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Report

How Multivariate Ejaculate Traits Determine Competitive Fertilization Success in *Drosophila melanogaster*

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Summary

Success in sperm competition, occurring whenever females mate with multiple males [1], is predicted to be influenced by variation in ejaculate quality and interactions among competing sperm [2]. Yet, apart from sperm number, relevant ejaculate characteristics and sperm-sperm interactions are poorly understood, particularly within a multivariate framework and the natural selective environment of the female reproductive tract. Here, we used isogenic lines of *Drosophila melanogaster* with distinguishable sperm to demonstrate and partition genetic variation in multiple sperm quality and performance traits. Next, by competing males from different lines, we show how rival sperm significantly influence each other's velocity and reveal that males with relatively slow and/or long sperm better displace rival sperm and resist displacement, thus avoiding ejection by the female from her reproductive tract. Finally, we establish fitness consequences of genetic variation in sperm quality and its role in securing a numerical advantage in storage by showing that offspring paternity is determined strictly by the representation of stored, competing sperm. These results provide novel insight into complex postcopulatory processes, illustrate that different ejaculate traits are critical at different biologically relevant time-points, and provide a critical foundation for elucidating the role of postcopulatory sexual selection in trait diversification and speciation.

Results and Discussion

Sperm competition is a near-universal phenomenon credited with driving rapid diversification of ejaculate traits in internally fertilizing species [2–6]. To understand the microevolutionary processes responsible for such diversification, we need to know how male and female traits functionally interact to contribute to variation in competitive fertilization success as well as the extent of their heritable variation. Such knowledge, however, has been elusive due to three formidable experimental challenges: (1) difficulties in observing sperm directly within their selective environment, the female reproductive tract, (2) challenges in discriminating among sperm from different males, and (3) a limited ability to examine ejaculate "quality" traits simultaneously in a multivariate approach. Consequently, investigations have predominantly assayed univariate ejaculate traits (e.g., sperm velocity) that are measured in vitro and in a noncompetitive context [7–10] (but see [11]). Constraints of such an approach on our understanding of postcopulatory sexual selection are revealed by a growing recognition that sperm competitive success involves complex ejaculate-female interactions [2, 6]. Moreover, ejaculates themselves constitute developmentally and functionally interacting traits that are likely to experience correlated evolution [12–14].

Our baseline understanding of sperm competition mechanisms in the model system *Drosophila melanogaster* has advanced due to the development of new methods of distinguishing two ejaculates within the female reproductive tract [11]. Here, we further develop this unique experimental material to examine within-population genetic variation in multivariate ejaculate traits and their contribution to fitness in the context of sperm competition within the female reproductive tract. For each of two genetically variable strains of *D. melanogaster*, expressing either green or red fluorescent protein in their sperm heads, we generated 100 isogenic lines ("isolines"; [15]) to partition genetic variation in sperm traits and patterns of sperm transfer, displacement, and storage, thus facilitating detailed multivariate and real-time spatiotemporal examinations of sperm performance and fate. After characterizing each line for ejaculate phenotypes in a standardized competitive context, we staged competitions between males from lines with contrasting sperm velocities to examine the effects of sperm length, sperm behavior, and sperm storage patterns on reproductive outcomes at three biologically relevant time-points after the second mating [11]: (1) immediately after females eject excess second-male and displaced first-male sperm (i.e., 1–5 hr after mating and before the first egg has entered the bursa for fertilization), (2) after 24 hr, when sperm displacement dynamics have subsided and sperm are competing for fertilizations, and (3) after 72 hr, which is the typical female remating interval and thus represents a reliable window to examine variation in paternity.

Repeatability in Ejaculate Traits and Female Sperm Handling

When experimentally competing focal males against standard males mating with standard females, we found significant repeatability (R) within isolines and across generations in sperm length ($R > 0.50$, $p < 0.0001$; Figure 1A; detailed results in Table S1 available online), sperm velocity ($R > 0.25$, $p < 0.002$; Figure 1B), number of sperm stored ($R > 0.23$, $p < 0.02$; Figure 1C), and in the distribution of sperm between female sperm-storage organs (i.e., seminal receptacle [SR] versus paired spermathecae; $R > 0.24$, $p < 0.001$; Figure 1D). Moreover, phenotypes of F_1 progeny from interisoline crosses were significantly correlated with the mean values of their corresponding parental lines for all four traits (see Supplemental Information), confirming that observed isoline differences were due to genetic variation rather than differential rearing conditions or inbreeding effects (e.g., [16]). Isoline males mated to virgin standard females further revealed significant isoline repeatability in the number of sperm transferred, egg-to-adult offspring viability and total number of progeny produced over a 10-day period (all $R > 0.24$, all $p < 0.002$; Supplemental Information).

Whereas heritability of some sperm traits has been reported in various taxa (reviewed in [17]), including *D. melanogaster*

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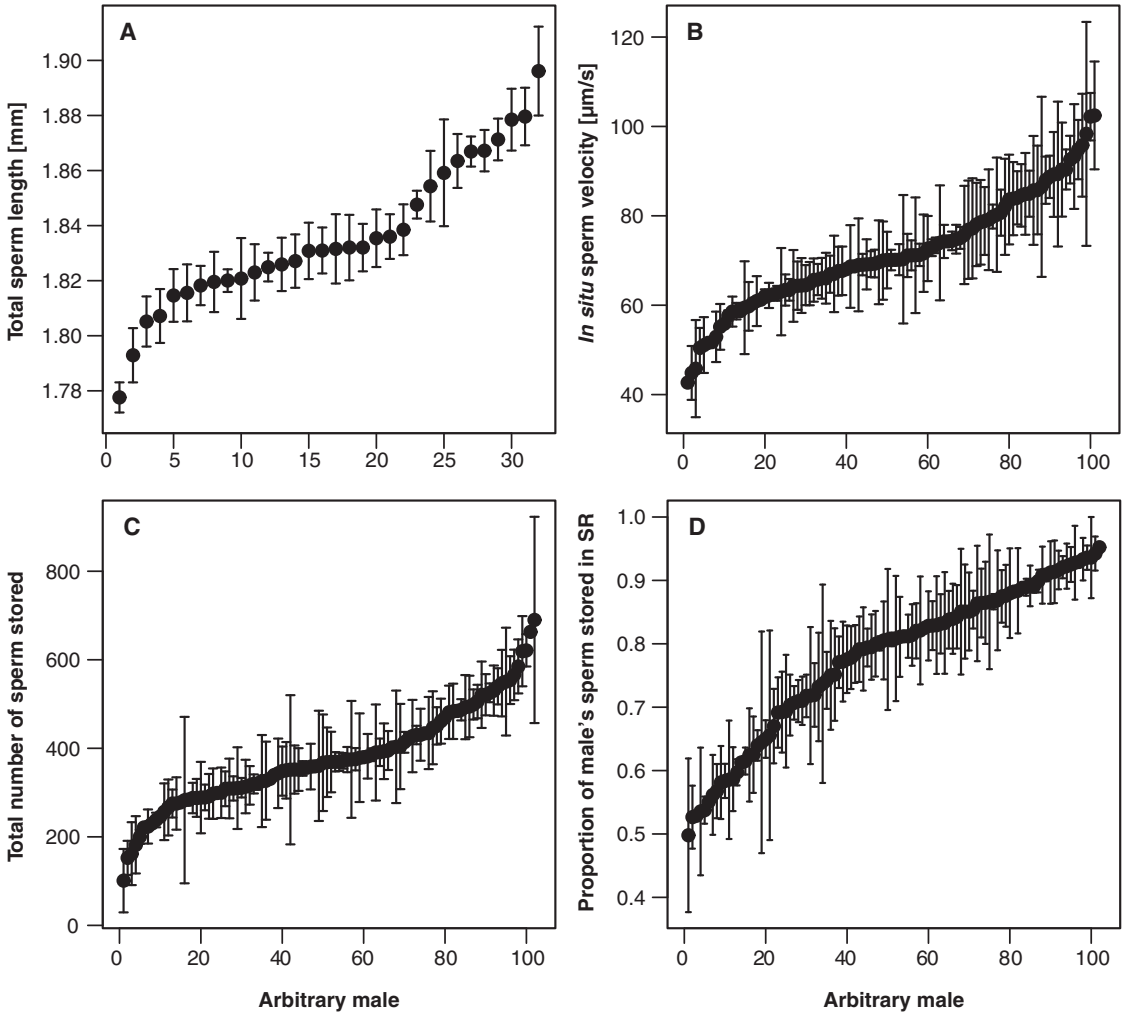

Figure 1. Within- and Between-Isoline Variation in Ejaculate and Sperm-Storage Characters

Total sperm length (A), sperm velocity (B), number of sperm in female sperm storage (C), and proportion of males' total sperm representation that is stored in female SR (D). Each point represents an individual isoline (2–4 males per isoline); error bars depict ± 1 SE. (A) is based on n = 32 isolines used for competitive experiments (generation 12), and (B)–(D) are based on the data from generation 8 since the inception of isolines (n = 101). For details, see Supplemental Information.

[18], here we present novel results on significant genetic variation in the number of sperm stored by females and of their distribution among the different sperm-storage organs, with important implications because genetic variation among individuals is a prerequisite for selection on any trait [19]. Genetic variation in sperm storage specifically is important because the relative number of sperm from competing males greatly influences male fertilization success [20]. Similarly, the location of sperm storage can be critical in *D. melanogaster* because displacement of resident sperm by the last male to mate occurs primarily in the SR [11] and because eggs are fertilized primarily by sperm stored in the SR [11, 21, 22]. The factors determining sperm distribution are not currently known, but associated among-isoline variation in accessory gland proteins (Acps) with concomitant variation in Acp-sperm-female interactions are a likely candidate, for example, resulting from segregation of different Acp loci between male isolines relative to the female receptor genotype fixed in the female isoline [6]. Investigations exploring the contribution of females and of male-female genotypic interactions to variation in ejaculate traits, sperm performance, and competitive fertilization success using these isolines are in progress.

Ejaculate Traits in Competing Males

The predictability of what sperm do within a given female background allowed us to subject focal males to competitive matings against specific male phenotypes. In fully factorial competitive matings between males from isolines with contrasting sperm velocities, we measured sperm velocity of both competing ejaculates simultaneously within the SR (Movie S1), immediately after female sperm ejection or 24 hr after mating, in two separate experiments using the same isolines but different sets of males. Controlling for isoline identity (n = 32), sperm-tag color and sperm density effects (see Experimental Procedures), sperm velocity of the competing males was highly correlated in both experiments (ejection: $r = 0.31$, $t = 4.50$, $p < 0.0001$, n = 132 male pairs; Figure 2; 24 hr: $r = 0.32$, $t = 3.57$, $p = 0.0005$; n = 165 male pairs). Further, the relative difference in sperm velocity between first and second males was significantly correlated among the two

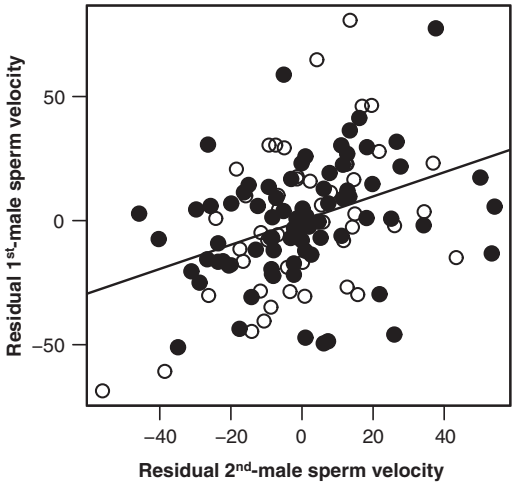

Figure 2. Competing Ejaculates Influence Each Other's Sperm Velocity

Relationship between first- and second-male sperm velocities in the ejection experiment ($r = 0.31$, $p < 0.0001$). Each data point depicts a pair of males (n = 132). Both axes are controlled for sperm-tag color (open circles, RFP second male; closed circles, GFP second male) and isoline identity.

experiments ($r = 0.37$, $t = 2.93$, $p = 0.005$; n = 86 male pairs from 31 isolines; Figure S1), suggesting strong sperm-sperm interactions that may be inherent to particular isoline combinations. These interactions were consistently accompanied by differences in sperm velocity between first- and second-male mating roles of individual males tested twice against the same competitor in reversed mating orders. Specifically, in the first-male compared to second-male role, fast sperm in competition against slow sperm were 13.4% ± 6.5% slower (n = 34 males) and slow sperm competing against fast sperm were 15.2% ± 7.0% faster (n = 35 males), whereas the differences for competitions among equal velocity phenotypes were <2.1%. It seems likely that the first-male sperm are more strongly influenced by those of the second male given the preponderance of both second-male sperm and seminal plasma within the SR, but this conjecture warrants further investigation. The influence of one male ejaculate phenotype upon that of his competitor was not restricted to sperm velocity, because we also found a significant relationship between the number of resident sperm in storage and that of second-male sperm transferred ($r = 0.22$, $t = 2.38$, $p = 0.02$, n = 147 male pairs from 32 isolines). The mechanism underlying this relationship remains unresolved, but it indicates a potentially more sophisticated, plastic adjustment in sperm transfer than simply in response to presence or absence of a previous male's sperm in storage [23], or relative to some variation in female "attractiveness" (despite being from a single isoline), with both competing males modulating sperm numbers in a correlated manner. In contrast, other traits such as copulation duration were unaffected by the competitor phenotype ($p > 0.49$ in all three experiments).

Overall, our results provide unprecedented resolution to the behavior of sperm in their selective environment, not only by measurements within the complex, three-dimensional female reproductive tract but also by simultaneous analysis of competing ejaculates. The significant influence of competing ejaculates on each other's sperm velocity emphasizes the importance of understanding sperm behavior in a competitive context rather than under noncompetitive in vitro conditions. It further confirms previous suggestions that one male's ejaculate may affect the performance of that of his competitor (e.g., [24]).

Effects of Ejaculate Traits on Sperm Competition Processes

Theoretical models predict that fertilization by stored sperm from different males can occur according to the relative sperm representation or with a bias toward one or the other male [25, 26]. However, direct empirical evidence for these predictions is largely lacking, and the processes determining the "fertilization set" from which sperm are drawn to fertilize the eggs remain largely unexplored, particularly in relation to variation in ejaculate quality. We thus competitively mated males of isolines with contrasting sperm velocities for spatiotemporal analyses on multivariate ejaculate traits.

Males of the low-velocity isolines transferred significantly more sperm than those of the high-velocity lines (slow, mean ± SE: 1,356.9 ± 49.9 sperm, n = 78 males; fast: 1,170.1 ± 51.1 sperm, n = 73 males; linear mixed-effects model [LME] controlling for sperm-tag color and isoline identity: $t = 2.71$, $p = 0.008$, n = 151 males from 32 isolines). Unsurprisingly, if the second male transferred a large ejaculate, the female retained relatively more of his sperm after ejection ($r = 0.54$, $t = 3.49$, $p = 0.002$, n = 62 pairs of males from 27 isolines; Table S2) but fewer of the resident first-male sperm ($r = -0.42$, $t = -2.47$, $p = 0.02$, n = 62 pairs of males from 28 isolines; Table S3; also see [11]). Of greater interest, in a multivariate analysis controlling for these sperm numbers, the proportion of second-male sperm (S_2) in storage immediately after sperm ejection increased if the second-male sperm were relatively longer ($r = 0.63$, $t = 4.32$, $p = 0.0002$, n = 62 pairs of males from 29 isolines; Figure 3A) and slower ($r = -0.42$, $t = -2.42$, $p = 0.02$; Figure 3B) than those of the first male. Similar long-sperm and slow-sperm advantages were obtained when focusing on the absolute numbers of second-male sperm entering storage or first-male sperm remaining in it (Tables S2 and S3) or the number of either male's sperm ejected by the female (Tables S4 and S5).

Overall, these results indicate that relatively long and slow sperm are at an advantage in entering or remaining in the "fertilization set" during the sperm storage and displacement phase of sperm competition [8], thereby gaining a chance of being used for fertilization later (see below). This slow-sperm advantage contrasts with previous studies, because most have documented a fast-sperm fitness benefit under both noncompetitive [27–29] and competitive conditions [30–32] (but see [33]). Similarly, the long-sperm combined with slow-sperm advantage, which was accompanied by a direct negative association between these two traits at the isoline level measured under standardized conditions ($r = -0.40$, $t = -2.38$, $p = 0.02$; n = 32 isolines), may appear counterintuitive. Previous studies in a range of taxa have reported either no association between sperm morphological traits and sperm velocity (e.g., [34–36]) or positive covariation [10, 37–39]. However, whereas these studies quantified noncompetitive in vitro sperm velocity of external fertilizers (e.g., fish) or internal fertilizers with very small sperm relative to the size of the female reproductive tract (i.e., mammals or birds), we measured competitive sperm behavior in vivo in an insect in which the SR is barely longer than an individual sperm [22]. Because sperm travel only a short distance [22], they may not be under selection to win the "race" into storage or to the egg as is typically hypothesized for other taxa.

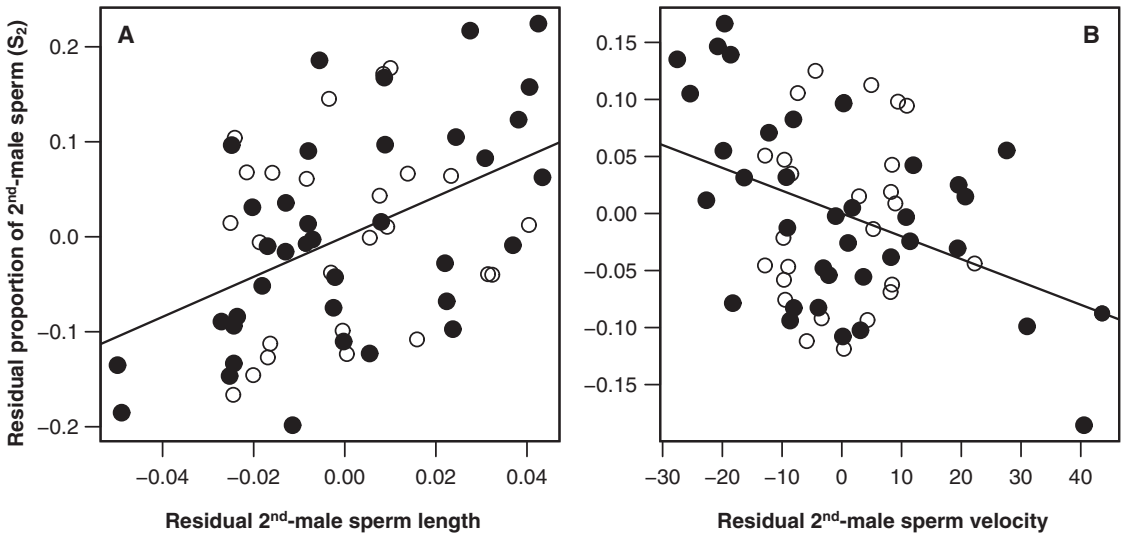

Figure 3. Effects of Sperm Length and Sperm Velocity on Relative Sperm Storage

Associations of S_2 immediately after female sperm ejection with second-male sperm length ($r = 0.40$, $p = 0.03$) (A), and second-male sperm velocity ($r = -0.54$, $p < 0.0001$) (B), controlling for sperm-tag color (open circles, RFP second male; closed circles, GFP second male), first-male sperm velocity and sperm length, the numbers of resident first-male sperm and second-male sperm transferred, and isoline ID.

Additionally, physical interactions among sperm and between sperm and the female may be of greater selective consequence than in larger-bodied internal fertilizers [3, 14, 40], consistent with the correlation in sperm velocity among males (see above).

We do not currently understand the precise mechanisms by which sperm velocity influences sperm displacement. Slower sperm may be more likely to remain in the SR (a long blind-ended tubule with a single entrance and exit), simply by approaching the proximal opening less frequently when moving back and forth within the organ [11], thus having a lower probability of being released during the dynamic processes of sperm displacement. Alternatively, sperm motility may be influenced by seminal fluid components or sperm density, possibly enhanced by sperm length effects, such that the velocity may be a consequence of variation in other ejaculate traits and not under direct selection itself. Although there are still gaps in our understanding of the selective causes of variation in sperm velocity, our results highlight the importance of investigating sperm structure-function relationships within the female tract and in a competitive context.

Comparative investigations of a variety of taxa have found a positive relationship between sperm length and the risk of sperm competition (these studies plus exceptions reviewed by [2, 4, 5]). By competing males from populations experimentally evolved to have either long or short sperm within females evolved to have long or short SRs (all traits bidirectionally exceeding natural variation), Miller and Pitnick [18] found that male and female morphologies interact and found a sperm competition advantage accrued by males with longer sperm increases with female SR length. Subsequent experiments with this same material suggested that longer sperm are better at displacing, and resisting displacement by, competitor sperm from the SR and better at occupying the proximal region of the SR, thus being more likely to be used for fertilization [41]. Here, we confirmed a longer-sperm advantage during the displacement process using natural variation in sperm length and while holding SR length constant (i.e., using standardized females). However, although we observed some sperm segregation between the SR regions, there was no sperm-length bias in location.

So far, we have shown that sperm length and velocity play a critical role in determining the composition of the fertilization set from which sperm are drawn to fertilize eggs. Subsequently, fertilization occurred strictly according to the relative representation of each male's sperm in storage. The proportion of progeny sired by the second male (P_2) decreased significantly with an increasing number of first-male sperm ($r = -0.50$, $t = -5.91$, $p < 0.0001$; n = 140 males from 32 isolines) and tended to increase with the number of second-male sperm at 72 hr after mating ($r = 0.17$, $t = 1.77$, $p = 0.08$) but was not associated with either sperm length or velocity (all $p > 0.19$; assuming that relative sperm velocities are correlated between time points as between the ejection and 24 hr experiments; see above). A further slope test controlling for confounding variables (Supplemental Information) yielded a slope of 1.14 (95% CI = 0.38–1.91) between P_2 and S_2 at 72 hr, thereby again indicating no significant fertilization bias. Although the lack of a fertilization bias is consistent with theoretical predictions [25, 26] and empirical data in *D. melanogaster* [11], our spatio-temporal analyses combined clearly indicate the importance of understanding the complex and temporally dynamic reproductive biology of the experimental organism before testing and interpreting putative biases in sperm use. Specifically, it is critical to discriminate processes and events (e.g., displacement and ejection) that determine the composition of the fertilization set from patterns of sperm use for fertilization after its establishment.

Conclusions

Numerous comparative analyses of diverse taxa have attributed much of the rapid evolutionary diversification of ejaculate and female reproductive tract characters to postcopulatory sexual selection [3, 4, 6, 42]. Here, we studied the microevolutionary processes that may give rise to such macroevolutionary patterns. By visualizing and identifying sperm, and by

using an isogenic line approach to partition genetic variation in ejaculate characteristics, we were able to study spatiotemporal effects of in vivo sperm behavior under competitive conditions. Overall, our results highlight the complexity and multifarious nature of postcopulatory processes, with significant interactions among competing ejaculates, and the role of different ejaculate quality traits at separate stages after mating. Relatively slow and long sperm were at an advantage during the sperm displacement phase, when sperm "compete" for membership in the fertilization set established by the time of female sperm ejection. Because subsequent fertilization success was determined solely by the numerical representation of each male's sperm, traits that maximize the chances of entering the fertilization set during the displacement phase are critical. Further investigations in different female backgrounds will allow us to also examine genetic variation in female contribution to these processes and the role of male-female interactions.

Experimental Procedures

For a detailed description of all procedures, see the Supplemental Information.

Experimental Material

To discriminate sperm from different males and quantify sperm motility in situ, we conducted all experiments with genetically variable LH_m populations of *D. melanogaster* that produce sperm with heads expressing either green (GFP) or red (RFP) fluorescent protein [11]. The GFP line also ubiquitously expresses GFP that permits unambiguous paternity assignment of progeny [43].

For each of the RFP and GFP outbred strains, we generated 100 isogenic lines and characterized them twice, four generations apart and under competitive conditions (i.e., against a standardized first male), for various ejaculate traits: (1) sperm length, (2) density-independent sperm velocity, (3) number of sperm stored per copulation, and (4) the proportion of a given male's total sperm representation in the female tract that reside in the SR (i.e., primary sperm-storage organ). All experimental males were derived from these isolines, and all females originated from a separate isoline of the wild-type strain (LH_m).

Sperm Competition Experiments

Based on the isoline ranking in our initial assays (see Figure 1B), we selected for each sperm-tag color the eight isolines with the highest and the eight isolines with the lowest mean sperm velocity, respectively, for fully factorial competitive matings between GFP and RFP lines with contrasting sperm velocities. Eight males per isoline were each randomly assigned to an isoline of the opposite color (four fast and four slow). Each male was then mated to two females, once as the first and once as the second male, with the corresponding competitor mated to the same females in reversed mating order.

We conducted three separate competitive experiments using the same isoline combinations but different sets of males and females, dissecting females at a given time point after the start of mating with the second male. In the "ejection experiment," we recorded the time from mating to female sperm ejection, number of each male's sperm ejected and retained, and velocity of stored sperm immediately after ejection. Combining ejected and retained sperm, we calculated the numbers of first-male sperm still in storage at the time of remating and of second-male sperm transferred. In the 24 hr experiment, we quantified the number and velocity of each male's sperm within the female SR 24 hr after mating. In the 72 hr experiment, we quantified all sperm still in storage 72 hr after remating and scored paternity for all progeny produced during that period (using ubiquitin GFP marker).

Statistical Analyses

Unless stated otherwise, we used general linear mixed-effects models (LME), controlled for isoline identity (random factor), sperm-tag color (fixed factor), and, in analyses involving sperm velocity, for the total number of sperm present in the distal sections of the SR to account for density dependence. Sample sizes varied between analyses due to missing data, particularly in the ejection experiment (for details see Supplemental Information).

Supplemental Information

Supplemental Information includes one figure, five tables, Supplemental Experimental Procedures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.06.059.

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