Female mediation of competitive fertilization success in Drosophila melanogaster

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How females store and utilize sperm after remating can generate postcopulatory sexual selection on male ejaculate traits. Variation in ejaculate performance traits is generally thought to be intrinsic to males, yet is likely to interact with the environment in which sperm compete (e.g., the female reproductive tract). Our understanding of female contributions to competitive fertilization success is limited, however, in part due to challenges of observing events within the reproductive tract of internally fertilizing species while discriminating among sperm from competing males. Here, we used females from crosses among isogenic lines of Drosophila melanogaster, each mated to two genetically standardized males (first with green- and second with red-tagged sperm heads) to demonstrate heritable variation in female remating interval, progeny production rate, sperm-storage organ morphology and a number of sperm performance, storage and handling traits. We then used multivariate analyses to examine relationships between this female-mediated variation and competitive paternity. In particular, the timing of female ejection of excess second-male and displaced first-male sperm was genetically variable and, by terminating the process of sperm displacement, significantly influenced the relative numbers of sperm from each male competing for fertilization and, consequently, biased paternity. Our results demonstrate that females do not simply provide a static 'arena' for sperm competition but rather play an active and pivotal role in postcopulatory processes. Resolving the adaptive significance of genetic variation in female-mediated mechanisms of sperm handling is critical for understanding sexual selection, sexual conflict, and the coevolution of male and female reproductive traits.

cryptic female choice | heritability | postcopulatory sexual selection | sperm ejection

Introduction

Because females of many species mate with multiple males within a reproductive cycle (1-3), sexual selection can continue after mating. When sperm from different males co-occur in the female reproductive tract, they compete for fertilization of the eggs and females may bias sperm use to favor some males over others. Such sperm competition and cryptic female choice are regarded as the postcopulatory equivalents of premating male-male competition and female choice, respectively (4, 5). This characterization, however, may be overly simplistic and belie differences between selection episodes that are critical for understanding selection dynamics.

Adaptations arising through premating versus postcopulatory sexual selection are likely to differ in phenotypic and genotypic complexity. With premating sexual selection, male armaments and ornaments tend to be complex somatic traits under the control of multiple genes [e.g., (6)], and female mate preferences predominantly have sensory and cognitive bases (7-9). In contrast, the principal target of postcopulatory sexual selection on males is the ejaculate (note: penis and copulatory courtship traits are excluded here for the sake of argument). Postcopulatory ornaments and armaments thus predominantly include single active molecules such as accessory gland proteins (Acps) that are controlled by single genes (10, 11), and traits borne by haploid single cells [e.g., sperm structures, membrane-bound proteins, energetics; (12, 13)]. The genetics of these traits are relatively unresolved (12, 14-17). The primary targets of postcopulatory sexual selection on females will be aspects of reproductive tract biochemistry, neurophysiology and morphology that interact with ejaculates and potentially bias paternity (5, 18-21). The genetics of cryptic female choice are also not well resolved [but see (22)]. Because ejaculate competition and processes of female sperm selection occur within the female reproductive tract, the relative competitiveness of ejaculates is predicted to be a function of ejaculate-female compatibility. If true, then sperm competition and cryptic female choice represent more of a continuum than dichotomous processes, especially [but not exclusively; e.g., (23-26)] in internally fertilizing species (20, 21).

Adaptations arising through premating versus postcopulatory sexual selection are also likely to fundamentally differ in the extent to which inter-sexual interactions influence their expression. Sex-specific, pre-mating traits are generally considered separate entities with distinct phenotypes and fitness consequences. In contrast, consider ejaculate processing and function within females. Seminal fluid is biochemically complex, with approximately 150 Acps being inseminated into female *Drosophila melanogaster* (27, 28). Most Acps are believed to have unique target receptors within the female (11), although to date only one has been identified [for sex peptide; (29)]. Moreover, phenotypic expression of some Acps follows modification (e.g., proteolytic cleavage) within the female, a process thought to require both male and female secretory contributions (11, 21). Likewise, sperm may complete maturation, capacitate, or otherwise undergo modification within the female. In some cases, these modifications are known to involve biochemical ejaculate-female interactions (21), with direct implications for competitive fertilization success [e.g., (30, 31)]. A major focus in the study of postcopulatory sexual selection has been to understand the evolution of ejaculate quality traits that are likely to influence competitive fertilization success, such as swimming velocity [reviewed by (32-34)]. Variation in these phenotypes has almost exclusively been assayed *in vitro* and interpreted as intrinsic to males. However, to the extent that ejaculate phenotypes are influenced by females and/or are the product of male-by-female interactions, ejaculate phenotypes in the narrow sense may not exist outside of the biochemically and structurally complex environment of the female reproductive

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Table 1. Additive (V_A) and non-additive (V_D) genetic variance components, phenotypic variance (V_P) and heritability (h^2) of female-mediated effects on ejaculate quality and handling, controlled for block and vial effects (for further details, see *Statistical analyses*). *LLR* = log-likelihood ratio used to calculate significance of heritability.

Trait	*N*	V_A	V_D	V_P	h^2	*LLR*	*P*
Thorax length[a]	484	1.29	0	5.44	0.24	8.2	0.09
Absolute SR length[a]	484	0.02	0.006	0.04	**0.50**	49.4	**<0.0001**
Relative SR length[a,b]	484	0.02	0.006	0.04	**0.54**	50.3	**<0.0001**
Day of remating	1585	0.06	0.03	0.46	**0.14**	18.2	**0.0001**
Progeny prior to remating (E)[c]	1572	329.6	0	340.5	**0.97**	386.6	**<0.0001**
Progeny prior to remating (P)[a,c]	487	436	0	485	**0.90**	46.3	**<0.0001**
Duration of copulation	1573	1.63	0	34.24	0.05	7.9	0.24
Resident sperm at remating	1115	9729	965	20815	**0.47**	61.8	**0.0003**
Number of sperm transferred	1104	0	0	65502	0.00	0.0	1.0
Time to ejection	1277	0.05	0	0.14	**0.36**	65.3	**<0.0001**
Mean sperm velocity[a]	536	130	0	1044	0.13	4.7	0.32
1st-male sperm stored	1272	599	108	4853	**0.12**	8.1	**0.044**
2nd-male sperm stored	1272	1955	0	10395	**0.19**	16.1	**0.001**
Total sperm stored	1228	2737	0	13697	**0.20**	28.0	**<0.0001**
S_2 (pre-ejection)[d]	1104	0.003	0	0.007	**0.36**	66.6	**<0.0001**
S_2 (post-ejection)	1272	0.001	0	0.010	**0.14**	20.1	**0.0005**
S_2 in SR (post-ejection)	1241	0.008	0	0.025	**0.29**	64.2	**<0.0001**
Prop. 1st-male sperm in SR	1293	0.008	0.0004	0.020	**0.43**	78.9	**<0.0001**
Prop. 2nd-male sperm in SR	1296	0.002	0	0.009	**0.19**	16.0	**0.001**
Second-male paternity (P_2)[a]	419	0.005	0.001	0.028	0.17	7.8	0.051

[a] based on one female per family (i.e., max. $N = 6$ per isoline cross)
[b] controlled for female thorax length as a fixed effect ($t = 2.42$, $P = 0.016$)
[c] (E) = ejection experiment, (P) = paternity experiment
[d] proportion of second-male sperm among all resident first-male sperm and the entire second-male ejaculate

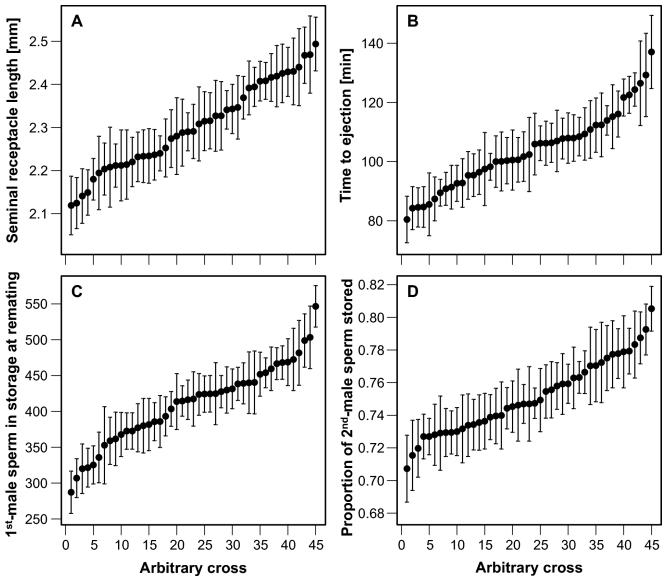

Fig. 1. Within- and between-cross variation in (A) seminal receptacle length, (B) time to female sperm ejection after the end of copulation, (C) the number of 1st-male sperm still in storage at the time of remating, and (D) the proportion of 2nd-male sperm among all sperm stored (i.e., S_2). Each point represents an individual isoline cross (for simplicity, the reciprocal crosses are combined by nuclear genotype); error bars depict ± SE. For statistics on heritability, see Table 1.

Table 2. Minimal adequate linear mixed-effects model explaining the variation in the proportion of second-male sperm among all sperm retained by the female (i.e., S_2), after sequential elimination of non-significant random and fixed effects (see Materials and Methods). For full model see Online Supplementary Table S5.

Fixed terms	Estimate ± SE	ddf	*t*	*P*
Time to ejection	0.17 ± 0.02	808.2	5.65	<0.0001
Resident sperm (1st male)	–0.62 ± 0.03	803.1	–19.14	<0.0001
Sperm transferred (2nd male)	0.55 ± 0.03	849.4	16.81	<0.0001
Random terms	**VC ± SE**	**df**	***LLR***	***P***
Maternal isoline	0.02 ± 0.004	1	0.00	0.004
Paternal isoline	0.01 ± 0.003	1	0.82	0.099
Residual	0.63 ± 0.027			

Parameter estimates standardized; ddf = denominator degrees of freedom estimated using Satterthwaite's approximation; *LLR* = log-likelihood ratio; VC = Variance component. $N = 855$ females from 90 diallel crosses derived from 10 isolines. Conditional model $R^2 = 0.38$.

tract. Rather, they may have to be considered a special case of gene-by-environment interactions [also see (35)].

Our knowledge of postcopulatory sexual selection and its role in maintaining variation and driving diversification therefore would be strengthened by investigation of genetically variable traits that influence competitive fertilization success and the respective contribution of the sexes to their expression, with assays conducted *in vivo* under competitive conditions. In a series of pioneering experiments using fixed-chromosome lines of *D. melanogaster*, Clark and colleagues (36-40) demonstrated male, female and male-by-female genotypic contributions to patterns

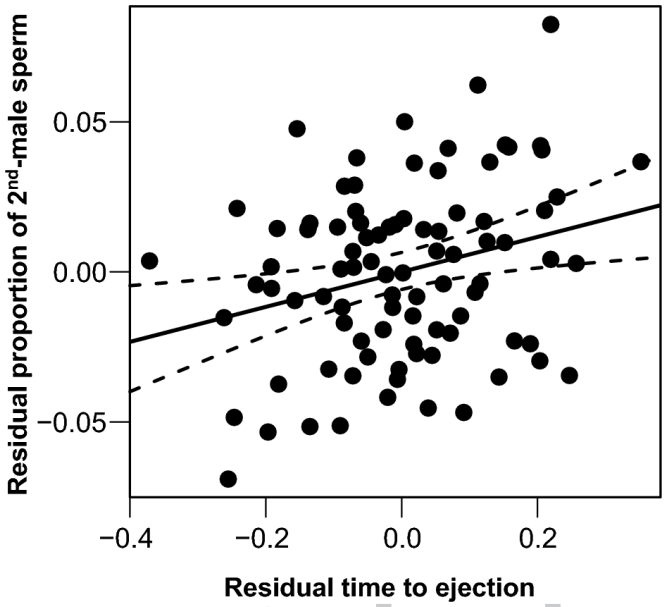

Fig. 2. Partial residual plot reflecting the significant relationship between the respective partial residuals of the time to female sperm ejection and the proportion of 2nd-male sperm in storage (S_2) post-ejection (t = 2.68, P = 0.009). Data points depict mean values for each of the 90 isoline crosses, and the dashed lines indicate the 95% confidence interval. Both axes are controlled for the number of first-male resident sperm at remating and the number of sperm transferred by the second male (full statistics of the multiple regression model in main text).

of sperm precedence [also see (41)]. We have expanded upon this approach using isogenic lines [inbred lines that approximate genetic clones, henceforth referred to as "isolines"; (42, 43)] of *D. melanogaster* expressing either green (GFP) or red fluorescent protein (RFP) in their sperm heads. The fluorescently-tagged sperm allow direct visualization of real-time and spatiotemporal *in vivo* sperm performance and fate while distinguishing between sperm from competing males (44, 45), thereby enabling the association of genotypic variation with sperm precedence traits and processes. We have recently documented heritable, strictly male-mediated variation (i.e., all females derived from a single isoline) in ejaculate traits, including sperm length, velocity and number, and how these traits significantly influence fertilization success at different stages following competitive matings (44). In the present paper, we examine strictly female-mediated additive and non-additive genetic variance in remating, progeny production, sperm performance and fate in *D. melanogaster* and its effects on competitive fertilization success among pairs of genetically standardized males (i.e., derived from two isolines). Investigations of male-by-female interactions in sperm performance and competitive fertilization success are in progress and will be the subject of a future report.

Results

Across 90 diallel crosses (45 nuclear genotypes), controlled for female genetic background and block and vial (=family) effects (see Materials and Methods), we found significant heritability for seminal receptacle (SR) length, remating interval, rate of progeny production prior to remating, time from copulation to female sperm ejection and for numerous female sperm-handling traits (Table 1; Fig. 1). The number of first-male sperm still in storage at the time of remating was significantly heritable (Table 1), but not significantly associated with SR length or with the number of progeny produced prior to remating ($|t| < 1.60$, $P > 0.11$, conditional model $R^2 = 0.25$). In the 72-h experiment, however, SR length covaried positively with the total number of sperm remaining in storage at the end of the three-day oviposition period (N = 453 families, $t = 4.61$, $P < 0.0001$, $R^2 = 0.15$) and in a heritable manner ($h^2 = 0.20$, $LLR = 10.20$, $P = 0.037$). Females with a relatively long SR also tended to store more sperm in the SR as the main sperm storage organ (N = 1169 females, $t = 1.89$, $P = 0.06$, $R^2 = 0.23$), but to remate sooner (N = 1398 females, $t = -3.09$, $P = 0.0005$, $R^2 = 0.15$) and to produce more progeny per unit of time, albeit not significantly (N = 1333 females, $t = 1.71$, $P = 0.09$, $R^2 = 0.49$). In contrast to first-male sperm, the number of sperm transferred by the second male was not affected by the female genetic background (Table 1) and, in a multivariate model (N = 960 females, $R^2 = 0.23$), it was independent of copulation duration ($t = 0.66$, $P = 0.51$), female thorax length ($t = 0.07$, $P = 0.95$) and SR length ($t = -0.25$, $P = 0.80$). However, the number of sperm retained from each male after female ejection was significantly heritable (Table 1).

Female genotypes also differed significantly in the interval between the end of copulation and the ejection of displaced first-male sperm and excess second-male sperm (Table 1). Ranging between a mean ± SEM of 55.3 ± 5.0 min and 134.0 ± 12.4 min among the 90 isoline crosses, this heritable variation played an important role in determining the relative fertilization success among the competing males. For example, controlling for SR length, first- and second-male sperm velocity, and the numbers of sperm competing for storage, a prolonged time to ejection significantly reduced the number of first-male sperm retained ($t = -6.11$, $P < 0.0001$; N = 682 females; online Supplementary Table S1), and significantly increased the proportion of resident sperm that were displaced (N = 682 females, $t = 5.73$, $P < 0.0001$; online Supplementary Table S2). Sperm velocity did not differ between female genotypes (Table 1) and had no significant influence on first-male sperm storage (Tables S1 and S2). The same results were obtained in a multiple regression analyses based on the mean values within crosses (Tables S1 and S2).

The number of second-male sperm retained was influenced by the relative sperm velocities among the competing ejaculates, with slower sperm being better at remaining in storage (online Supplementary Table S3), thus confirming an earlier report (44). We obtained qualitatively similar results when focusing on the proportion of all transferred second-male sperm that remained in storage, except there was no significant effect of SR length (online Supplementary Table S4). Despite the above sperm-velocity effect on second-male sperm storage, the proportion of second-male sperm among all retained sperm (i.e., S_2) was explained by the time to ejection and the numbers of first- and second-male sperm competing for access to storage (Table 2; online Supplementary Table S5). These results were consistent in a multiple regression analysis based on the mean values within crosses (N = 90 crosses; time to ejection: $t = 2.68$, $P = 0.009$; first-male sperm: $t = -6.06$, $P < 0.0001$; second-male sperm: $t = 3.20$, $P = 0.002$; model $R^2 = 0.32$; Fig. 2), as well as with each predictor analyzed separately (all $|t| > 8.39$, $P < 0.0001$).

Combining the experimental units at the family (vial) level and controlling for block effects and female genetic background, the relative numbers of sperm from each male remaining in storage after female sperm ejection significantly influenced competitive fertilization success: the paternity share of the second male, measured by the proportion of progeny produced after remating that were sired by the second male (P_2), increased with the number of second-male sperm retained (N = 389 families within 90 crosses, $t = 2.95$, $P = 0.003$), controlling for the number of first-male sperm ($t = 1.52$, $P = 0.13$) and SR length ($t = -1.57$, $P = 0.12$; model $R^2 = 0.11$). SR length further had no significant effect on S_2 among the sperm still in storage after 72 h of oviposition (N = 464 families, $t = -1.74$, $P = 0.08$, $R^2 = 0.09$), but it increased the absolute sperm numbers still in storage after

that period for both the first (N = 464 families, t = 3.36, P = 0.001, R^2 = 0.11) and second males (N = 464 families, t = 3.45, P = 0.0006, R^2 = 0.18). Similar results were obtained in regression analyses using mean values within each of the 90 crosses.

Discussion

Our results reveal within-population heritable variation in female SR length, remating interval, rate of progeny production, time from copulation to sperm ejection and aspects of sperm storage. In addition, the variable female genetic background significantly affected competitive fertilization success between standardized competitor males, with functional associations established. For example after remating, sperm of the last male move into the female's sperm-storage organs and start displacing resident sperm from the previous male back into the bursa, with displacement rates higher for the SR than the spermathecae (45). The female terminates this storage and displacement process 1–5 h after mating by ejecting all the sperm located in the bursa, which include any excess sperm from the second male and all displaced first-male sperm (45). As predicted *a priori*, the timing of sperm ejection had a particularly strong effect on the absolute and relative numbers of each male's sperm remaining in storage, thereby determining the fertilization set (i.e., the sperm able to compete for egg fertilization). Females with relatively late ejection retained a disproportionate number of second-male compared to first-male sperm, presumably because the sperm of the second male had more time to achieve entry into the sperm-storage organs and to displace first-male sperm residing there. In fact, our data indicate that this bias was primarily driven by displacement of first-male sperm rather than variation in second-male sperm storage, both in terms of absolute numbers displaced and the proportion of each male's total sperm mass that was ejected. The potential adaptive significance of sperm ejection time is evident in its direct influence on paternity, which was determined by the relative numbers of sperm in the fertilization set [also see (44-46)].

Once the fertilization set is established, female *D. melanogaster* may not be able to further bias competitive fertilization *per se*, given that sperm for fertilization in this species derive primarily from the SR and in direct proportion to their representation (46). This pattern of sperm use contrasts starkly with that of *D. simulans*, in which females may directly influence relative fertilization success even after sperm ejection. In this species, sperm for fertilization derive equally from the spermathecae and SR and each sperm-storage organ exhibits a significant bias: favoring first-male sperm in the SR and second-male sperm in the spermathecae, with females able to shift toward one or the other storage organ depending on the mating order of males differing in quality (46, 47). Nevertheless, we did also find in the present study genetic variation in female remating interval and progeny production rate [also see (48-50)], both of which can generate postcopulatory sexual selection on males.

Previous experimental evolution research with *D. melanogaster* found heritable variation in SR length and revealed that the evolution of longer SRs drove the evolution of longer sperm [e.g., (51)]. This latter result was attributed to a demonstrated interaction between SR length and sperm length that influenced competitive fertilization success (51). Longer sperm were found to be superior to shorter sperm in displacing, and resisting displacement by, competing sperm (52) [also see (44)], with this advantage increasing with SR length (51). In the absence of systematic variation in sperm length, SR length variation was unrelated to the pattern of sperm precedence (53). Here, we similarly found significant heritable variation in SR length and the lack of any relationship to the second-male paternity share (P_2) in the absence of sperm length variation. We did, however, find that females with relatively long SRs remated faster, tended to produce progeny at a higher rate during that period, and stored more sperm initially and had more sperm remaining in storage after three days of oviposition than females with a shorter SR, all of which may contribute to postcopulatory sexual selection on males (53). The underlying mechanisms for these relationships currently remain unresolved. It is possible that females with longer SRs are more strongly influenced by male seminal proteins that are known to mediate various aspects of female sperm storage, receptivity, and oviposition (10, 11), because the longer organ receives or retains more seminal plasma and/or because it possesses more seminal fluid protein receptors. Alternatively, SR length may be genetically correlated with female quality and thus fecundity, with highly fecund females remating faster and more frequently than females of poor quality [e.g., (54-56); but see (57, 58)].

In addition to sperm ejection time, females could potentially have impacted composition of the fertilization set, and hence P_2, by influencing either the number of sperm transferred during copulation [e.g., (59)] or the behavior of sperm (i.e., swimming velocity). Sperm velocity has been found to be a critical determinant of fertilization success in diverse taxa, with faster sperm having an advantage in some taxa [e.g., (60, 61)] and slower sperm having an advantage in others (44, 62). In *D. melanogaster*, slower sperm have been shown to be superior at displacing and resisting displacement by faster sperm, with sperm velocity significantly influenced by male genotype (44). However, we found no significant female genetic variation for copulation duration or the number of sperm transferred, supporting the contention that these phenomena are under male control in *D. melanogaster* and related species [(63) and references therein]. The absence of a relationship between the number of sperm transferred and female genetic background further reinforces the interpretation that the number of sperm entering or remaining in storage is primarily attributable to female effects rather than to differential male allocation relative to the female genotype (see above). Similarly, we found that neither female genetic background nor SR length significantly affected sperm velocity. This negative result is potentially important; although a few previous investigations have shown significant female and/or male-by-female interaction effects on sperm velocity (23-26), these studies have all been conducted *in vitro*, with externally fertilizing species, and were not designed to explore genetic variation.

It is important to note that variation in reproductive phenotypes attributed to female-mediated genetic variation in the present study (where competing male genotypes were held constant), and attributed to male-mediated genetic variation in a previous study [(44); where female genotypes were held constant], may be explained in part or entirely by genetic variation in male-by-female interactions (22, 37-39, 64). An investigation in progress will soon sort this out. Such interaction between the sexes is predicted by genetic compatibility models of sexual selection [e.g., (65, 66)] and is expected to often be mediated by physiological interactions between ejaculates and female reproductive tracts [e.g., via seminal fluid proteins and female receptors for them; (21)]. Irrespective of the adaptive significance, genetic variation in male and female reproductive characters identified in investigations of our isolines likely represent some of the mechanisms underlying previous demonstrations of genetic male-by-male and male-by-female interactions in sperm precedence [e.g., (37-39, 41)].

Cryptic female choice is defined as "nonrandom paternity biases resulting from female morphology, physiology or behavior that occur after coupling" (67), and our results meet those criteria. Nevertheless, because our investigation was designed to reveal strictly female-mediated genetic variation in traits relevant to postcopulatory sexual selection, which necessitated standardizing

the genetic contribution of competing males (18), the implications of our results for understanding directional postcopulatory sexual selection cannot yet be fully ascertained. Specifically, the demonstrated associations between female genetic variation and patterns of non-random reproductive success represent male mating order biases. Unless male mating order correlates with differential male quality, the identified genetic variation will be selectively neutral [at least in the absence of male-by-female interactions; also see (36)]. Indeed, some of the most convincing demonstrations of cryptic female choice/sperm choice have shown fertilization bias patterns based on MHC loci genotype (68, 69) or that are consistent with adaptation to avoid selfing [e.g., (70)] or inbreeding [e.g., (71, 72)], which also may fail to generate directional sexual selection (18). Notably, sperm ejection by female fowl *Gallus gallus domesticus* has been shown to be adaptively plastic, with the probability of ejection occurring and the proportion of the ejaculate ejected being greater for subordinate than dominant males (73). However, further investigation exploring the relationships between variation in male and female "sperm competition" phenotypes (e.g., sperm number, sperm length, sperm velocity, SR length, ejection time) is needed to clarify the adaptive significance of female-mediated variation revealed here.

Materials and Methods

Experimental material

To discriminate sperm from different males and quantify sperm motility *in vivo*, all experiments were conducted with LH_m populations of *D. melanogaster* that express a protamine labeled with either green fluorescent protein (GFP) or red fluorescent protein (RFP) in sperm heads [backcrossed for 6 generations to wild type; see (45) for transformation and fitness assay details]. The GFP line also ubiquitously expresses GFP, thus permitting paternity assignments on progeny (e.g., P_2).

All experimental flies were derived from isogenic lines ["isolines"; (42, 43)] generated for each sperm-tag color by 15 generations of fullsib inbreeding. The experimental males were F_1 progeny from crosses among a single pair of isolines per sperm-tag color (i.e., virgin females from one and males from the other isoline in each cross). Based on isoline characterization under standardized conditions [standard female and competitor male; (44)], we selected isolines with intermediate values for sperm length, sperm velocity and ejaculate size. Our two hybrid isolines did not differ significantly in sperm length (GFP, N = 15 males: mean ± SEM = 1.86 ± 0.01 mm; RFP, N = 15 males: 1.84 ± 0.02 mm; $t_{28} = 1.21$, $P = 0.24$).

To vary female genetic background, we crossed single pairs of virgin males and females of 10 different RFP isolines in all non-self combinations (i.e., 90 diallel crosses with 45 different nuclear genotypes, all independent of the RFP standard competitor male). In each of two blocks, separated by two generations, we used flies from three separate male-female pairs for each cross, and for each pair we assayed five F_1 females (i.e., 90 crosses × 2 blocks × 3 families × 5 females = 2,700 females). Three females per family were used in the ejection experiment and two females in the 72-h experiment (see below). All flies were maintained at low densities in vials with standard cornmeal-molasses-agar medium supplemented with yeast, collected as virgins upon eclosion and aged for three days before their first mating. All males were used only once; all females were mated to two males of opposite sperm-tag color.

Sperm competition experiment

We investigated reproductive outcomes at two biologically relevant time-points after the second mating (45): (i) immediately after female sperm ejection (i.e., <5 h after mating and before the first egg has entered the bursa for fertilization), and (ii) after 72 h, which is the typical female remating interval and thus represents a reliable window to examine variation in paternity. We conducted both experiments using the same isoline crosses but different sets of males and females: each female was mated with a virgin GFP male and, two days later, with a virgin RFP male, with additional 6-h remating opportunities on days 3–4 for any refractory females. For each mating, we recorded the copulation duration, removed the males from the mating vials immediately after the end of copulation and dissected the females at a given time point after mating.

In the "sperm ejection experiment," we isolated females in glass three-well spot plates beneath glass coverslips immediately after mating to the second male and checked for ejection every 10 min for up to 5 h using a stereomicroscope. We recorded the time to ejection, immediately removed females from the wells and transferred the ejected masses to saline on slides. Subsequently, we anaesthetized these females under CO_2, gently dissected the reproductive tract into 20 µl of enhanced Grace's Supplemented Insect Medium at room temperature and captured a 10-sec movie at 400× magnification using an Olympus DP71 cooled, color digital camera mounted onto an Olympus BX-60 fluorescent microscope equipped with a red-green dual filter. We analyzed sperm velocity within the seminal receptacle (SR), using the Manual Tracking plugin for ImageJ v. 1.44j (National Institutes of Health, USA). We restricted our analyses to the SR because this is the primary sperm storage organ (45, 74) and because tracking individual sperm for multiple frames in the spermathecae is not generally possible.

In the "72-h experiment," we transferred each female daily to a new vial until freezing it 72 h after remating for later dissection and quantification of sperm. We reared all progeny and assigned paternity based on the presence/absence of the ubiquitin GFP marker. We further measured the length of the thorax and the SR of one of the frozen females per family (i.e., 6 females per cross). We dissected the reproductive tract into PBS on a microscopic slide and covered it with a glass coverslip, placed on top with clay at the corners allowing flattening of the SR to two dimensions without stretching. We measured SR length using ImageJ at 200× magnification under an Olympus BX-60 microscope with Nomarski DIC optics.

For all dissected females of both experimental units, we counted the sperm of both competitors across the different organs of the female reproductive tract (bursa copulatrix, SR, and paired spermathecae) and determined the total number of sperm for each male in all female sperm-storage organs combined, the proportion of total sperm derived from the first (S_1) or second male (S_2), respectively, and the proportion of each male's total sperm representation in the female tract that reside in the SR. Combining these counts with those of the ejected masses further allowed us to calculate the number of first-male sperm still in storage at the time of remating, sperm displacement, second-male sperm transfer and the number and proportion of each male's sperm ejected.

Statistical analyses

We performed all analyses using the statistical software package R version 2.15.2 (R Development Core Team 2011), with S_2 and P_2 values normalized by arcsine/square-root transformations and the time to ejection log-transformed to meet the parametric requirements of the statistical models. Unless stated otherwise, we used general linear mixed-effects models (R package *lmer*) with restricted maximum likelihood (REML). We controlled for random block effects and for the female genetic background by including the random maternal and paternal isoline effects (i.e., general combining ability), the random isoline cross effects (i.e., specific combining ability), the random diallel reciprocal effects, and the replicate family (vial) nested within the isoline cross. Fixed effects were included as necessary and are mentioned in the text or listed in the tables.

After examining the results deriving from the full models, we performed stepwise model selection by comparing mixed models using likelihood ratio tests (maximum likelihood, ML) and refitting the final, minimum adequate models with REML (75), first removing non-significant random effects and then non-significant fixed effects. Model diagnostics revealed no evidence for overdispersion in any of our analyses based on the Pearson residuals [i.e., the sum of the squared Pearson residuals divided by the residual degrees of freedom (75); all < 0.8], for serious collinearity among fixed effects given the correlation structure in the model outputs (all < 0.6), or for non-Gaussian distributions of the residuals. To estimate denominator degrees of freedom and *P*-values of the fixed effects, we used Satterthwaite's approximation (implemented in the R package *lmerTest*), which resulted in nearly identical *P*-values as with Bayesian probability estimates (function *pvals.fnc* in the *languageR* package). *P*-values of random effects were calculated based on log-likelihood ratio tests comparing models with and without the random effect of concern. To further investigate the relationships revealed by mixed models, we performed multiple regression analyses based on the within-cross means. Most associations were stable across these different levels and are thus likely to be biologically relevant rather than statistical artifacts. Finally, for each mixed model we report the total variance explained by the fixed and random effects combined [i.e., conditional R^2; (76)], and for multiple regression analyses the multiple R^2, as indicators of the model goodness-of-fit.

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