired by each. Overall, we found an average of 3.1 (± 0.28 SE) sires per brood based on 779 progeny. Fifteen offspring were excluded from this analysis because we were unable to unambiguously assign them to a full-sibling group. To examine the extent to which our sampling resulted in an underestimate of multiple paternity, we expanded the number of offspring genotyped in each of four broods. We found an increase in the number of fathers present in one of the four broods (Table 2).

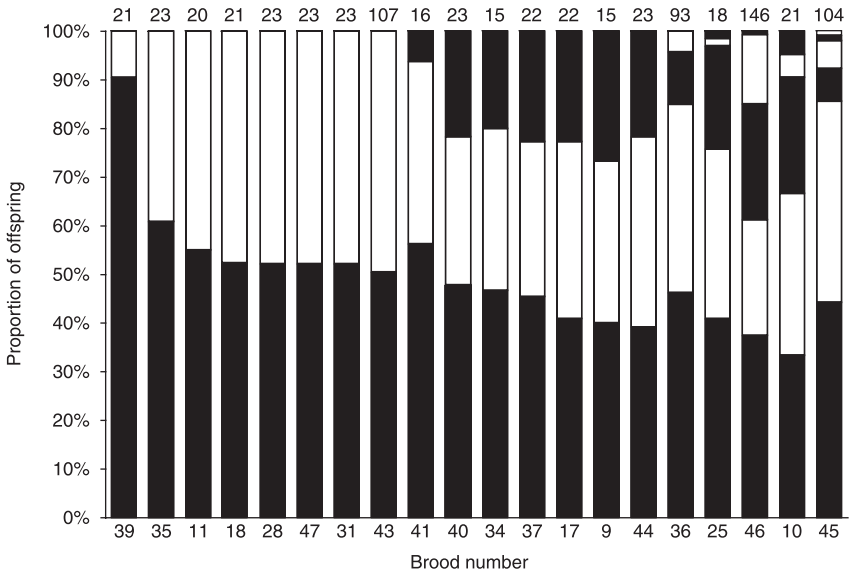

Fig. 2 Patterns of multiple paternity in the broods of 20 wild-caught female *Drosophila mojavensis* based on indirect paternity analysis (Goodnight & Queller 1999). Each bar represents an individual brood with the total sample size indicated at the top. The alternative shading indicates the proportion of offspring sired by each male. For example, in brood 41 we identified three different fathers that sired 56%, 38%, and 6% of the offspring, respectively.

Table 2 Sample size and number of fathers detected per brood

	Brood 43		Brood 36		Brood 46		Brood 45	
Sample size	23	107	23	93	22	146	22	104
Number of fathers	2	2	4	4	5	5	3	6

The relatively few number of sperm transferred by male *Drosophila mojavensis* during a single copulation imposes a limit on female reproductive output. In principle, this constraint could lead to a positive correlation between overall sperm load and the number of sires present within a brood. Overall, we found no evidence for a positive relationship between the number of sires and total number of progeny in our data (Spearman's $\rho = 0.083$, $P = 0.728$).

Sperm utilization

We found little evidence for bias in female sperm utilization among males. In seven of the eight broods where only two fathers were detected, both sired approximately equal proportions of offspring (for all tests $\chi^2 < 1.1$, $P > 0.290$, 1 d.f.), including one of the broods with expanded sampling (brood 43, $N = 107$, $\chi^2 = 0.009$, $P = 0.923$, 1 d.f.). Only brood 39 showed evidence of heterogeneity in sperm usage with paternity strongly skewed towards one of the two sires ($N = 21$, $\chi^2 = 13.8$, $P < 0.001$, 1 d.f.). The average proportion of offspring sired by the most common male in each of these eight broods was 0.582 (± 0.047 SE). Excluding brood 39 this estimate becomes 0.536 (± 0.013 SE).

Sperm stratification based on mating order can be an important component of temporal sperm utilization (Simmons 2001). We examined the four broods with expanded sampling to test for temporal variation in sperm utilization. We partitioned our data into three general groups: early eclosing (eclosed day 1–4), mid eclosing (eclosed day 5–9), and late eclosing (eclosed day ≥ 10). For each of the broods, we compared the number of offspring sired by the two most frequent males at each of the three time points (Table 3). Only one of the four broods showed evidence for significant heterogeneity in sperm usage with respect to time of eclosion (brood 45; Table 3).

Discussion

Species of *Drosophila* have long served as important model organisms for the study of sexual selection (Bateman 1948; Markow 2002). However, many of the species-specific details have been determined using controlled laboratory conditions and may not accurately reflect conditions in nature. Our study provides an assessment of basic aspects of reproductive behaviour in a natural population of *Drosophila mojavensis*, enabling a direct comparison with the wealth of laboratory data available for this species. These data facilitate comparisons with a handful of similar studies in other species of *Drosophila*, providing an assessment of the relationship between sperm loads, remating frequency, incidence of multiple paternity, and sperm utilization patterns among species of *Drosophila*. We discuss these issues below.

Sperm loads, mating frequency, and multiple paternity

We observed a large range of brood sizes produced by ovipositing females sampled in our study (Fig. 1). Nevertheless, the reproductive output of wild-caught females is low compared to what has been reported for *D.*

Table 3 Sperm usage across time

	Offspring counts				
	Eclosed early (days 1–4)	Eclosed mid (days 5–9)	Eclosed late (days 10+)	χ^2	Probability
Brood 43					
Male 1	0	33	21	0.449	0.503
Male 2	0	29	24		d.f. = 1
Brood 36					
Male 1	9	21	13	3.14	0.208
Male 2	3	17	16		d.f. = 2
Brood 46					
Male 1	5	33	5	4.14	0.126
Male 2	1	19	8		d.f. = 2
Brood 45					
Male 1	16	17	13	6.46	0.040
Male 2	23	6	14		d.f. = 2

melanogaster (Stalker 1976a) or *D. pseudoobscura* (Snook & Markow 2002). Even the most productive females in our study produce fewer offspring than the averages reported for wild-caught *D. melanogaster* females (~300 offpsring; Stalker 1976a; Gromko & Markow 1993). These findings are consistent with laboratory observations that male *D. mojavensis* transfer relatively few sperm per copulation (68.9 ± 8.1 based on laboratory progeny counts; Markow *et al*. 1990), limiting female reproductive output relative to other *Drosophila* species that transfer many more sperm. Even so, females from species that receive many more sperm may also experience some limitation in nature. Females of *D. melanogaster* have been observed to produce over 1000 progeny when provided with a continuous source of mates in the laboratory (Friberg & Arnqvist 2003), considerably more than their wild-caught counterparts (Stalker 1976a; Gromko & Markow 1993).

We found evidence for multiple mating for every female examined, with a maximum of 6 and an average of 3.1 (± 0.28 SE) males per brood (Fig. 2). This estimate suggests that female remating is very frequent in nature and results in consistently high levels of multiple paternity. Given the potential for male-induced sperm limitation, it seems likely that frequent remating is essential for females to maximize reproductive output. If multiple mating often involves different males, then this constraint could generate a positive correlation between total sperm load and the number of fathers per brood. This simple expectation would be sensitive to a number of factors including partial usage of sperm stores prior to capture and/or between sequential matings, patterns of sperm displacement among males, and multiple copulations with the same male. We found no evidence for a positive correlation between the numbers of sires and overall brood size (Spearman's $\rho = 0.083$, $P = 0.728$), suggesting total sperm load is strongly influenced by one or more of these factors. In laboratory populations, ovipositing and remating have been observed to overlap (Markow 1988). In the case of females mating with only two males, we found patterns consistent with no partial utilization between mating bouts. However, it is reasonable to assume that this behaviour influences sperm loads when several males have been mated with. Likewise, both strong displacement among males and/or frequent repeated mating with a single male seem less likely in the case of two males, but is much more difficult to evaluate in broods with many more fathers present (see *Sperm utilization* below).

Our estimate of multiple paternity is higher than recently reported estimates for *D. simulans* (~1.4 males per brood; Schlötterer *et al*. 2005) and *D. buzzatii* (~2.2 males per brood; Bundgaard *et al*. 2004). Both *D. buzzatii* and *D. mojavensis* show a higher remating frequency than *D. simulans* in the laboratory, suggesting some qualitative agreement between behaviour in natural and experimental conditions. Unfortunately, we cannot determine with certainty if the incidence of multiple paternity in *D. mojavensis* is higher or lower relative to *D. melanogaster*. Similar studies of paternity for broods from wild-caught *D. melanogaster* females are variable with respect to the number of fathers detected (Harshman & Clark 1998; Imhof *et al*. 1998; Jones & Clark 2003). Harshman & Clark (1998) reported the mean number of sires per brood as 1.8 with a maximum of four fathers using a maximum-likelihood approach. Reanalysis of these data using a Bayesian framework provided a slightly higher estimate of 2.4 fathers per brood (Jones & Clark 2003). Imhof *et al*. (1998) used five to six microsatellite markers and detected four to six fathers per brood based on paternal allele counts in a sample of four broods. As pointed out by Imhof *et al*. (1998), frequent remating would be surprising given the reported cost of mating for

D. melanogaster females (Chapman *et al*. 1995). Nevertheless, this latter estimate exceeds levels of multiple paternity estimated for both *D. mojavensis* and *D. buzzatii*.

Based upon laboratory remating frequency, it could be argued that even higher numbers of sires should have been observed in *D. mojavensis*. How can we explain the observation that wild-caught *D. melanogaster* females contain sperm from as many, and possibly more mates, than wild-caught female *D. mojavensis*? First, the patchy distribution of breeding sites in *D. mojavensis* (Breitmeyer & Markow 1998) could result in elevated allele sharing within a population, thus reducing our power to discriminate among potential fathers. Indeed, there is some evidence for a reduction in observed heterozygosity in the Organ Pipe population (Tables 1, C. L. Ross & T. A. Markow, unpublished). Second, we may have failed to sample some sires represented at very low frequency among the offspring (Table 2). Third, reproductive tract incompatibilities observed in *D. mojavensis* (Knowles & Markow 2001) could lead to assortative fertilization, causing an underestimate of the number of matings by females when using paternity analyses. Female *D. mojavensis*, along with other cactophilic species of *Drosophila*, are known to differentially utilize sperm from different males, depending upon genotypic similarity (Markow 1982, 1997).

Species differences in the abundance and distribution of breeding sites also are expected to influence remating patterns. *D. melanogaster* and *D. mojavensis* have very different reproductive ecologies. *D. melanogaster* is a dietary generalist with a worldwide distribution. Primarily a human commensal, *D. melanogaster* is found throughout urban sites wherever there is decaying fruit or vegetable matter, as well as in orchards, wineries and food processing facilities (Powell 1997). For this species, while a single resource patch consists of one piece of fruit, these patches are typically found within 'super patches' (Krijger & Sevenster 2001). In contrast, *D. mojavensis* is a specialist, breeding in the necrotic tissue of specific cactus hosts found in the deserts of North America (Heed 1982). Their breeding sites, or patches, consist of individual necrotic cacti, and are among the least frequently encountered resources known for any *Drosophila* (Breitmeyer & Markow 1998). What do such ecological differences imply for the reproductive biology of these species in nature? For *D. melanogaster*, females should have greater opportunities to utilize sperm stores than females of *D. mojavensis*. Since female receptivity is negatively associated with usage of existing sperm loads in *D. melanogaster* (Gromko & Markow 1993) but not in *D. mojavensis* (T. A. Markow, unpublished), greater resource availability could translate into higher remating rates in *D. melanogaster* relative to *D. mojavensis* when considering natural populations. Such differences may not be apparent in common laboratory environments where remating behaviours typically have been measured.

Sperm utilization

Multiple mating by females provides the opportunity for interejaculate competition. Given the diversity of female remating rates, comparisons among various *Drosophila* species provide a useful framework to study postcopulatory sexual selection (Markow 2002). In *Drosophila*, sperm precedence usually favours the last male to mate and is rooted in a process such as sperm displacement, incapacitation, or other competitive mechanism (Simmons 2001). However, we found little evidence for strong skew in sperm utilization among males within a given brood. In seven of the eight broods with two males represented, both sires were found in equal proportions (Fig. 2). One simple interpretation of this pattern is that when a female mates with two males, she stores approximately equal numbers of sperm from each with little or no differential displacement or incapacitation among males. Thus, it is likely that both males achieved the same number of copulations with the female and that sperms loads from the first male were not partially utilized prior to remating. If true, the intensity of interejaculate competition among males within a given female could be fairly weak in this system. However, after several bouts of remating, females presumably are no longer sperm limited, and the dynamics of precedence among ejaculates could change dramatically. One brood with two fathers represented did show a very strong skew towards one male sire (brood 39, Fig. 2; $\chi^2 = 13.8$, $P < 0.001$, 1 d.f.). Whether this is the result of some form of precedence or partial utilization of stored sperm prior to remating cannot be determined with these data.

Last-male sperm precedence related to timing of ovipositing has been observed in *D. mojavensis* in the laboratory and could contribute to sperm competition among males if oviposition opportunities are limited in nature. In doubly mated females Markow *et al*. (1990) showed that eggs oviposited on the first day after insemination show the strongest skew in paternity (~80% of the progeny sired by the last male) while both sires were represented at approximately equal frequency a few days later (Markow *et al*. 1990). We examined sperm utilization patterns across time in four broods. One of the four broods showed significant heterogeneity in the usage of different male's sperm across time (Table 3). In this brood, early- and mid-eclosed flies showed the most variation among sires while late-eclosed progeny were sired in proportions more or less equal to the overall patterns (i.e. pooled samples). With a sample of only four broods we cannot rigorously evaluate the evidence for or against temporal variation in sperm utilization in nature. Nevertheless, this pattern of moderate heterogeneity in sperm utilization early in reproduction, followed by more or less equal mixing later is in general agreement with observations from laboratory matings (Markow 1982, 1988).

Others have used a Bayesian framework to estimate the number of sires per female and the degree of sperm

displacement in wild-caught females (Jones & Clark 2003; Bundgaard *et al*. 2004; Schlötterer *et al*. 2005). This method uses a Markov chain Monte Carlo (MCMC) to generate a posterior distribution for the relevant parameters of the sperm competition model developed by Harshman & Clark (1998), including the number of males per brood (α) and the proportion of sperm displaced (β) by the last male to mate (assumed to be the most frequent male within a brood). When used on our data, this method provided an estimate of multiple paternity ($\alpha = 3.2$) that was in good agreement with our calculations based on likelihood-based tests of within-brood relatedness and sperm precedence estimates more or less consistent with equal mixing of sperm ($\beta = 0.47$; analyses not shown). Nonetheless, estimating precedence with this approach relies upon several assumptions, including no female sperm limitation or use of prior male's sperm before remating. These assumptions are clearly problematic for the *D. mojavensis* system. In general, sperm precedence likely depends on a number of species–specific details including interaction of factors such as oviposition opportunities, remating intervals, and sperm longevity. In the absence of these data it seems premature to draw conclusions about the processes responsible for variation in fertilization success among sires within a brood from a wild-caught female of any species.

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Supplementary material

The supplementary material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2847/MEC2847sm.htm

Table S1 Multilocus genotypes for 814 flies

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