Current Biology 19, 751–757, May 12, 2009 ©2009 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2009.03.036

Report

Seminal Fluid Protein Allocation and Male Reproductive Success

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Summary

Postcopulatory sexual selection can select for sperm allocation strategies in males [1, 2], but males should also strategically allocate nonsperm components of the ejaculate [3, 4], such as seminal fluid proteins (Sfps). Sfps can influence the extent of postcopulatory sexual selection [5–7], but little is known of the causes or consequences of quantitative variation in Sfp production and transfer. Using *Drosophila melanogaster*, we demonstrate that Sfps are strategically allocated to females in response to the potential level of sperm competition. We also show that males who can produce and transfer larger quantities of specific Sfps have a significant competitive advantage. When males were exposed to a competitor male, matings were longer and more of two key Sfps, sex peptide [8] and ovulin [9], were transferred, indicating strategic allocation of Sfps. Males selected for large accessory glands (a major site of Sfp synthesis) produced and transferred significantly more sex peptide, but not more ovulin. Males with large accessory glands also had significantly increased competitive reproductive success. Our results show that quantitative variation in specific Sfps is likely to play an important role in postcopulatory sexual selection and that investment in Sfp production is essential for male fitness in a competitive environment.

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Results and Discussion

In insects, seminal fluid proteins (Sfps) produced in the male accessory glands significantly increase male fitness: for example, by promoting sperm storage, temporarily increasing female egg-laying rate and decreasing female sexual receptivity [10], and thus increasing progeny production and delaying sperm displacement and/or competition [11]. However, Sfp production is limited (L.K.S. et al., unpublished data; [12–14]); hence, males should allocate Sfps prudently. We therefore predicted that major factors likely to influence Sfp transfer would be the quantity of Sfps that a male has available and a male's ability to adjust Sfp transfer in response to the potential level of postcopulatory competition.

Selection on Accessory Gland Size Affects Sfp Production and Transfer, as well as Male Competitive Reproductive Success

To experimentally manipulate the Sfp investment potential of males, we artificially selected for large (L) or small (S) accessory glands in replicate pairs of populations. Unselected (U), but otherwise identically cultured, populations were also maintained (giving six lines in total: L1, L2, U1, U2, S1, and S2; see Experimental Procedures). Artificial selection produced a consistent and significant divergence in male accessory gland size. The accessory gland size of L males was significantly larger than that of either S or U males, but we detected no correlated responses in terms of body size or testis size (Figure 1, as well as Figure S1, available online). Unexpectedly, selection for reduced accessory gland size was ineffective, resulting in no decrease in S lines (Figure 1) and no significant difference between S and U lines (Figure S1A). Thus, although it was possible to select for significantly increased accessory gland size, there was a minimum stable size.

To quantify Sfp production and transfer in the selected lines, we used enzyme-linked immunosorbent assays (ELISAs; see Experimental Procedures and Supplemental Data). We measured two Sfps predicted to influence male competitive reproductive success: sex peptide (a.k.a. Acp70Aa) and ovulin (a.k.a. Acp26Aa). Both sex peptide and ovulin increase female egg production, though by different mechanisms [8, 15–17], and sex peptide additionally causes dramatically decreased receptivity [18, 19]. As expected, changes in accessory gland size altered the quantity of sex peptide produced; L male accessory glands contained significantly more sex peptide than either U or S male accessory glands, and no significant difference was observed in sex peptide between S and U male accessory glands (Figure 2A). However, although the trend was in the same direction for ovulin production, there was no statistically significant difference among the lines (Figure 2B). Thus, it cannot be assumed that responses to increased accessory gland size are consistent among different Sfps in terms of the quantity of protein produced. A potential mechanism underlying the differences between sex peptide and ovulin responses to selection is the difference in Sfp sites of synthesis within the accessory glands. The accessory gland is composed of about 1000 main cells and 40 secondary cells per gland [20]. Sex peptide is synthesized only in the main cells

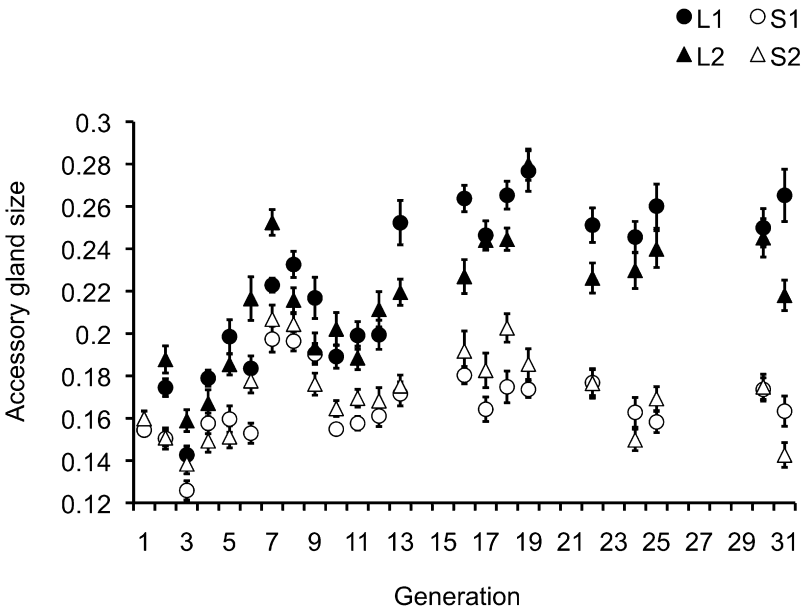

Figure 1. Response to Bidirectional Selection on Accessory Gland Size

There was a significant response to selection for increased accessory gland size (mm^2; mean ± SE), L1 and L2 showing realized heritabilities (measured over generations 1 to 13) of 0.405 ($F_{1,11}$ = 11.08, p = 0.007) and 0.301 ($F_{1,11}$ = 5.29, p = 0.042), respectively. However, accessory gland size failed to respond to selection for decreased size (realized heritabilities of −0.152 and −0.124 for S1 and S2, respectively; $F_{1,11}$ < 3.04, p > 0.1 for both). Number of pairs of accessory glands measured for each line at each generation = 22–26.

[21], whereas ovulin is synthesized in both the main and the secondary cells [13]. Our selection for overall accessory gland size could have disproportionately affected proteins produced only in the main cells, such as sex peptide. It will be interesting in the future to compare how different selection pressures affect Sfp production. For example, one could test whether Sfps evolve differently when under selection generated by different levels of male-male competition and how any changes compare to those occurring under selection for accessory gland size (as tested here).

Because we detected no difference between U and S males in accessory gland size or Sfp production, we focused on comparing the S and L lines for measures of Sfp transfer. We found a striking difference in the amount of sex peptide transferred by L versus S males: L males transferred significantly more sex peptide to females than did S males (Figure 3A). There was, however, no significant difference in ovulin transfer among the different lines (Figure 3B). Moreover, the trend for ovulin was in the opposite direction from that for sex peptide (Figures 3A and 3B). The pattern of Sfp transfer associated with differences in accessory gland size was therefore similar to—though more pronounced than—that of Sfp production (Figure 2) and was consistent across matings in which a competitor male was present as well as those in which no competitor male was present (Figures 3A and 3B; see also Competition Affects Mating Duration and Sfp Transfer below). Thus, despite having larger accessory glands, L males did not transfer a uniformly larger ejaculate: they transferred increased amounts of sex peptide but not of ovulin, resulting in changes to the sex peptide:ovulin ratio received by females. Selection on accessory gland size did not significantly alter mating duration (Figure 3C; however, mating duration was influenced by the presence of rival males: see Competition Affects Mating Duration and Sfp Transfer). Therefore, differences between S and L males in the amount of sex peptide transferred during mating were not explained by divergence in mating duration and were instead probably due to differences in sex peptide production or allocation.

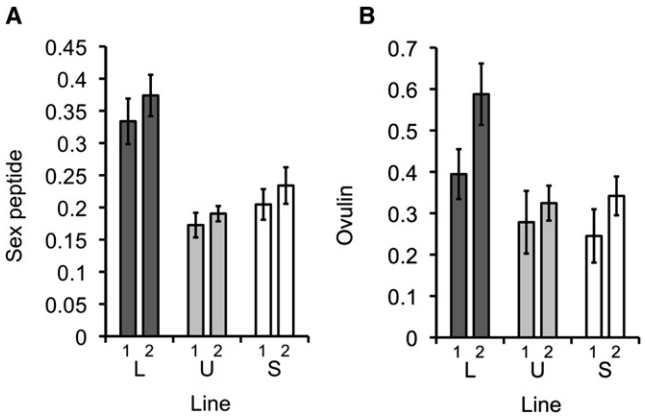

Figure 2. Quantity of Sex Peptide and Ovulin Produced by Accessory Glands of Selection Line Males

(A) Sex peptide production (mean ± SE in relative units) was significantly higher in the L males than in both U and S males; sex peptide production did not significantly differ between U and S males (selection effect, $F_{2,3}$ = 24.10, p = 0.014; L versus S, z = 5.15, p < 0.0001; L versus U, z = 6.61, p < 0.0001; S versus U, z = −1.45, p = 0.31).

(B) Ovulin production (mean ± SE in relative units) did not differ significantly among L, U, and S males (selection effect, $F_{2,3}$ = 3.1, p = 0.187). There were trends for increased ovulin in the L lines, but no comparisons were significant (L versus S, z = 2.19, p = 0.072; L versus U, z = 2.12, p = 0.087; S versus U, z = 0.08, p = 0.99).

Relative units were based on a standard curve consisting of serial dilutions of an extract of male accessory glands. N = 9–10 males per line.

To test whether increased transfer of sex peptide benefitted L males, we measured the competitive reproductive success of selection line males in two ways. First, we tested the L, U, and S males in a two-mating sperm displacement ability assay [22]. Females were mated first to a competitor male, then to a selection line male 48 hr later. Females, as well as the competitor males, were homozygous for the recessive *sparkling*^{poliert} (*spa*^{pol}) eye phenotype. Consequently, the paternity of the offspring produced could be assessed visually (see Experimental Procedures). We found no significant differences in sperm displacement ability among L, U, and S males ($F_{2,3}$ = 1.28, p = 0.40). Thus, under these conditions, there was no evidence that L line males benefitted from increased sex peptide transfer by increasing their ability to displace a prior male's sperm (although the accessory gland and testis size did account for some variance in sperm displacement ability: see Supplemental Data).

Second, we conducted a multiple-mating competition assay over a ten-day period. Selection line males were housed with *spa*^{pol} females and *spa*^{pol} competitor males. We measured the reproductive success (number of progeny sired) of the males and sampled mating frequencies. We found no overall

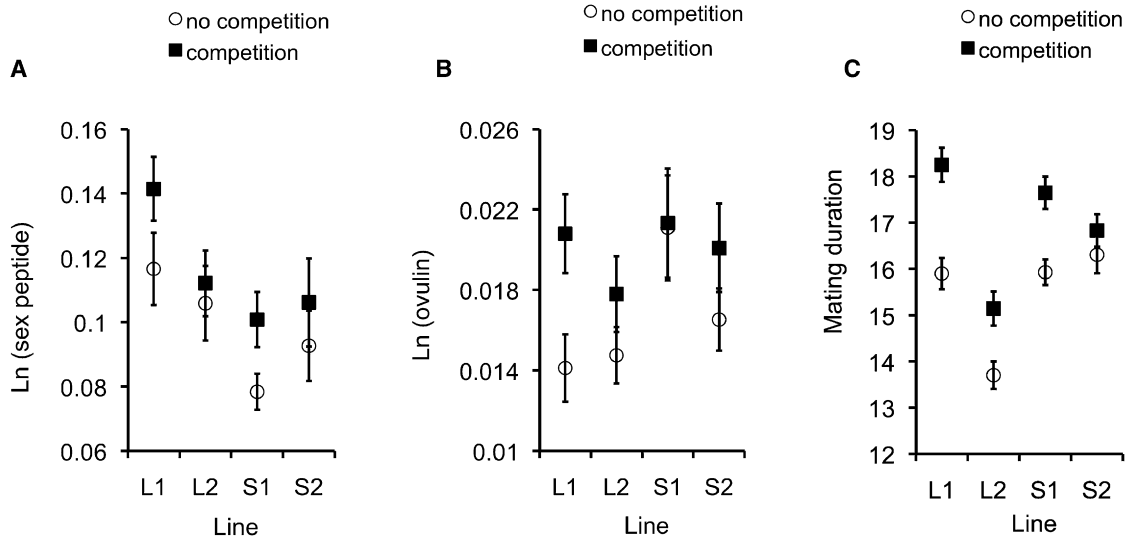

Figure 3. Ovulin and Sex Peptide Transfer and Mating Duration of Selection Line Males in Social Environments with and without Competition

(A) L males transferred significantly more sex peptide than did S males (mean ± SE; $F_{1,17} = 8.67$, p = 0.0091), and sex peptide transfer was significantly increased when a competitor was present ($F_{1,334} = 6.81$, p = 0.0094). N = 29–66 per replicate line per treatment.

(B) There were no differences in ovulin transfer between L and S lines (mean ± SE; $F_{1,17} = 2.24$, p = 0.152), but ovulin transfer was significantly increased when a competitor was present ($F_{1,415} = 5.55$, p = 0.019). N = 51–61 per replicate line per treatment.

(C) Selection on accessory gland size did not affect mating duration ($F_{1,17} = 2.51$, p = 0.130). However, mating duration (mean ± SE; minutes) was significantly longer when a competitor male was present ($F_{1,636} = 42.90$, p < 0.0001). The interaction between selection and the presence or absence of a competitor was not significant ($F_{1,636} = 3.04$, p = 0.082). N = 78–88 per replicate line per treatment.

There were no significant interactions between the presence or absence of a competitor and selection regime on the quantity of sex peptide or ovulin transferred (sex peptide $F_{1,334} = 0.12$, p = 0.73; ovulin $F_{1,415} = 1.30$, p = 0.25). Relative units were based on a standard curve consisting of serial dilutions of an extract of male accessory glands. We transformed these units by adding one and taking the natural log of the sum (see Experimental Procedures).

differences among L, U, and S males in the number of matings obtained by selection line males relative to competitor males ($\chi^2 = 4.2$, df = 5, p = 0.52; data not presented), showing that there were no detectable differences between treatments in premating competitive ability. However, selection regime had a significant effect on the number of progeny sired. L males sired significantly more offspring than did either U or S males, and there was no significant difference between U and S males in progeny production (Figure 4). Thus, the reproductive success of males paralleled the pattern of differences in accessory gland size, sex peptide production, and sex peptide transfer to females (L > U = S). This suggests that males gained significant fitness benefits from the ability to transfer larger quantities of specific Sfps, such as sex peptide. The mechanism for this increased reproductive success is likely to occur via the increased transfer of sex peptide and/or other Sfps that elevate egg production, delay the onset of sperm competition, and/or function in sperm defense in the L males. Although sex peptide has no direct effect on sperm displacement ability, it benefits males primarily through its ability to decrease female receptivity [18, 19]. This results in a higher "per mating" share of reproduction for males in multiple-mating situations, because the intermating interval is increased [11]. It is also possible that other Sfps that play roles in sperm defense [23–25] are produced and transferred in higher quantities by the L males. Candidates include Acp36DE, which is essential for sperm storage [26] and consequently for sperm competitive ability [27], or CG9997, 1652/56, 17575, and Acp29AB, which affect sperm retention in storage [28, 29]. The only antibodies currently available for these Sfps cross-react with other proteins, thus making their ELISAs difficult to interpret (L.K.S. et al., unpublished data; [30, 31]). However, once highly specific antibodies are obtained, it will be important to look at quantitative variation in a range of Sfps to explore their effect on postcopulatory sexual selection.

Competition Affects Mating Duration and Sfp Transfer

To test whether males can plastically allocate Sfps, we measured the Sfp transfer by accessory gland selection line

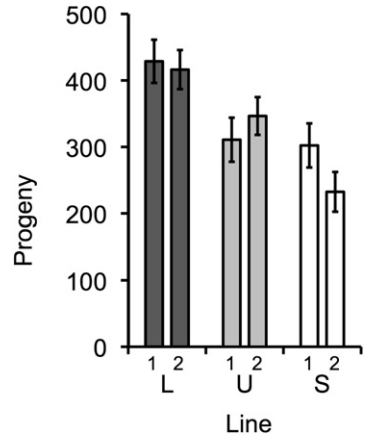

Figure 4. Total Progeny Sired by Selection Line Males when in Competition for Ten Days with spa^{pol} Males

Selection regime significantly affected the number of progeny sired (mean ± SE; $F_{2,3} = 9.81$, p = 0.048), L males siring significantly more offspring than both U and S males (L versus U, $z = 2.59$, p = 0.0226; L versus S, z = 4.42, p < 0.001). There were no significant differences in the number of progeny sired by U and S males ($z = 1.81$, p = 0.16). N = 22–30 for each replicate line.

males that had been exposed to different potential levels of sperm competition. Male social environments were experimentally varied in the 24 hr prior to and including mating. Males experiencing "competition" were housed in pairs, and males experiencing "no competition" were housed alone (see Experimental Procedures). This experimental manipulation could vary both sperm competition "risk" and "intensity" [32, 33]; hence, we refer to the "level" of sperm competition only.

The presence of a rival competitor male had a strong and consistent effect on both Sfp transfer and mating duration. Significantly more sex peptide and ovulin were transferred to females, and matings were significantly longer, when a competitor male was present prior to and during mating (Figure 3). These results support the hypothesis that males tailor the quantity of Sfps transferred in relation to the potential level of competition. Recent theory has addressed how Sfp allocation could be affected by various factors, including sperm precedence (i.e., first or second male), the relative influence of sperm versus Sfps on fertilization success [3], and the exploitation of the Sfps of rival males [4]. Moreover, models of sperm allocation (e.g., [32, 33]) could be applicable to Sfps wherever increased Sfp quantities directly influence the outcome of sperm competition in the same way as sperm numbers. However, *D. melanogaster* males benefit from sex peptide transfer, and potentially ovulin transfer, primarily by increasing their paternity prior to female remating and, hence, before sperm competition occurs [11]. The consequences of this specific effect for ejaculate allocation have not yet been explored directly by formal theory. Nevertheless, our results are consistent with the hypothesis that it is advantageous for a male to increase the transfer of receptivity-inhibiting and short-term fecundity-enhancing Sfps when his mate is likely to encounter subsequent mating attempts by competitor males. Recent findings of Bretman et al. [34] support this idea: female postmating responses (increased egg production and decreased receptivity) were significantly stronger when their mates had been housed with competitors prior to mating. Our results suggest that differences in Sfp transfer are likely to be the underlying mechanism, because these postmating responses are stimulated by Sfps [35] such as sex peptide [18, 19] and ovulin [17]. Bretman et al. [34] also found that the increases in female postmating responses result entirely from prior exposure of males to competitors and not to the presence of competitors at the time of mating. Hence, males who are most successful in premating competition do not induce greater postmating responses, indicating that such males do not transfer increased levels of Sfps. Our results are therefore consistent with the strategic allocation of Sfps by males and not with higher Sfp transfer by the most successful premating competitors.

It will be important to determine whether, in addition to Sfp allocation, male *D. melanogaster* strategically allocates sperm. Sex peptide is known to bind to sperm in the mated female [36], but it is not currently known at what stage this occurs (i.e., pre- or postinsemination) and, hence, whether sperm numbers and sex peptide transfer efficiency are linked. Ovulin, and at least some of the sex peptide, is transferred free from sperm, and short-term sex peptide responses are shown by females mated to males that lack sperm [35, 36]. Thus, Sfp transfer efficiency is unlikely to be obligately linked to sperm number. However, determining the form of any association between Sfp and sperm quantities will be crucial for testing ejaculate composition and allocation theory [3, 4].

Consequences of Variation in Sfp Investment

Although there are clear male reproductive benefits associated with the ability to transfer large quantities of some Sfps, accessory gland size was close to a minimum level in our starting lab population: selection for smaller accessory glands was unsuccessful. Accessory gland size might be subject to truncation selection if a minimal investment is required for avoiding too much depletion of Sfps from small accessory glands over successive matings. Sfp depletion leads to dramatically decreased male fertility and paternity assurance [12, 14]; thus, there is likely to be strong selection on males to avoid depletion. However, accessory glands could be costly to develop, maintain, or fill with Sfps, in which case accessory gland size could trade off against other life history traits. So far, there is no evidence of any such tradeoffs in terms of development time or virgin male survival in our accessory gland selection lines (C. Fricke and T.C., unpublished data). However, tradeoffs could have been minimized in our selection lines by rearing conditions that reduced competition for resources, including low densities of flies and excess food. This may have permitted the evolution of larger accessory glands in the L lines without the costs that would usually inhibit such investment under natural or standard lab-cage conditions [37, 38].

The receipt of Sfps such as sex peptide can be costly to females [39, 40] and can potentially mediate sexually antagonistic coevolution [41]. In certain experimental evolution studies, rapid changes in female resistance to male-induced harm have been observed (e.g., [42, 43]). In these studies, male-male interaction was eliminated or reduced, through enforced monogamy [42] or female-biased sex ratios [43], respectively. Our results suggest that under such conditions, males would plastically (i.e., immediately) reduce the level of Sfp transfer, which would reduce mating costs to females. Selection on female resistance would therefore immediately be relaxed even before evolutionary changes in males occurred. Thus, plastic Sfp allocation could potentially select for rapid intersexual coevolution.

Conclusions

Our results show that, in *D. melanogaster*, Sfp allocation is plastic, can evolve rapidly under selection, and is more complex than has hitherto been considered. It will be important to determine whether Sfp allocation is as taxonomically widespread as sperm allocation [44]. Testing this should be possible (L.K.S. et al., unpublished data), because the functions of specific Sfps are known in species ranging from arthropods to mammals [6]; antibodies to Sfps have been developed in several species (e.g., fruit flies [9, 30, 31], carp [45], bulls [46], and humans [47]); and bioinformatic, proteomic, and RNA-interference tools that aid the discovery and characterization of new Sfps are becoming increasingly available [48–50]. More theory is also needed for predictions of how males should invest in, as well as allocate, the Sfps that play important roles in postcopulatory sexual selection [3, 4] and of, crucially, how females should evolve in response to Sfp allocation. A potential application of our work is the manipulation of males used in biological and genetic insect-pest management. Males released for pest control are often poor in both acquiring mates and inducing the postmating behavioral changes that are stimulated by Sfps [10, 51]. Our results demonstrate the potential for increasing the reproductive competitiveness of mass-reared males by selecting on

accessory gland size or by selecting on the ability of males to induce female postmating responses.

Experimental Procedures

Stocks

The Dahomey wild-type (WT) stock used in these experiments is as previously described [43]. Competitor males were from a Dahomey WT stock into which we had introduced the recessive *spa*^{pol} eye mutation [11]. For all experiments, fly food was supplemented with live yeast granules.

Artificial Selection for Large and Small Accessory Glands

For initiation of the selection scheme, 50 Dahomey males per replicate (two replicates each for L, S, and U lines) were each housed with two virgin females in sugar-yeast-maize medium vials (1% agar, 8.5% sucrose, 2% yeast, 6% maize meal, and 2.5% Nipagin). After 1 day, males were removed and housed individually for 3–7 days so that they could replenish their Sfps. Females were discarded after they laid eggs for several days. For propagation of the L and S lines, male accessory glands were dissected and the perimeters measured [22]. Scoring was blind with respect to line identity, and repeated scoring on the same samples gave 96% repeatability. Virgin progeny were collected from eight families per replicate of males with the largest or smallest accessory glands for L and S lines, respectively, for propagation of the subsequent generation. Eight vials per U line were chosen at random. For subsequent generations, virgin males and females from the eight highest- and lowest-ranked families (for the L and S lines, respectively), as well as the randomly chosen U families, were housed in single-sexed family groups of ~12 and aged for 3–10 days. Virgin females from families ranked 1, 3, 5, and 7 were mated to virgin males from families 2, 4, 6, 8, and vice versa, ensuring that there were no matings between full sibs. Individual males were housed with two females, allowed to mate for one day, then maintained alone for replenishment of accessory glands, as above. Twenty-five males per line were dissected per generation (L and S lines) and scored and selected as above (no selection was imposed in generation 20, 21, 23, 26–29, or 31–37). After 40 generations, lines were kept under relaxed-density, unselected conditions at 18°C in bottle culture.

Responses to Accessory Gland Selection: Morphology and Sfp Production

The direct response of accessory gland size to high and low selection was measured as part of the selection process as described above (25 males per line per generation). To test for correlated responses and changes relative to the U lines, we measured accessory gland size, body size (using wing area as a proxy), and testis size of males that were the offspring of females from generations 16 and 38. Measures of wing or testis size were the averages of the size of the left and right of these organs for a given individual, wherever possible. To measure the quantity of sex peptide and ovulin produced by the males, we dissected the accessory glands and performed ELISAs, as described below.

Mating Duration and Sfp Transfer in Response to Competition and Accessory Gland Selection

To test the quantity of Sfps transferred to females during mating, we raised males from the L and S lines, as well as Dahomey females, at a standardized density of 100 larvae per sugar-yeast medium vial [43]. Virgin Dahomey females and selection line males were collected on ice and stored 5 per vial in single-sex groups. Three days later, males of each line were placed either 1 ("no competition" treatment) or 2 ("competition" treatment) per vial, with the use of ice anesthesia, 24 hr before matings took place. On the day of the matings, one female was introduced into each of the male vials and the duration of mating was recorded. Twenty-five minutes after the start of mating, females were aspirated into microcentrifuge tubes, flash frozen in liquid nitrogen, then stored at −80°C for subsequent ELISAs (see below). Matings that lasted for less than 10 min or more than 25 min were removed from the data set. Experiments were conducted in six blocks between generations 47 and 55 after the establishment of the selection lines (selection was relaxed after generation 40, but the differences in accessory gland size between L and S males were still present: e.g., generation 47, $F_{1,2} = 29.8$, $p = 0.032$; generation 49, $F_{1,2} = 57.6$, $p = 0.017$). Final sample sizes for mating duration analyses were 78–88 per replicate line per treatment. For Sfp transfer analysis, sample sizes were 51–61 and 29–66 per replicate line per treatment for ovulin and sex peptide, respectively.

Effects of Accessory Gland Size Selection on Male Reproductive Success

For both reproductive success assays, we competed selection line males (offspring of generation 16 flies) against rival *spa*^{pol} males. Sugar-yeast fly medium was used throughout. First, we tested sperm displacement ability. Single *spa*^{pol} females were placed with single *spa*^{pol} males, and the males were removed after a single, observed mating (day 1). On day 2, single two-day-old selection line males were introduced to each female and observed until mating occurred. Males were removed after mating. Females were transferred to new food vials on days 3, 4, 6, 8, 10, 13, 15, and 17. The number of progeny produced from eggs laid between day 3 and day 17 was counted and scored for eye phenotype. Sperm displacement ability was calculated as a/(b+1), in which a is the number of progeny sired by the second male to mate and b is the number of progeny sired by the first male [22].

Second, we tested male reproductive success in a multiple-mating competition assay lasting ten days. One-day-old selection line males were housed in groups of ten in vials with sugar-yeast medium. Experimental vials were then set up, each containing two selection line males, two virgin *spa*^{pol} males, and two *spa*^{pol} virgin females (sample sizes were as follows: L1 = 23, L2 = 22, U1 = 22, U2 = 28, S1 = 30, and S2 = 27). These groups of flies were maintained for ten days, and transferred onto fresh sugar-yeast food on days 1, 4, and 8. Vials were observed for matings, at 20 min intervals over a 3 hr period on days 2, 4, 7, and 9, and the eye phenotype (WT or *spa*^{pol}) of the mating male was recorded. Offspring produced during the ten-day period were counted, and paternity was assessed by recording of eye color phenotype. The number of matings obtained and progeny sired in competition provide measures of pre- and postmating competitive ability.

ELISAs for Sex Peptide and Ovulin

ELISA methods are described in more detail in the Supplemental Data. In brief, flies were dissected and ground in Dulbecco's Phosphate Buffering Solution with protease inhibitors. The protein samples from male accessory glands or female lower reproductive tracts were then aliquoted into wells on four replicate ELISA plates, two for ovulin and two for sex peptide. The plates were incubated overnight at 4°C, with shaking. On the following day, the plates were incubated with block, then with primary antibody against ovulin or against sex peptide, then with horseradish-peroxidase-conjugated secondary antibody, each for one hour at room temperature, with shaking. The level of ovulin or sex peptide was detected through a reaction of the horseradish-peroxidase with 3,3′,5,5′-tetramethylbenzidine substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). The reaction was stopped by the addition of 100 μl 1 M H_3PO_4 after the wells developed a deep blue color or after 30 min. Optical density at 450 nm (OD_{450}) was determined with a Molecular Devices kinetic microplate reader. The OD_{450} value of a blank well was subtracted from the OD_{450} values of all of the other samples on its plate. Resulting OD_{450} values for the samples on one plate were regressed against OD_{450} values of the same samples from the replicate plate. Points with residuals greater than three standard deviations were considered to have low repeatability and were removed. The OD_{450} values of the two replicate plates were averaged and converted to male accessory gland equivalents through a standard curve generated from male accessory glands of Canton S males.

Statistical Analysis

Data analyses were performed with Excel, JMP (ver. 5, SAS Institute), and R (ver. 2.8.0) software in Mac OS X. The realized heritability for each accessory gland selection line was calculated as the regression of cumulative response on cumulative selection differential measured over the first 13 generations. Analyses of accessory gland sizes, Sfp production, Sfp transfer, mating duration, sperm displacement ability, and reproductive success (i.e., the number of progeny sired) were performed with the use of linear mixed effects models (nlme package in R). For accessory gland sizes, Sfp production, sperm displacement ability, and reproductive success, the fixed effect was selection regime (L, U, or S) and the random effect was replicate within regime. For Sfp transfer and mating duration, competition was an additional fixed effect, and line within regime was nested within block for random effects. For the additional sperm displacement ability analysis (see Supplemental Data), the fixed effects were accessory gland size, testes size, wing area, and selection regime. Extreme outliers were detected with Grubb's tests, and data points with p values < 0.0001 were excluded from further analysis (two excluded for sex peptide data, four for ovulin, and three for mating duration). For multiple comparisons in mixed effects models, we used Tukey tests in the multcomp package in R. Mating

frequency data were analyzed with Chi-square tests. Progeny counts from the ten-day multiple-mating competition assay were transformed to the power of 1.2 for improvement of normality. Sperm displacement ability data were normalized by a cube-root transformation. Accessory gland, testis, and body size data were Box-Cox transformed as necessary. Sfp transfer data were log transformed for improvement of normality (1 was added to all data points, making them positive). Analyses on untransformed data produced qualitatively identical results.

Supplemental Data

Supplemental Data include Supplemental Results and Experimental Procedures and one figure and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)00887-2.

Acknowledgments

Funding was provided by the Natural Environment Research Council (studentship to J.L.), the Biotechnology and Biological Sciences Research Council (research grant to T.C. and S.W.), the Royal Society (University Research Fellowship to T.C.), the National Institutes of Health (Ruth L. Kirschstein National Research Service Award fellowship 1F32GM074361 to L.K.S., research grant 1R01HD38921 to M.F.W.), the Human Frontiers Science Program (Short-Term Fellowship to S.W. while in M.F.W.'s lab at Cornell University), and the Lloyd's Tercentenary Foundation (Fellowship to S.W.). We thank Andy Barnes, Mara Lawniczak, Yasmine Driege, James Boone, Claudia Fricke, and Dave Gerrard for assistance with experiments; Bregje Wertheim for invaluable guidance on statistical analysis; Eric Kubli for sex peptide antiserum; Judy Appleton and Lisa Daley for advice on ELISA methods; Andrew Clark for use of his microplate reader; Tom Pizzari for advice on the manuscript; and two anonymous reviewers for helpful comments.

Received: January 15, 2009
Revised: March 2, 2009
Accepted: March 3, 2009
Published online: April 9, 2009

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