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Supplemental Information

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

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Supplemental Inventory

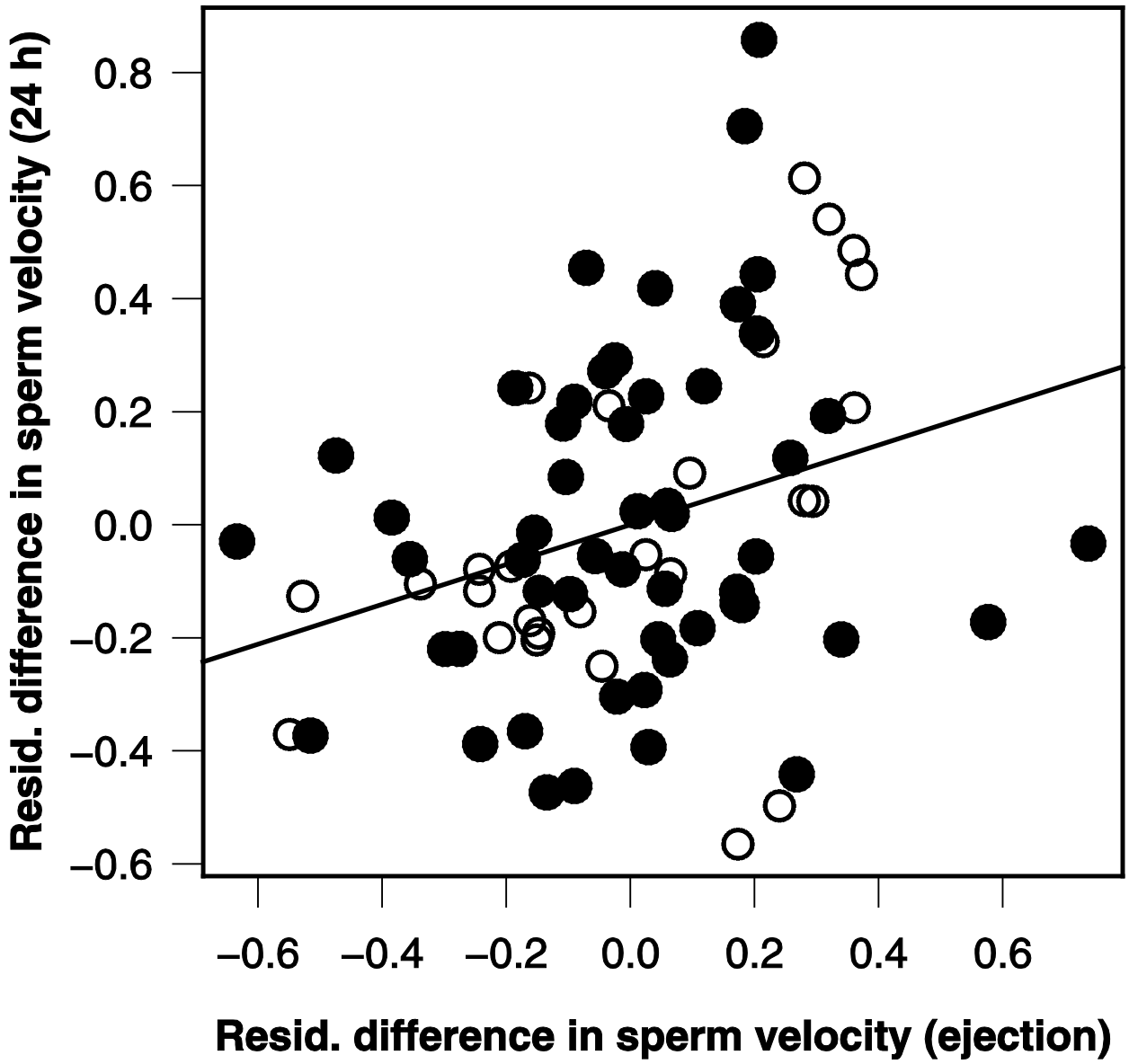

Figure S1. Relative Differences between First- and Second-Male Sperm Velocity across Time Points, Related to Figure 2

Association between the relative differences in sperm velocity (i.e., (2^{nd} male – 1^{st} male) / (2^{nd} male + 1^{st} male)) of the ejection experiment and 24-hr experiment. Each data point depicts a pair of isolines that were tested in both experiments ($r = 0.37$, $t = 2.93$, $p = 0.005$; $n = 86$ males from 31 isolines). Both axes are controlled for sperm-tag color (fixed factor; open circles: second male was RFP; closed circles: second male was GFP) and isoline ID (random factor).

Table S1. Repeatability of Sperm Quality and Sperm Storage Traits, Related to Figure 1

Repeatability values within and between generations as measured after 24 hrs or, in the case of the ejection experiment, immediately after mating. All focal males were the second of two males, mated two days after a standard competitor, and all values were controlled for sperm-tag color effects and, in the case of sperm velocity, for sperm density in the SR, using a linear mixed-effects model approach for repeatability analysis [1]. All analyses are based on data from 2–4 males per isoline. *P*-values in parentheses.

Trait	**Within generation 4 (*n* = 174 isolines)**	**Within generation 8 (*n* = 101 isolines)**	**Between generations (*n* = 101 isolines)**	**Ejection experiment (*n* = 32 isolines)**
Sperm velocity	0.294 (<0.0001)	0.251 (0.0001)	0.298 (0.002)	0.209 (0.020)
Sperm storage	0.367 (<0.0001)	0.328 (<0.0001)	0.230 (0.020)	0.194 (0.016)
Sperm distribution	0.245 (<0.0001)	0.241 (0.0001)	0.318 (0.001)	0.193 (0.039)
Sperm length*	0.541 (<0.0001)	0.497 (<0.0001)	0.717 (<0.0001)	

*Sperm length data are listed for comparison of intra- and inter-generational repeatability but were obtained in generations 12 and 26, respectively, and restricted to the 32 isolines used in the competitive experiments, with 5 sperm measured in each of 5 males per isoline. In generation 12, we assayed all 32 isolines, in generation 26, we remeasured only the 16 GFP lines due to the high repeatability in the first assay and expectedly high heritability from previous studies (e.g., [2]).

Table S2. Influences of the Number of Second-Male Sperm Retained after Ejection

Results of a linear mixed-effects model explaining the variation in the number of second-male sperm retained in storage after female sperm ejection.

Fixed terms	**Estimate**	**SE**	**df**	***t***	***p***	***r***
Sperm velocity (1^{st})	0.80	0.39	30	2.07	**0.047**	0.35
Sperm velocity (2^{nd})	–1.43	0.56	30	–2.56	**0.016**	–0.42
Resident sperm (1^{st})	–2.46	2.45	30	–1.01	0.323	–0.18
Sperm transferred (2^{nd})	0.08	0.02	30	3.49	**0.002**	0.54
Sperm length (1^{st})	–966.94	429.95	30	–2.25	**0.032**	–0.38
Sperm length (2^{nd})	365.27	356.75	24	1.02	0.316	0.20
Sperm-tag color	–58.60	22.68	24	2.58	**0.016**	0.47

Random terms	**Var. comp.**	**SE**	**df**	***LRT***	***p***
Isoline ID	492.15	22.18	1	0.30	0.583
Residual	4420.72	66.49	1		

Parameter estimates are on the raw scale except the number of resident sperm, which was square-root transformed; boldface indicates significant values. *LRT* = log-likelihood ratio test, Var. comp. = Variance component; $n = 62$ male pairs from 27 isolines.

Table S3. Influences of the Number of First-Male Sperm Retained after Ejection

Results of a linear mixed-effects model explaining the variation in the number of first-male sperm retained in storage after female sperm ejection.

Fixed terms	**Estimate**	**SE**	**df**	***t***	***p***	***r***
Sperm velocity (1^{st})	0.00	0.21	29	–0.02	0.986	0.00
Sperm velocity (2^{nd})	–0.05	0.31	29	–0.17	0.867	–0.03
Resident sperm (1^{st})	8.22	1.33	29	6.16	**<0.001**	0.75
Sperm transferred (2^{nd})	–0.03	0.01	29	–2.47	**0.020**	–0.42
Sperm length (1^{st})	455.41	233.08	25	1.95	0.062	0.36
Sperm length (2^{nd})	–441.56	177.57	29	–2.49	**0.019**	–0.42
Sperm-tag color	20.45	11.47	25	1.78	0.087	0.34

Random terms	**Var. comp.**	**SE**	**df**	***LRT***	***p***
Isoline ID	0.000	0.000	1	0.000	0.999
Residual	0.001	0.377	1		

Parameter estimates are on the raw scale except the number of resident sperm, which was square-root transformed; boldface indicates significant values. *LRT* = log-likelihood ratio test, Var. comp. = Variance component; $n = 62$ male pairs from 28 isolines.

Table S4. Influences on the Number of Second-Male Sperm Ejected by the Female

Results of a linear mixed-effects model explaining the variation in the number of second-male sperm ejected by the female.

Fixed terms	**Estimate**	**SE**	**df**	***t***	***p***	***r***
Sperm velocity (1^{st})	–1.01	0.40	25	–2.53	**0.018**	–0.45
Sperm velocity (2^{nd})	1.67	0.62	25	2.72	**0.012**	0.48
Resident sperm (1^{st})	2.48	2.73	25	0.91	0.371	0.18
Sperm transferred (2^{nd})	0.91	0.03	25	33.46	**<0.001**	0.99
Sperm length (1^{st})	1062.05	438.13	25	2.42	**0.023**	0.44
Sperm length (2^{nd})	–213.52	412.30	22	–0.52	0.610	–0.11
Sperm-tag color	–52.73	24.71	22	–2.13	**0.044**	–0.41

Random terms	**Var. comp.**	**SE**	**df**	***LRT***	***p***
Isoline ID	946.84	30.77	1	0.90	0.343
Residual	3984.99	62.97	1		

Parameter estimates are on the raw scale except the number of resident sperm, which was square-root transformed; boldface indicates significant values. *LRT* = log-likelihood ratio test, Var. comp. = Variance component; $n = 55$ male pairs from 25 isolines.

Table S5. Influences on the Number of First-Male Sperm Ejected by the Female

Results of a linear mixed-effects model explaining the variation in the number of first-male sperm ejected by the female.

Fixed terms	**Estimate**	**SE**	**df**	***t***	***p***	***r***
Sperm velocity (1^{st})	0.11	0.25	22	0.44	0.662	0.09
Sperm velocity (2^{nd})	–0.05	0.39	22	–0.14	0.894	–0.03
Resident sperm (1^{st})	23.32	1.77	22	13.19	**<0.001**	0.94
Sperm transferred (2^{nd})	0.02	0.02	22	1.05	0.305	0.22
Sperm length (1^{st})	–522.04	277.39	25	–1.88	0.072	–0.35
Sperm length (2^{nd})	351.79	228.35	22	1.54	0.138	0.31
Sperm-tag color	–13.13	13.70	25	–0.96	0.347	–0.19

Random terms	**Var. comp.**	**SE**	**df**	***LRT***	***p***
Isoline ID	0.00	0.00	1	0.00	1.000
Residual	1865.40	43.19	1		

Parameter estimates are on the raw scale except the number of resident sperm, which was square-root transformed; boldface indicates significant values. *LRT* = log-likelihood ratio test, Var. comp. = Variance component; $n = 55$ male pairs from 25 isolines.

Supplemental Experimental Procedures

Experimental Material

To discriminate sperm from different males and quantify sperm motility *in situ*, all experiments were conducted with genetically variable 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 [3] for transformation and fitness assay details). The GFP strain also ubiquitously expresses GFP, thus permitting paternity assignments on progeny (P_1 and P_2, respectively).

For each of the RFP and GFP outbred strains, we used isogenic lines ("isolines"; [4]), generated by four generations of fullsib inbreeding. The fifth and all subsequent generations of each isoline were initiated by allowing 4–6 females to lay 50–80 eggs in a vial with standard cornmeal-molasses-agar medium supplemented with yeast. All males used in the experiments were derived from these lines, and all females from a separate isoline originating from the wild-type strain (LH_m), raised at 50 individuals per vial to minimize female size variation as a potential confounding effect on sperm transfer and utilization (e.g., [5, 6]). All flies were maintained at low densities in vials with standard cornmeal-molasses-agar medium supplemented with yeast. They were all 3 days old on the day of their first experimental mating, and each male had mated once with a non-experimental female the previous day to avoid potential virgin effects [7].

Characterization of Isolines

Sperm performance assays were conducted twice under competitive conditions in generations 4 and 8, respectively. Three 3-day-old males per isoline were each mated second to a 5-day-old wild-type isoline female 2 days after the female had been inseminated once by a "standard competitor" with a reciprocal fluorescent label. All standard competitor males were derived from a single, non-focal GFP or RFP isoline, respectively. The standardized remating interval of 2 days further minimized variation in the competitive conditions because the time to remating is likely to affect the dynamics of sperm competition, for example by the number of first-male sperm still in storage. For each focal mating, we recorded the copulation duration and removed the males from the mating vials immediately after the end of copulation.

Twenty-four hours after the second mating, we anaesthetized 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 using an Olympus DP71 cooled, color digital camera mounted onto an Olympus BX-60 fluorescent microscope at 400× magnification. We measured slice-by-slice instantaneous linear velocities (µm/s) for 10 haphazardly selected sperm per male 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 [3, 8] and because tracking individual sperm for multiple frames in the spermathecae is not generally possible. However, since RFP sperm were often masked by GFP sperm, we captured two movies per slide in rapid succession, using a red-green dual pass filter and a red filter, respectively. We tracked sperm for approximately 0.5–1.0 s, depending on the duration for which sperm remained in the focal plane and swam continuously without rapid turns and stops. More detailed analyses of sperm behavior, including these characteristic turns and stops as well as mass movements, are part of ongoing research in our laboratory.

We subsequently 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.

In 32 isolines used in the sperm competition experiments (see below), we also examined sperm velocity, storage and distribution immediately after female sperm ejection. Female *D. melanogaster* typically eject excess second-male sperm and displaced first-male sperm 1–5 hrs after copulation [3, 8], and sperm velocity might play an important role during this phase of sperm displacement. We thus repeated the above assays in an additional generation, but instead of dissecting females after 24 hrs, we dissected them immediately after they ejected the sperm mass (see below).

The same 32 isolines were also assayed under single-mate conditions, with four 3-day-old non-virgin males/isoline each mated with two 3-day-old virgin females on successive days. Two randomly chosen females per male isoline and day were flash-frozen 60 min after the start of mating (i.e., peak of second-male sperm storage and first-male displacement and well before females eject excess sperm and begin to lay eggs [3]), the other two were allowed to lay eggs for 10 days, with females transferred to a new oviposition vial daily and all progeny reared. We determined sperm transfer in the 60-min treatment, and egg-to-adult viability and single-mate productivity in the 10-d treatment. All three traits were significantly repeatable within isolines (sperm transfer: $R = 0.35$, $p = 0.0002$; egg-to-adult offspring viability: $R = 0.27$, $p = 0.001$; total number of progeny: $R = 0.25$, $p = 0.002$). We further used the mean egg-to-adult viability per isoline to correct the paternity estimates in the paternity experiment above [9, 10].

Finally, these 32 isolines were assayed for sperm length, with five sperm measured from each of five males per isoline. Following ether anesthetization of a male, we dissected one seminal vesicle into phosphate-buffered saline (PBS) on a subbed microscope slide and then ruptured it with a fine probe. Sperm were dispersed in the droplet before drying the slide at 60 °C, fixing in methanol:acetic acid (3:1), rinsing in PBS and mounting under a coverslip in glycerol and PBS (80/20 v/v). We measured dark-field images of five sperm per male at 200× magnification by tracing with the segmented line tool of ImageJ. For inter-generational repeatability analyses, we remeasured sperm length in only the 16 GFP isolines because it is well established that sperm length is highly heritable in *D. melanogaster* [2].

Isoline Hybrids

To confirm that the within-isoline repeatability reflected genetic variation rather than variation in rearing conditions or differential inbreeding effects (e.g., [11]), we generated 12 "hybrid isolines" (three per sperm-tag color and sperm-velocity type) and compared their ejaculate traits with their respective parental lines (all examined under the same standardized conditions as above). All F_1 progeny of these isoline crosses were expected to be highly heterozygous, thus showing no inbreeding effects. Additionally, we hypothesized that if the isoline repeatability reflects fixed genetic variation, crosses between fast-sperm isolines or between slow-sperm isolines should also exhibit fast or slow sperm, respectively, but in the case of differential inbreeding, the trait values of these crosses should be random relative to their parental lines. Of all 12 isoline combinations, one produced no progeny, thereby resulting in 11 available isoline crosses. Controlling for sperm-tag color, the means of the hybrid F_1 lines were significantly

correlated with the mean values between their corresponding parental lines for sperm velocity (r = 0.62, t = 2.37, p = 0.045), total number of focal-male sperm in storage (r = 0.64, t = 2.47, p = 0.036), the proportion thereof stored in the SR (r = 0.73, t = 3.24, p = 0.01), and the proportion of second-male sperm among all stored sperm (i.e., S_2; r = 0.79, t = 3.88, p = 0.005). Based on these results, combined with the inter-generational repeatability, we conclude that the observed differences between isolines are the result of repeatable genetic variation.

Sperm Competition Experiments

Based on the isoline ranking in our previous assays, we selected for each sperm-tag color the 8 isolines with the fastest and the 8 isolines with the slowest mean sperm velocity, respectively, for fully factorial competitive experiments between GFP and RFP lines. Eight males per isoline were each randomly assigned to 8 different isolines of the opposite color (4 fast-sperm and 4 slow-sperm isolines, respectively). Each male was then mated to two females, once as the first and once as the second male, with the corresponding competitor being mated to the same females in reversed mating order, resulting in n = 256 pairs of males across n = 128 unique combinations of isolines.

We conducted three separate competitive experiments using the same isoline combinations but different sets of males and females. In each experiment, the first of two matings was with virgin females and the first male of each competitive male pair. Two days later, each female remated once with the second male of each pair, with additional 6-hr remating opportunities on days 3–5 for any refractory females. Thus, each male pair competed twice in different females and in reversed mating order. 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 remating.

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 hours using a stereomicroscope. We recorded the time to ejection and immediately transferred the ejected masses to saline on slides and dissected the females to capture 10-sec movies for measurements of sperm velocity and to quantify 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 (for details see *Characterization of isolines* above).

In the 24-hr experiment (“sperm-storage experiment”), we dissected females 24 hrs after remating and immediately captured 10-sec movies to measure sperm velocity and quantify sperm within the female reproductive tract. The 24-hr period corresponds to the time when sperm displacement dynamics have subsided (i.e., ejection has occurred) and sperm are competing for fertilizations (i.e., egg-laying is underway [3]).

In the 72-hr experiment (“paternity experiment”), we transferred females daily to a new vial until freezing them 72 hrs after mating for later dissection and quantification of sperm. We reared all progeny from all three vials and assigned paternity based on the presence/absence of the ubiquitin GFP marker. A 72-hr period reflects the typical female remating interval and thus represents a reliable window to examine variation in paternity [3].

Statistical Analyses

We performed all analyses with the statistical software package R version 2.12.2 (R Development Core Team 2011). Unless stated otherwise, we used general linear mixed-effects models (LME) with restricted maximum likelihood (REML), controlling for isoline identity as a random factor and sperm-tag color as a fixed factor (which also determined the mating order), but for simplicity we will omit these terms in the in-text results as they were included to control for confounding effects rather than due to biological interest for the interpretation of the results. We arcsine/square-root transformed proportions and square-root transformed the number of resident (first-male) sperm in the ejection experiment due to heteroscedasticity in the residuals. Since preliminary data suggested that sperm velocity is highly density-dependent in *Drosophila*, we further controlled analyses involving sperm velocity for the total number of sperm present in the distal section of the SR, except for analyses that already contained numbers of transferred or stored sperm to avoid substantial collinearity. 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 [12].

To test whether female sperm use was proportional to the relative sperm representation in storage or biased towards one or the other male [13], we determined the slope of the relationship between S_2 and P_2, with a slope of 1 indicating no fertilization bias and a slope different from 1 indicating a fertilization bias [14, 15]. To calculate the slope while controlling for sperm-tag color and isoline identity, we performed a mixed model with P_2 as the response variable, isoline as the random factor, and sperm-tag color, the isoline means of S_2 and individual S_2 values centered about these means as the fixed effects, including the interaction between them [16].

Sample sizes varied between analyses due to missing data, particularly in the ejection experiment. Additionally, we excluded all females with no second-male sperm in storage and all females that laid no fertile eggs between the two copulations and had no first-male sperm in storage (i.e., indicating no first-male sperm transfer rather than complete sperm displacement). Similarly, the total sperm counts across the ejected masses (n = 172) were bimodally distributed, with n = 60 samples containing a mean ± SE of 52.2 ± 8.2 (range = 2–268) sperm and the other n = 112 samples counting 1005.6 ± 37.0 (378–2639) sperm. Since there was no difference between these groups of males in the number of second-male sperm remaining in the female reproductive tract (431.2 ± 18.3 and 437.5 ± 12.7 sperm, respectively), the small ejected masses were likely to have broken apart during processing, which was also confirmed by the lack of a gelatinous autofluorescent component on many of these slides. We thus excluded these ejection data assuming incomplete sperm quantification. Finally, we excluded n = 35 females from all analyses using data on sperm ejection, sperm retention/displacement, or S_1/S_2 from the ejection experiment because they were confirmed not to have ejected a sperm mass within the 5-hr window allotted to minimize the risk of female desiccation (i.e., bursa still full of sperm and no ejected mass found in wells). However, these females were used in analyses involving the total numbers of first-male resident sperm or sperm transferred by the second male.

Supplemental References

1. Nakagawa, S., and Schielzeth, H. (2010). Repeatability for Gaussian and non-Gaussian data: a practical guide for biologists. Biol. Rev. *85*, 935-956.
2. Miller, G.T., and Pitnick, S. (2002). Sperm-female coevolution in *Drosophila*. Science *298*, 1230-1233.
3. Manier, M.K., Belote, J.M., Berben, K.S., Novikov, D., Stuart, W.T., and Pitnick, S. (2010). Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. Science *328*, 354-357.
4. Parsons, P.A., and Hosgood, S.M.W. (1968). Genetic heterogeneity among the founders of laboratory populations of *Drosophila*. I. Scutellar chaetae. Genetica *38*, 328-339.
5. Lüpold, S., Manier, M.K., Ala-Honkola, O., Belote, J.M., and Pitnick, S. (2011). Male *Drosophila melanogaster* adjust ejaculate size based on female mating status, fecundity and age. Behav. Ecol. *22*, 184-191.
6. Amitin, E.G., and Pitnick, S. (2007). Influence of developmental environment on male- and female-mediated sperm precedence in *Drosophila melanogaster*. J. Evol. Biol. *20*, 381-391.
7. Bjork, A., Dallai, R., and Pitnick, S. (2007). Adaptive modulation of sperm production rate in *Drosophila bifurca*, a species with giant sperm. Biol. Lett. *3*, 517-519.
8. Nonidez, J.F. (1920). The internal phenomena of reproduction in *Drosophila*. Biol. Bull. *39*, 207-230.
9. Gilchrist, A.S., and Partridge, L. (1997). Heritability of pre-adult viability differences can explain apparent heritability of sperm displacement ability in *Drosophila melanogaster*. Proc. R. Soc. B *264*, 1271-1275.
10. Droge-Young, E.M., Manier, M.K., Lüpold, S., Belote, J.M., and Pitnick, S. (2012). Covariance among premating, post-copulatory and viability fitness components in *Drosophila melanogaster* and their influence on paternity measurement. J. Evol. Biol., doi: 10.1111/j.1420-9101.2012.02540.x.
11. David, J.R., Gibert, P., Legout, H., Pétavy, G., Capy, P., and Moreteau, B. (2005). Isofemale lines in *Drosophila*: an empirical approach to quantitative trait analysis in natural populations. Heredity *94*, 3-12.
12. Zuur, A.F., Ieno, E.N., Walker, N.J., Saveliev, A.A., and Smith, G.S. (2009). Mixed Effects Models and Extensions in Ecology with R (New York, NY: Springer).
13. Parker, G.A., and Pizzari, T. (2010). Sperm competition and ejaculate economics. Biol. Rev. *85*, 897-934.
14. Parker, G.A. (1990). Sperm competition games: raffles and roles. Proc. R. Soc. B *242*, 120-126.
15. Parker, G.A., Simmons, L.W., and Kirk, H. (1990). Analysing sperm competition data: simple models for predicting mechanisms. Behav. Ecol. Sociobiol. *27*, 55-65.
16. Singer, J.D. (1998). Using SAS PROC MIXED to fit multilevel models, hierarchical models, and individual growth models. J. Educ. Behav. Stat. *24*, 323-355.