

Resolving Mechanisms of Competitive Fertilization Success in *Drosophila melanogaster*

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Our understanding of postcopulatory sexual selection has been constrained by an inability to discriminate competing sperm of different males, coupled with challenges of directly observing live sperm inside the female reproductive tract. Real-time and spatiotemporal analyses of sperm movement, storage, and use within female *Drosophila melanogaster* inseminated by two transgenic males with respectively green (GFP) and red (RFP) sperm heads allowed us to unambiguously discriminate among hypothesized mechanisms underlying sperm precedence, including physical displacement and incapacitation of “resident” sperm by second males, female ejection of sperm, and biased use of competing sperm for fertilization. We find that competitive male fertilization success derives from a multivariate process involving ejaculate-female and ejaculate-ejaculate interactions, as well as complex sperm behavior *in vivo*.

Remating with different males by females generates sexual conflict over paternity (1) and sets the stage for postcopulatory sexual selection (2–4), which can drive diversification of both male and female biochemistry, physiology, morphology and behavior (4, 5). Most investigations of postcopulatory sexual selection have focused on the pattern of sperm precedence, such as the proportion of progeny sired by the second of two males subsequent to female remating (P_2). However, without knowledge of underlying mechanisms, these patterns reveal little about the intensity of selection or sex-specific adaptation (4–6). Consequently, even with *D. melanogaster* there is contention over the mechanisms giving rise to the roughly 80% last male sperm precedence observed (7–12). Our understanding of these and other phenomena has been constrained by the technical challenge of directly observing sperm dynamics within the female reproductive tract and our limited ability to discriminate between sperm of different males (7, 13, 14).

We have overcome these challenges by transforming *D. melanogaster* to express a GFP- or RFP-labeled protamine in sperm heads, which can be easily observed and unambiguously differentiated within the female reproductive

tract (fig. S1 and S2, movies S1 to S3). These lines enable direct visualization of sperm competition *in vivo*, in real time and over extensive periods of time, allowing us to discriminate among alternative hypothesized mechanisms of sperm precedence. Multiple indices of male fitness relevant to sperm/ejaculate function were assayed, with transgenic males compared to each other and to a wild type LH_m strain (into which the GFP and RFP constructs were backcrossed for six generations). Although some significant differences were found, with transformed lines performing less well, equivalent to or better than the wild type in different fitness assays, all three strains fell within the typical range of values reported in the literature for all assays (figs. S3 to S7), suggesting there was no dysfunction of transformant sperm. Moreover, results of the sperm precedence experiments reported below are unbiased, because GFP and RFP males (i) were competed using a reciprocal mating order design and (ii) did not differ in female remating interval, P_1 or P_2 in those experiments (15).

We quantified (i) spatiotemporal patterns of sperm storage and use by the female following remating, (ii) the extent and timing of sperm ejection by females and (iii) the influence of remating on resident sperm motility. Unless otherwise specified, in all experiments 3 d old, virgin LH_m females were randomly assigned to all treatment groups, initially mated to a GFP or an RFP male, and beginning three days later (the typical remating latency for *D. melanogaster*), provided a daily, six-hour opportunity to remate to a male of the alternative genotype (reciprocal male mating order balanced). All copulations were observed, and copulation durations recorded (15).

To quantify temporal dynamics of sperm fate, females in two experimental replicates were flash-frozen at randomly-assigned times after the start of the second mating (ASM): 0 min (immediately after second male mounting), 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 2 hr, 8 hr, and 24 hr, with three additional time points in the second replicate: 90 min, 48 hr and 72 hr. For the last three time treatments of the second replicate (24 hr, 48 hr, 72 hr), all progeny were reared, and the proportion sired by the second male (P_2) was determined by assaying all sons for green or red sperm.

Frozen females were dissected, and red and green sperm were counted in the bursa (the site of insemination), paired spermathecae (including spermathecal ducts), and the seminal receptacle (SR), distinguishing between the proximal and distal halves. For illustrative purposes, results from both replicates and male mating orders are combined in Fig. 1 (individual replicates and male mating orders shown in figs. S8 to S10; see SOM for tests of differences), with sample sizes per time treatment ranging from 30 to 50 females (total $N = 543$; table S1).

We revealed two different mechanisms by which resident sperm are displaced from the sperm-storage organs following remating, hence contributing to last male sperm precedence. First, there was an early release of some resident sperm from storage into the bursa, a process that does not involve second-male sperm. Second, resident sperm appear to have been physically displaced over time from both the SR and the spermathecae by incoming second male sperm. Wild type females store approximately 400 sperm at maximum in their SR and 130 sperm in the paired spermathecae (Fig. 1). Upon remating (i.e., time 0 ASM), resident sperm were depleted from this maximum capacity due to sperm use for fertilization over preceding days to $149 (\pm 12)$ sperm in the SR and $108 (\pm 11)$ sperm in the spermathecae; resident sperm were rarely observed in the bursa (Fig. 1) or in the spermathecal ducts at time 0. Insemination of second-male sperm began as early as 5 min ASM ($N = 25/46$ or 54% of the time), and in 19% of cases where second-male sperm had not yet been transferred ($N = 5/26$), some resident sperm (17 ± 5) were observed in the bursa 5-10 min ASM (Figs. 1 and 2). We also saw first-male sperm in the bursa in 14% of observations where second-male sperm had been transferred but had not yet entered storage ($N = 5/37$). This release of resident sperm was presumably female mediated and triggered by male accessory gland proteins (Acps) and/or mechanical stimulation of copulation.

First-male sperm continued to be displaced as second-male sperm entered storage, soon after insemination (the SR earlier than the spermathecae) (Fig. 1) (16). Sperm transfer peaked at 15 min ASM (1412 ± 66 sperm, or approximately 2.7 times the female's storage capacity), after which second-male sperm steeply declined in the bursa, while resident sperm steadily increased. Although sperm storage leveled off around 45 min ASM, the first- and second-male sperm proportions continued to change, with displacement peaking 60-90 min ASM (Fig. 1), when 26% of resident stored sperm had been relocated into the bursa. While this process is likely to displace some of the second male's own sperm (17), it will also increase the proportion of second-male sperm in storage (as observed) due to their over-representation in the bursa. This outcome also depends on active motility within the storage organs (movies S1 to S3) and adequate mixing of

first- and second-male sperm, consistent with our observations (fig. S11, movies S4 and S5). Competing sperm were highly stratified during and immediately following copulation, with resident sperm concentrated in the distal half of the SR (Fig. 2, B and C), but dynamic mobility of sperm in storage increased mixing over time (Fig. 2D and fig. S11).

The question of why males ejaculate many more sperm than can be stored has long puzzled researchers, given that the (1.8 mm) sperm of *D. melanogaster* are many times longer than any distance they travel within the female, and storage appears to be a rapid and efficient process (18). We postulate that excess sperm, and perhaps their mobility (movie S6), are adaptations to sperm competition in this species, specifically to physical displacement of resident sperm. Indeed, the size of the second male's ejaculate was significantly correlated with the amount of resident sperm displaced from storage, and the strength of this relationship increased substantially between 45 and 60 min ASM (fig. S12; 45 min ASM: $N = 45$, $F = 6.85$, $R^2 = 0.137$, $P = 0.012$; 60 min ASM: $N = 34$, $F = 30.95$, $R^2 = 0.49$, $P < 0.0001$). These results also suggest that ejaculate size is evolving via sexual selection mediated by sperm competition.

Sperm storage and displacement dynamics cease when the female ejects excess second-male and displaced first-male sperm from her reproductive tract. To establish that females are ejecting sperm, as previously inferred (13), we doubly mated wild type females to GFP and RFP males and, within 30 min after copulation, transferred females in groups of up to five (mean = 4.7) into $N = 20$ cubes constructed of glass coverslips. Cubes were deconstructed 3 hr later and the coverslips were examined under fluorescence. We found several ejected sperm masses per cube, highly stereotypical in form (e.g., Fig. 3), consisting of mixed (but predominantly second-male) sperm in association with the gelatinous mating plug. To better quantify ejection, wild type females were isolated in glass 3-well spot plates beneath coverslips immediately after mating to GFP males, checking for ejection every 10 min for up to 5 hr using a stereomicroscope. Ejected masses were immediately transferred to saline on slides and examined under epifluorescence to confirm the presence of GFP sperm, and females were dissected to examine the bursa for sperm. We found that 84% of females ($N = 61$) ejected sperm within five hours of mating (mean \pm se = 181 ± 11 min), and none of these females had any sperm in their bursa, nor had they laid any eggs. Additionally, examination of first eggs laid in media vials in the time series experiment (above) were never associated with large amounts of sperm, reinforcing that active sperm ejection by females is typical, if not universal.

After ejection, remaining stored sperm comprise the "fertilization set" (19), or the population of sperm directly engaging in sperm-sperm competition. To delineate which

storage organs house the fertilization set, we compared simple linear regressions of P_2 (proportion offspring sired by second male) on the proportion of second-male sperm in storage (S_2) (Fig. 4) (15). S_2 was found to be a highly significant predictor of P_2 for the SR ($F = 25.55$, $P < 0.0001$, $R^2 = 0.259$) but not the spermathecae ($F = 0.26$, $P = 0.614$, $R^2 = 0.004$), reinforcing previous indirect evidence (20) that sperm in the SR constitutes the more immediate fertilization set. Proportional representation in the proximal versus distal regions of the SR may further contribute to differential fertilization success and has been shown to be influenced by sperm length (21, 22). However, sperm length here did not systematically vary between first and second males, and sperm proportions did not differ regionally in the SR in the hours immediately following mating (fig. S11).

We finally tested if each male's sperm in the SR post-ejection was equally competitive, akin to a "fair raffle" (19) with sperm used for fertilization in direct proportion to their prevalence. Two results suggest that they were: (i) *in vivo* sperm motility did not change upon remating and (ii) sperm were used in direct proportion to their abundance. First, we examined if female remating induces a change in motility of GFP resident sperm in the SR by quantifying their velocity and percent motility (i) 5 min ASM to an RFP male, (ii) 60 min ASM to an RFP male, (iii) 60 min after the first mating and (iv) 3 d after the first mating (15). Percent motility and sperm velocity showed no significant changes, suggesting that in the SR, neither several days of storage nor female remating had any detrimental effect on sperm survival or motility (fig. S13; movies S5 and S6). Second, if sperm competition was disproportionately and systematically biased toward one male over another ("loaded raffle"), the slope of the regression of P_2 on S_2 would be either significantly less than 1 (favoring the first male) or greater than 1 (favoring the second male). However, the slope of the reduced major axis regression was not significantly different from 1 (slope = 1.203, $t = 0.81$, d.f. = 68, $P = 0.422$; Fig. 4A), supporting the fair raffle hypothesis.

Our observations of *in vivo* motility and fine-scale spatiotemporal patterns of sperm fate corroborate some previously conjectured mechanisms underlying competitive fertilization success, including sperm displacement (10, 12, 23) and sperm ejection by females (13), but fail to support other proposed mechanisms, such as sperm incapacitation (9–11). We cannot exclude the possibility that Acp-mediated incapacitation contributes to the early release of some resident sperm from storage. However, we would then expect to see dead or dying sperm in storage and in the bursa, yet neither sperm velocity nor sperm motility in storage changed upon remating. Our results complement other investigations of sperm mortality of competing ejaculates *in vivo* (13) and *in vitro* (24), suggesting that incapacitation makes little to no

contribution to sperm precedence in *D. melanogaster*. The present investigation reveals that postcopulatory sexual selection in *D. melanogaster* includes the early release of resident sperm from storage, the heterogeneous distribution of competing sperm proportions in different storage organs, fair raffle sperm use in the SR, and the surprising level of sperm mobility within female sperm-storage organs (movies S1 to S5). Most importantly, a complex and dynamic array of processes underlying patterns of sperm precedence (e.g., P_2) can now be dissected in targeted, detailed genetic analyses to reveal specific behavioral, physiological and biochemical mechanisms relevant to postcopulatory sexual selection.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1187096/DC1

Materials and Methods

SOM Text

Figs. S1 to S13

Table S1

References

Movies S1 to S6

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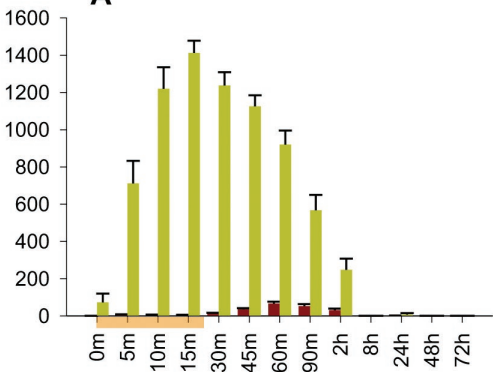
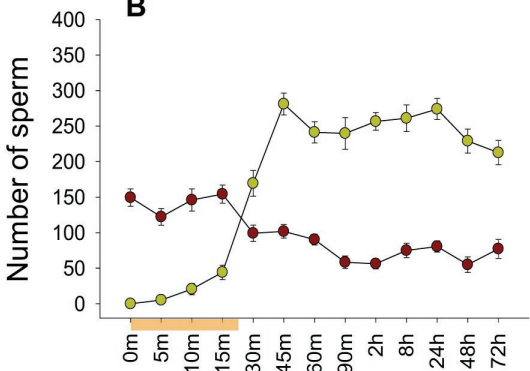
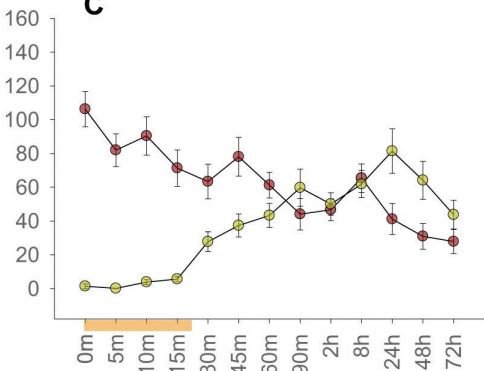
Include this information when citing this paper.

Fig. 1. Numbers of first-male (red) and second-male (yellow) sperm in the (A) bursa, (B) seminal receptacle and (C) spermathecae for thirteen time points ASM, averaged over two experimental replicates and for both reciprocal mating orders. Error bars represent SE.

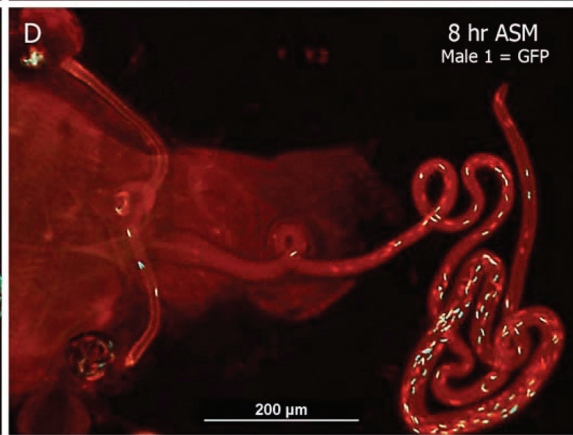
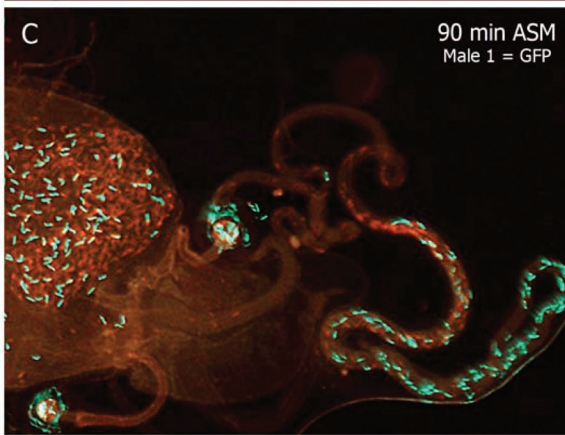
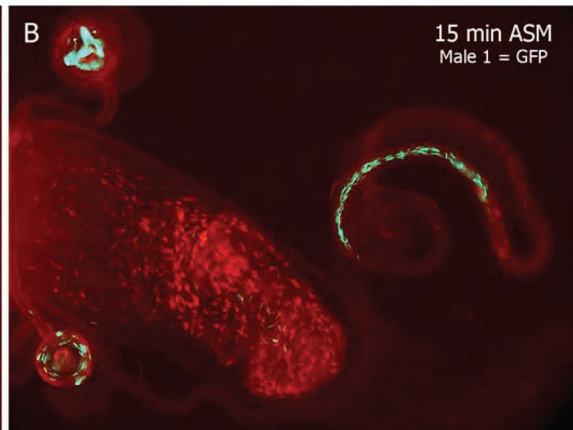
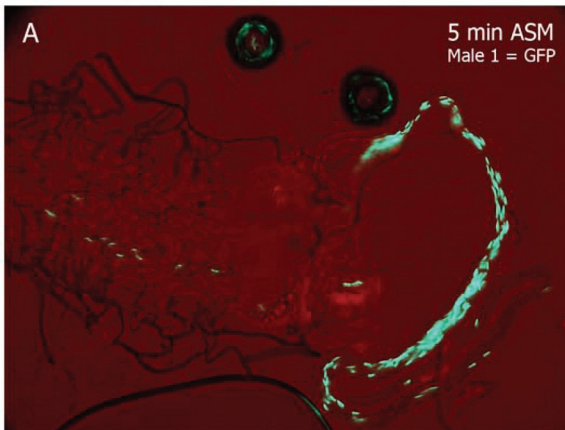
Fig. 2. Representative female reproductive tracts at (A) 5 min, (B) 15 min, (C) 90 min and (D) 8 hr ASM, showing relative abundances and locations of first-male (indicated as GFP or RFP) and second-male sperm.

Fig. 3. Ejected sperm mass containing both first-male (GFP) and second-male (RFP) sperm under (A) differential interference contrast and (B) fluorescence.

Fig. 4. Relationship between P_2 and S_2 (both variables arcsine square root transformed) in the (A) seminal receptacle and (B) spermathecae. Seminal receptacle: $y = 1.203x - 0.191$ (reduced major axis regression); spermathecae: $y = 0.0383x + 1.144$ (simple linear regression).

A**B****C**

Time after start of remating
(ASM)



A



B

