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EJACULATE DEPLETION PATTERNS EVOLVE IN RESPONSE TO EXPERIMENTAL MANIPULATION OF SEX RATIO IN *DROSOPHILA MELANOGASTER*

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We assessed the extent to which traits related to ejaculate investment have evolved in lines of *Drosophila melanogaster* that had an evolutionary history of maintenance at biased sex ratios. Measures of ejaculate investment were made in males that had been maintained at male-biased (MB) and female-biased (FB) adult sex ratios, in which levels of sperm competition were high and low, respectively. Theory predicts that when the risk of sperm competition is high and mating opportunities are rare (as they are for males in the MB populations), males should increase investment in their few matings. We therefore predicted that males from the MB lines would (1) exhibit increased investment in their first mating opportunities and (2) deplete their ejaculates at a faster rate when mating multiply, in comparison to FB males. To investigate these predictions we measured the single mating productivity of males from three replicates each of MB and FB lines mated to five wild-type virgin females in succession. In contrast to the first prediction, there was no evidence for differences in productivity between MB and FB line males in their first matings. The second prediction was upheld: mates of MB and FB males suffered increasingly reduced productivity with successive matings, but the decline was significantly more pronounced for MB than for FB males. There was a significant reduction in the size of the accessory glands and testes of males from the MB and FB regimes after five successive matings. However, the accessory glands, but not testes, of MB males became depleted at a significantly faster rate than those of FB males. The results show that male reproductive traits evolved in response to the level of sperm competition and suggest that the ability to maintain fertility over successive matings is associated with the rate of ejaculate, and particularly accessory gland, depletion.

KEY WORDS: **Accessory glands, *Drosophila melanogaster*, ejaculate allocation, sexual selection, testis size.**

In the widespread, perhaps ubiquitous, situations in which females mate with more than one partner, males must compete for access to females and for access to fertilization opportunities (Parker 1970, 1998). Males may increase their success in sperm competition by making a greater investment in sperm production, by increasing gamete size or number (Parker 1990a), by investing in different sperm types (Cook and Gage 1995), by delaying remating by females (Gillott 2003), or by donating nuptial gifts (Engqvist and Sauer 2001). Although individual sperm are expected to be energetically cheap to produce (Parker 1970), spermatophores and

whole ejaculates may be energetically expensive (Dewsbury 1982; Wedell et al. 2002). In these situations males are expected to evolve strategies to allocate their ejaculates according to the level of sperm competition that they experience and the frequency of their access to mates (Parker 1970, 1990a,b).

The response to selection arising from sperm competition has been assessed by testing for increased ejaculate investment with elevated sperm competition risk (the probability that females in the population have mated or will mate again) and/or with elevated sperm competition intensity (the average number of males competing for a given set of eggs; Parker 1982). The data are consistent with these ideas, with the finding that males in polyandrous mating systems have relatively larger testes (primates, Harcourt et al. 1981; birds, Møller and Ninni 1998; amphibians, Jennions and Passmore 1993; butterflies, Gage 1994; Karlsson 1995; bats, Hosken 1997; fish, Stockley et al. 1997) and are hence capable of producing more sperm (Gage 1994; Møller 1988).

Intraspecific studies have documented the evolution of plastic responses to the perceived threat of sperm competition (Wedell and Cook 1999), have investigated the ejaculate investment of different male morphs (Gage et al. 1995), or have measured evolved responses to artificial selection (Hosken and Ward 2001). When sperm competition is analogous to a fair raffle, high sperm number is advantageous in assuring paternity (Parker 1990a) and theory predicts that where sperm competition risk is high, males should increase sperm number (Parker 1990b). Intraspecific studies provide direct evidence that males show plastic responses and allocate their ejaculates according to the degree of sperm competition risk (e.g., in snails, Oppliger et al. 1998; insects, Gage and Baker 1991; Simmons et al. 1993; Cook and Wedell 1996; Wedell and Cook 1999; Martin and Hosken 2002; and birds, Pizzari et al. 2003) or according to sperm competition intensity (e.g., in bushcrickets, Simmons and Kvarnemo 1997; and fish, Pilastro et al. 2002). Support for the prediction that sperm competition selects for increased investment in sperm also comes from intraspecific studies of male polymorphisms, in which morphs likely to experience high levels of sperm competition invest more in testis mass and ejaculates (Gage et al. 1995; Simmons et al. 1999). In certain contexts however, males are predicted to decrease their sperm expenditure, for example, when the number of competing male ejaculates exceeds two and hence the probability of paternity is low (Parker et al. 1996; Ball and Parker 1997), as occurs in gobiid fishes (Pilastro et al. 2002). Intraspecific ejaculate allocation has also been investigated using artificial selection. For example, Hosken and Ward (2001) placed replicate populations of the yellow dung fly, *Scathophaga stercoraria,* under monogamous or polyandrous conditions and documented a rapid evolution of testis size consistent with the expectations of sperm competition theory.

An important assumption underlying predictions about strategic ejaculate allocation is that ejaculates are limiting (Dewsbury 1982; Wedell et al. 2002). Hihara (1981) observed that over five successive matings, the accessory glands of male *Drosophila melanogaster* became depleted, which caused a decrease in the number of eggs laid by the fourth- and fifth-mated females, and in the fertility of fifth-mated females. The latter drop in fertility was associated with accessory gland depletion and not with sperm depletion, suggesting that accessory gland products were the main limiting factor (Hihara 1981). Mating frequency will also influence the expected number and size of ejaculates a male has to deliver. Together with the plastic responses to sperm competition described above, this suggests that the ejaculate allocation patterns of males will be shaped by the mating system and degree of sperm competition, yet to date there have been few explicit experimental tests of this idea. It is also important to note that, as yet, theory has rarely made separate predictions for sperm versus other ejaculate components. It would be important to separate them if, for example, these two components of the ejaculate trade-off against one another.

Here we use experimental evolution to examine the ejaculate allocation strategies of *D. melanogaster* males evolving under differing levels of sperm competition. We manipulated mating opportunities and the risk and intensity of sperm competition by varying the adult sex ratio. Three replicate lines each of male-biased (MB) and female-biased (FB) regimes were created (as described in Wigby and Chapman 2004). During the nine-day adult sex ratio biased period of each generation, mating opportunities for males in the MB lines were significantly fewer than for males in FB lines (a sample of 3 h of observations per day over six observation days gave an average of 0.22 matings per male for MB males and 0.69 matings per male for FB males, Wigby and Chapman 2004). Extrapolating these figures to give the total number of matings during the nine-day selection period gives an average for MB and FB males respectively of (1) 1.32 and 4.14 matings if we assume no mating during the dark, (2) 1.98 and 6.21 matings if there is 50% mating during the dark (Willmund and Ewing 1982), and (3) 2.64 and 8.28 matings if mating occurs irrespective of the dark (Fujii et al. 2007). Note that matings were sampled during peak mating periods and so in each case the extrapolated figures are likely to be significant overestimates.

Regardless of which estimate is used, MB males mate infrequently but face a high risk and intensity of sperm competition when they do mate (each MB female mates on average three times as often as each male and is therefore multiply inseminated). MB males should therefore be selected to increase their reproductive success by transferring large ejaculates, particularly in their first matings (Parker 1998). Our first prediction was therefore that MB males would have higher productivity in their first matings than FB males. The mating rate estimates also reveal that selection for MB males to maintain fertility over rapid, successive matings might also be weak, as opportunities for MB males to mate multiply

are relatively rare during the selection procedure. Our second prediction was therefore that MB males would show weaker partitioning of ejaculates (and hence more ejaculate exhaustion) with successive partners than would FB males. To test these predictions, males were given the opportunity to mate with five wild-type females in succession in a series of single pair matings. We then measured the productivity (number of offspring) from each of these matings. To test whether any exhaustion was due to ejaculate depletion, we compared the effect of successive matings on the reduction in size of the testes and the accessory glands (i.e., the structures that produce the ejaculate).

Materials and Methods

STOCKS AND CULTURES

Wild-type stock

The Dahomey wild-type stock used in these experiments was collected in 1970 and has been maintained since in four population cages with overlapping generations. Each Dahomey stock cage was supplied with three bottles (189 mL each) containing 70 mL of sugar-yeast (SY) food (100 g autolysed yeast powder, 100 g sugar, 20 g agar, 30 mL nipagin (10% solution), 3 mL propionic acid, in 1 L water) every week. Bottles were removed after 28 days. Population cages, selection lines and experiments were maintained at 25°C in nonhumidified rooms on a 12:12 h light:dark cycle. Females for the experiment were obtained by collecting eggs from population cages. Petri dishes filled with a grape juice medium (50 g agar, 600 mL red grape juice, 42.5 mL nipagin (10% solution), 1.1 L water) and smeared with live yeast paste were placed inside each population cage to serve as oviposition substrates. Eggs laid on these dishes were then placed at standard density in SY bottles (Clancy and Kennington 2001).

Males with an evolutionary history of maintenance at biased sex ratios (i.e., high and low levels of sperm competition)

Males for the experiments were obtained from the selection lines described in Wigby and Chapman (2004). Briefly, in these lines the intensity of sexual selection and sexual conflict was varied by altering the adult sex ratio. Each generation, three replicate lines each of MB (75 males and 25 females) and FB (25 males and 75 females) sex ratio treatments were propagated. The sex ratio in the MB lines was changed to 70 males and 30 females from generation 53 to ease propagation. Flies were provided access to water and were fed ad libitum with two vials of SY food with added live yeast every two or three days. Nine days after the cages were set up, eggs were collected. The majority of eggs were allowed to hatch before larvae were collected, thus minimizing selection on early egg hatchability. Larvae were raised at standard density to minimize environmentally determined differences in adult body size. All adults were allowed to eclose over two days before being allocated to the same sex ratio treatment and replicate number as their parents for the next generation.

EXPERIMENTAL PROCEDURES

PROGENY SIRED BY MALES FROM THE MB AND FB LINES MATED TO FIVE WILD-TYPE VIRGIN FEMALES IN SERIES

To test the ability of males to allocate ejaculates over successive matings, we mated males from the MB and FB lines to five wild-type virgin females in succession and recorded the number of progeny produced from each of those matings. The experiments were performed in three blocks using males from different generations of selection; generation 60 (Block 1), 65 (Block 2), and 67 (Block 3). Progeny data were not recorded for replicate 3 of each regime in Block 2. SY vials seeded with live yeast were used throughout. Males used in experiments were housed in groups of 10 in vials and aged for 10 days after eclosion (the interval at which eggs were normally sampled during selection). Dahomey wild-type virgin females were anaesthetized on ice and aged for five days in groups of 10–12 and then housed individually for the 24 h prior to experiments.

Individual males were randomly allocated to a wild-type female and allowed to mate once. Males that mated successfully were then transferred to another vial containing a virgin female. This was repeated up to five times. Males failing to mate within 1 h were transferred to a new vial containing another virgin female. A second consecutive failed mating was considered a "fail" and such males were not given further opportunities to mate. The total number of progeny produced by each of the five females mated to each male was recorded for 15 days following each mating. Females were transferred to fresh food vials on days 2, 3, 4, 6, 8, 10 (Block 1) and days 2, 4, 7, 10 (Blocks 2 and 3).

TIME TO MATING AND MATING DURATION OF MALES FROM THE MB AND FB LINES MATED TO FIVE WILD-TYPE VIRGIN FEMALES IN SERIES

For each of the matings described above, we recorded the time from the introduction of the male into each vial until the time when mating began (to the nearest minute). We also recorded the duration of each mating (in minutes).

ACCESSORY GLAND AND TESTIS SIZE OF MALES FROM THE MB AND FB LINES MATED TO FIVE WILD-TYPE VIRGIN FEMALES IN SERIES

We examined the degree to which five successive matings reduced the size of the accessory glands and testes of the MB and FB males. Males were collected from generation 86 and after completing five successful matings (using the protocol described above), were immediately frozen at –80°C. For comparison, 20 control males per line were not mated, but otherwise treated the same.

Males were dissected as in Bangham et al. (2002) in phosphate-buffered saline, and images of the accessory glands and testes were captured from a compound microscope. All dissections were coded blind. Images were measured and analyzed using the NIH Object Image program (ver. 1.62n3 by Norbert Vischer, available at http://simon.bio.uva.nl/object-image.html). Body size was measured as wing area (Gilchrist and Partridge 1999). The wings of males from all selection treatments were mounted on glass slides and measured as above. Analysis was performed on the average of both wings, accessory glands, or testes of each male, or single measures if only one was available (for wings, 6/111 were single measures; for accessory glands, 0/198, and for testes, 18/194).

Statistical analysis was performed using JMP statistical software (ver. 5, SAS Institute Inc.) and the nlme package of R software (Pinheiro et al. 2006).

All data were analyzed with a linear mixed effects ANOVA model (by REML), specifying selection regime (FB and MB) and the five wild-type virgin females in series as fixed effects. Females were treated as a factorial variable to explore when differences between the regimes occurred. To account for the repeated measurements on individual males and the blocking structure of the experiment, the individual males nested in replicate and Block were included as random effects. Data for zero progeny values were removed from the analysis. Different variance strata were applied to replicates within regime for the progeny data to account for nonconstant variance (such weighting improved the models although it did not change the result). Both time to mating and mating duration were log-transformed in the analyses to address the skew.

Results

PROGENY SIRED BY MALES FROM THE MB AND FB LINES MATED TO FIVE WILD-TYPE VIRGIN FEMALES IN SERIES

The total number of progeny sired by males mated to 5 virgin females in series declined significantly for both MB and FB regimes over the series (factor [female], $F_{4,669} = 36.1, P < 0.0001$; Fig. 1). There was also a significant interaction effect between female in the series and regime (factor [female] by regime, $F_{4,669} = 3.7$, $P = 0.006$), showing that the decline in progeny produced by MB males over the series of females was significantly more rapid than the decline for FB males (Fig. 1). There were no significant differences between the regimes in the number of offspring sired by males with females 1, 2, or 3, but FB males sired significantly more offspring than MB males with females 4 and 5 in the series (Table 1). However, there was no difference in the total number of progeny sired across all females for either regime (linear mixed effects model [by REML] with different standard deviations per stratum, individual males nested in replicate and Block as random effects, $F_{1,12} = 1.16, P = 0.30$). The number of males that failed to mate with five females in succession did not differ between regimes (all blocks combined, $\chi^2_6 = 0.024, P > 0.99$).

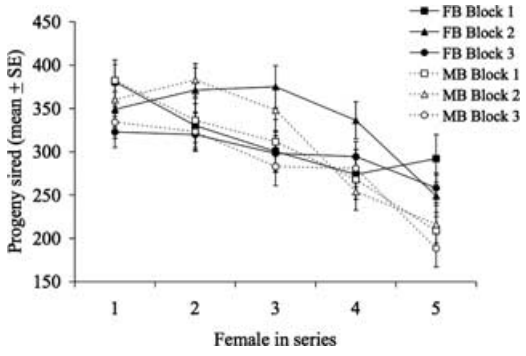

Figure 1. Mean (± SE) total number of progeny produced by males from the MB and FB lines mated in series to five virgin wild-type females. Replicates have been combined for both regimes. Progeny were collected for individual females for 15 days. The counts are standardized for female laying time.

TIME TO MATING AND MATING DURATION OF MALES FROM THE MB AND FB LINES MATED TO FIVE WILD-TYPE VIRGIN FEMALES IN SERIES

The time taken to initiate each mating did not significantly differ between females 1–5 in the series and did not differ between regimes (ANOVA, factor [female], $F_{4,786} = 0.76$, $P = 0.55$, regime, $F_{1,12} = 0.02$, $P = 0.8917$, factor [female] by regime, $F_{4,786} = 1.37$, P = 0.24, online Supplementary Material Fig. S1). There was a significant decline in mating duration with females 1–5 for both FB and MB males, but no significant difference between the regimes (online Supplementary Material Fig. S2, Table S1).

ACCESSORY GLAND AND TESTES SIZES OF MALES FROM THE MB AND FB LINES MATED TO FIVE WILD-TYPE VIRGIN FEMALES IN SERIES

There were no significant differences between the nonmating males from MB and FB lines in body size or in relative and absolute accessory gland and testis size (online Supplementary Material Table S2). Males from both regimes that mated five times had significantly smaller accessory glands and testes (absolute size) than males than nonmating males (Fig. 2; Table 2). However, there was a significant interaction between mating status and regime for accessory gland size, and although there was a slight tendency for the same effect in testis size, it was not significant (Table 2). This analysis shows that the reduction in accessory gland size after five matings was significantly greater for MB males than for FB males.

Table 1. Effect sizes of linear mixed effects ANOVA model (by REML) on the total number of progeny sired by FB and MB males mated to five wild-type virgin females in series. The total number of progeny produced was recorded for 15 days following each mating. Female and regime were set as fixed effects, and individual males nested in replicate and block as random effects. Zero progeny values were removed from the analysis. Different variance strata were applied to account for differences in variation between replicates within regime (such weighting improved the model although it did not change the result).

Factor	Value	SE	df	*t*	*P*
Intercept	355.65	14.60	669	24.35	<0.0001
Female 2	−8.02	13.89	669	−0.58	0.5637
Female 3	−37.90	14.36	669	−2.64	0.0085
Female 4	−45.84	14.68	669	−3.12	0.0019
Female 5	−84.87	15.70	669	−5.41	<0.0001
Regime	8.62	18.93	12	0.46	0.6571
Female 2: Regime FB vs. MB	−4.47	20.25	669	−0.22	0.8255
Female 3: Regime FB vs. MB	−5.02	20.71	669	−0.24	0.8085
Female 4: Regime FB vs. MB	−44.45	21.34	669	−2.08	0.0376
Female 5: Regime FB vs. MB	−70.34	22.32	669	−3.15	0.0017

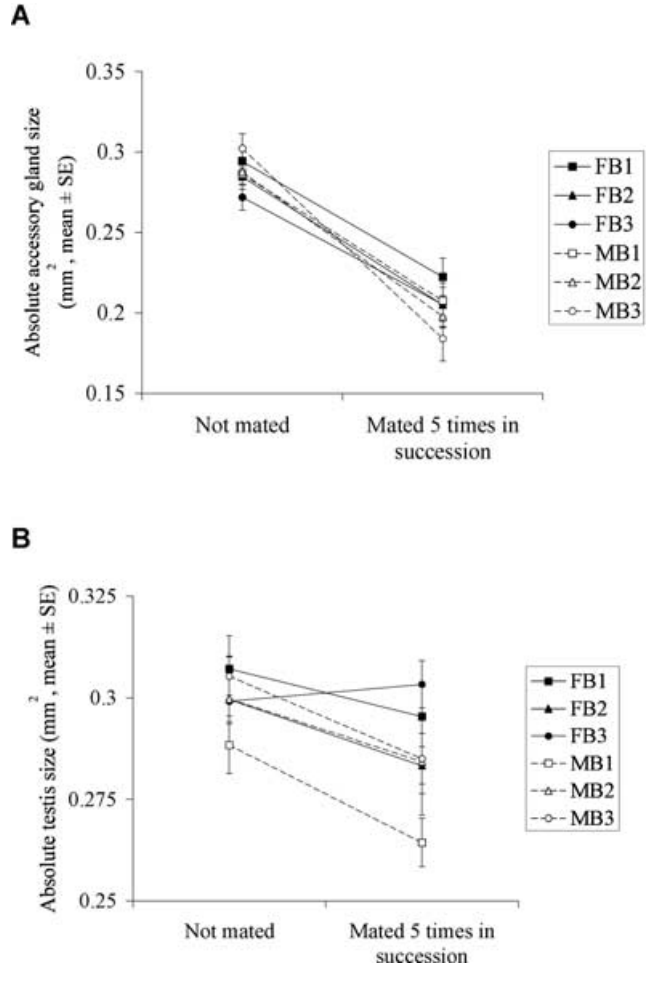

Figure 2. (A) Mean (±SE) accessory gland size (mm^2) and (B) testes size (mm^2) for males from FB and MB lines that were either not mated or mated to five females in series. Replicates have been combined for each block.

Discussion

We found that males that had different evolutionary histories of sperm competition risk/ intensity and mating opportunities differed significantly in their ejaculate allocation patterns. There was no evidence to support the first prediction that males from the MB and FB regimes differed in their first mating investment. This could mean that the prediction is wrong, or that in these experiments males from both regimes transferred sufficient sperm and seminal fluid to maximally inseminate their first mates, masking any such effect. However, the second prediction that MB and FB males would differ in their rate of ejaculate depletion was supported: although males from both selection regimes suffered reduced fertility when mating with five virgin females in succession, the mates of MB males that had evolved under a high level of sperm competition declined in progeny production at a significantly faster rate than did mates of FB males. This resulted in significantly more offspring being sired in matings 4 and 5 by FB as compared to MB males. There were significant reductions in accessory gland and testis size after five matings for males of both regimes. However, the degree of accessory gland reduction was significantly greater for MB than FB males. Our results suggest that MB males decline in fertility faster than FB males because they exhibit faster rates of ejaculate, and particularly accessory gland, depletion.

The failure of our first prediction to be upheld, suggests alternative explanations. Theory predicts that when multiple male ejaculates compete, there may be selection for decreased ejaculate investment (Parker et al. 1996; Ball and Parker 1997). MB line females are estimated to mate on average at least four times and FB females at least 1.4 times during the selection period (data from Wigby and Chapman 2004). Hence, although individual MB

Table 2. Effect sizes of mixed effects ANOVA of (A) absolute accessory gland size and (B) absolute testis size (log transformed) of males from replicate MB and FB lines that were not mated or that were mated to five females in series, with status (not mated vs. mated five times in succession without recovery), selection regime, and status × regime (fixed effects) and replicate line nested within selection regime (random effect).

Source of variation	Value	SE	df	*t*	*P*
(A) Accessory Gland Size					
Intercept	0.25	0.003	190	78.34	<0.0001
Status	0.04	0.003	190	14.82	<0.0001
Regime	0.001	0.003	4	0.43	0.6685
Status × Regime	−0.006	0.003	190	−2.01	0.0457
(B) Testis Size					
Intercept	−1.23	0.01	183	−105.5	<0.0001
Status	0.02	0.008	183	2.95	0.0036
Regime	0.02	0.01	4	1.51	0.1319
Status × Regime	−0.01	0.01	183	−1.65	0.0997

males mate infrequently (estimated average from 1.32 to 2.64, see above), when they do, their ejaculates are in competition. Therefore MB males could have decreased their ejaculate expenditure, which is consistent with their lack of differences in first mating productivity and their lower productivity in subsequent matings. However, we would expect this strategy to increase fitness only with access to multiple mating opportunities (Parker et al. 1996; Ball and Parker 1997), which our estimates show may not generally be the case for MB males. An alternative possibility is that the low productivity of MB males across successive matings reflects a cost of sperm competition. For example, MB males may transfer ejaculate at a greater rate and become ejaculate depleted sooner than FB males, but might show increased fitness in a competitive environment, a possibility that would be interesting to test.

There was no evidence that differences in sperm competition levels had selected for differences in accessory gland or testis size, in contrast to findings in the yellow dung fly, *Scathophaga stercoraria* (Hosken and Ward 2001). This could indicate that the two types of experimental protocols produced differences in the intensity versus risk of sperm competition. MB and FB males differed in the degree to which their accessory gland size decreased following five matings, suggesting that the failure of MB males to maintain mate productivity was associated with accessory gland protein depletion. This interpretation is consistent with previous observations of the loss of fertility in serially mated males (Hihara 1981). However, testis size was also reduced as a consequence of consecutive matings, and the interaction with selection regime was only just nonsignificant (Table 2). Hence a lesser contribution of sperm depletion to the lower productivity of MB males in matings 4–5 is likely. Reductions in testis size with consecutive matings have been reported in *Scathophaga stercoraria* (Ward and Simmons 1991) but not *Cyrtodiopsis dalmanni* (Rogers et al. 2005).

Virgin females were used throughout these experiments. Under certain conditions, males would be expected to allocate more ejaculate to virgin than to mated females (Ball and Parker 2007), but it is unclear whether, if this were the case here it would alter the pattern of our results. This could be tested in the future by asking whether there is any significant interaction in ejaculate allocation between female mating status and male line.

Mating duration showed a tendency to decline over successive matings but it did not explain variation in progeny number and did not differ significantly between regimes. The observed decline in mating duration could be adaptive for females if they were able to reduce mating costs by disengaging earlier with males that are unable to provide sufficient ejaculate. Ejaculate transfer could also occur earlier or be shorter in successive matings because less ejaculate remains to be transferred. There were also no significant differences in time to mating. MB males did not increase the interval between matings to compensate for reduced ability to fertilize females and males from both regimes did not differ, or show an increase, in mating failure rate (i.e., matings that produced no progeny) with successive matings. These results suggest that males did not lose their willingness or ability to mate multiply.

Previous work on various *Drosophila* species has documented variation in fertility across multiple matings. For example, *D. melanogaster* males have been observed to mate 3–5 times before showing reduced fertility (Lefevre and Jonsson 1962; Hihara 1981). Observations consistent with ejaculate partitioning have also been observed. For example, males of *D. hydei* and *D. buzzati* sire few offspring per copulation but maintain high fertility in up to six matings (Markow 1985). Interspecific variation in ejaculate allocation is also reported by Pitnick and Markow (1994), who suggested that selection may favor a bet-hedging strategy whereby males that partition ejaculates can realize greater reproductive success.

Conclusion

In summary, we have found evidence for differences in ejaculate delivery patterns in lines selected under biased adult sex ratios. In serial matings, males with an evolutionary history of exposure to high levels of sperm competition exhibited faster declines in fertility than did males exposed to lower levels of sperm competition. Our evidence suggests that this effect was due to differences in rates of ejaculate, particularly accessory gland, depletion.

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Supplementary Material

The following supplementary material is available for this article:

Figure S1. Median (±interquartile range) mating duration of each mating for females 1–5 mating with MB and FB males. Replicates have been combined for regimes within each block.

Figure S2. Median (±interquartile range) time to mating of each mating for females 1–5 mated to FB and MB males. Replicates have been combined for regimes within each block.

Table S1. Effect sizes of linear mixed effects ANOVA model (by REML) on the mating duration (log transformed) of FB and MB males mated to 5 wild-type virgin females in series. Female and regime were set as fixed effects, and individual males was nested in replicate and Block as random effects.

Table S2. Mixed effects ANOVA of (a) body (wing), (b) relative testis and (c) relative accessory gland size of unmated males from replicate MB and FB lines, with selection regime (fixed effect) and replicate line nested within selection regime (random effect).

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