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Sperm depletion in relation to developmental nutrition and genotype in *Drosophila melanogaster*

Erin L. Macartney,[1,2,3,*] Valérian Zeender,[2,4,*] Abhishek Meena,[2,5] Alessio N. De Nardo,[2] Russell Bonduriansky,[1] and Stefan Lüpold[2]

[1] *Evolution and Ecology Research Centre, School of Biological, Earth and Environmental Sciences, University of New South Wales, Sydney, NSW 2052, Australia*

[2] *Department of Evolutionary Biology and Environmental Studies, University of Zurich, Zurich CH-8057, Switzerland*

[3] *E-mail: e.macartney@unsw.edu.au*

[4] *E-mail: valerian.zeender@uzh.ch*

[5] *Department of Biological Sciences, Indian Institute of Science Education and Research Mohali, Mohali 140306, India*

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Nutrient limitation during development can restrict the ability of adults to invest in costly fitness traits, and genotypes can vary in their sensitivity to developmental nutrition. However, little is known about how genotype and nutrition affect male ability to maintain ejaculate allocation and achieve fertilization across successive matings. Using 17 isogenic lines of *Drosophila melanogaster*, we investigated how variation in developmental nutrition affects males' abilities to mate, transfer sperm, and sire offspring when presented with successive virgin females. We found that, with each successive mating, males required longer to initiate copulation, transferred fewer sperm, and sired fewer offspring. Males reared on a low-nutrient diet transferred fewer sperm than those reared on nutritionally superior diets, but the rate at which males depleted their sperm, as well as their reproductive performance, was largely independent of diet. Genotype and the genotype × diet interaction explained little of the variation in these male reproductive traits. Our results show that sperm depletion can occur rapidly and impose substantial fitness costs for *D. melanogaster* males across multiple genotypes and developmental environments.

KEY WORDS: Fitness, GEI, genotype, mating propensity, nutrition, sperm number.

The energetic and nutritional costs of synthesizing sperm and other components of ejaculates can be considerable, and males of many species can therefore suffer ejaculate depletion across successive matings (Dewsbury 1982; Preston et al. 2001; Torres-Vila and Jennions 2005; Linklater et al. 2007; Muller et al. 2016). Ejaculate depletion could have important consequences for male fitness, limiting their ability to capitalize on additional mating opportunities or reducing the fitness gains from multiple mating. The rate of ejaculate depletion could also shape male reproductive strategies. For example, males could be selected to adjust their mating behavior to mitigate ejaculate depletion, sacrificing mating opportunities to maintain full ejaculate transfer across successive matings (Macartney et al. 2020). The rate of ejaculate depletion could depend on nutrient availability as well as genetic effects on resource allocation per mating (Hopkins et al. 2019). Yet, we still know little about how male nutrition and genotype interact to affect sperm transfer and fertilization success across successive matings.

Sexual traits that have been selected for increased expression are often costly to produce and maintain (Andersson 1994), and environmental factors such as nutrient availability or stress can alter males' ability to invest in such traits. Males that experience a low-nutrient diet have reduced metabolic resources (i.e.,

[*] These authors share first authorship.

low phenotypic condition), and are less able to invest in costly fitness traits (Nur and Hasson 1984; Grafen 1990; Iwasa et al. 1991; Andersson 1994). Yet, sexual traits are also thought to have high levels of additive genetic variation compared to nonsexual ("metric") traits (Houle 1992; Pomiankowski and Møller 1995; Rowe and Houle 1996; Ward 2000; Simmons and Kotiaho 2002), and genotype can interact with environmental effects to influence male condition (Hunt et al. 2004b; Hill 2011). For example, certain genotypes may be better able to cope with environmental stressors such as nutrient limitation, and genotypes can also vary in resource allocation to different traits. Thus, genotype could interact with diet to influence male investment in costly sexual traits (Gienapp and Merilä 2010; Ingleby et al. 2010). However, the roles and relative importance of environment, genes, and their interaction in shaping male reproductive traits remain poorly understood (Bonduriansky et al. 2015).

Environment and genotype could affect both precopulatory sexual traits that function in sexual display and male-male combat, and postcopulatory sexual traits that function in sperm competition and fertilization (Ward 2000; Simmons and Kotiaho 2002; Evans et al. 2015; Kahrl and Cox 2015; Macartney et al. 2018). Sperm number is highly important in the outcome of sperm competition (Parker 1970, 1990; Parker and Pizzari 2010), and sperm production also exhibits strong condition dependence in response to variation in nutrient availability (Macartney et al. 2019). However, very few studies have quantified both dietary and genetic effects on sperm production (but see Evans et al. 2015), and even fewer studies have investigated effects on the amount of sperm transferred at mating (Rahman et al. 2013; Melo et al. 2014; O'Dea et al. 2014; Kaldun and Otti 2016; but see Engqvist 2008; Vermeulen et al. 2008; Perry and Rowe 2010).

Although numbers of sperm produced and stored are informative of male potential to invest in sperm transfer, they do not provide complete information on male postcopulatory investment strategies. For example, males of low condition may have a reduced ability to produce large quantities of sperm, but if they rarely achieve mating, they may strategically allocate relatively more sperm to a single mating compared to high-condition males (Rowe and Arnqvist 1996; Danielsson 2001; Fricke et al. 2015; De Nardo et al. 2021). Such conditional tactics in postcopulatory reproductive investment have been demonstrated in the ladybird, *Adalia bipunctata*, where food-deprived (i.e., low-condition) males transfer relatively more sperm compared to well-nourished (i.e., high-condition) males, whereas the latter transfer larger spermatophores with relatively more nonsperm components (Perry and Rowe 2010). Although competitive fertilization success ultimately depends on multiple ejaculate traits (Fitzpatrick et al. 2012; Lüpold et al. 2012; Lymbery et al. 2018), sperm number is a particularly useful trait to study strategic investment in ejaculates across successive matings due to its high plasticity, methodological tractability, and potential for direct quantification of costs and fitness consequences.

Drosophila species have extraordinarily long sperm, which are likely to result in a high cost of sperm production (Pitnick et al. 1995; Pitnick 1996; Lüpold et al. 2016). Further, in *D. melanogaster*, the number of sperm transferred at mating can be influenced by the availability of protein during development (McGraw et al. 2007), and sperm depletion can occur rapidly (Linklater et al. 2007). However, it remains unknown whether nutrient availability in the larval diet affects the rate of sperm depletion experienced by adult males across successive matings, whether these effects are genotype dependent, or how they influence male fitness. To address these questions, we used 17 independent *D. melanogaster* isolines, each reared on three larval diets varying in nutrient concentration. First, we carried out a "sperm-depletion assay" in which we quantified mating latency, mating duration, and the number of sperm transferred across successive matings with virgin females. Next, we conducted a "short-term fitness assay" to investigate male reproductive performance across successive matings, including male effects on female fecundity and refractory period (both known to be mediated by seminal-fluid proteins; Avila et al. 2011), sperm competitiveness, and male recovery from sperm depletion.

We predicted that (i) males reared on a larval diet with a lower nutrient concentration would transfer fewer sperm overall and experience a steeper rate of sperm depletion compared to males reared on higher nutrient concentrations. This prediction was based on the assumptions that males reared on low nutrients would have lower nutrient stores compared to males reared on higher nutrients, and that these males would therefore incur relatively higher costs of sperm production and have smaller total sperm reserves (Lüpold et al. 2016). Moreover, males reared on a low-nutrient larval diet might invest relatively more in earlier matings if they are less attractive to females and therefore anticipate fewer mating opportunities (see De Nardo et al. 2021). We also predicted that (ii) isolines would exhibit broad-sense genetic variation in male mating latency, mating duration, and sperm transfer (e.g., see Lüpold et al. 2012), and that effects of isoline and diet would interact, reflecting a genotype-by-environment interaction for males' ability to invest in reproduction. We further predicted that (iii) males that mated more often would sire more offspring overall (Bateman 1948), but as a consequence of ejaculate depletion, later copulations would have lower marginal gains in the number of offspring sired, either through a reduced female refractory period and/or lower ability to fertilize eggs (sensu Manning 1962; Markow et al. 1978; Gromko et al. 1984; also see Douglas et al. 2020). For the reasons given in prediction (i), this effect could be more pronounced for low-condition males. Lastly, we predicted that (iv), given their relatively higher costs of sperm production (Lüpold et al. 2016), low-condition males should be

less able to replenish their sperm reserves and thus suffer a higher fitness cost after some refractory period.

Materials and Methods

STUDY ANIMALS

We conducted two assays, one on sperm depletion ("sperm-depletion assay") and the other on the short-term fitness consequences of frequent mating likely due to sperm and/or seminal fluid depletion ("short-term fitness assay"), using *Drosophila melanogaster* (strain LHm) that were genetically engineered to express green fluorescent protein (GFP) in their sperm heads and ubiquitously in somatic cells, thus facilitating the quantification of sperm transferred to females and the paternity of offspring relative to a non-GFP competitor (Manier et al. 2010; Lüpold et al. 2012). The flies used in the sperm-depletion assay were derived from independent isogenic lines (i.e., approximate "clones"), created from a large outbred population of GFP flies in 2010 (from approximately 1000 adult individuals with overlapping generations) by an initial 15 generations of full-sibling inbreeding (i.e., expected homozygosity of 96%; Falconer and Mackay 1996) and subsequent maintenance in small isogenic groups. Each "isoline" used in the experiment was created by crossing males of one isogenic line and virgin females from another isogenic line to generate heterozygous, but still quasi-clonal individuals. There was no overlap of the isogenic lines used to create the isolines for this experiment. The isolines enabled us to subject the same genotypes to different treatments simultaneously, replicated across multiple individuals. Although these isolines have not been sequenced, clear between-line phenotypic variation in reproductive traits, including ejaculate traits, have been reported for them (Lüpold et al. 2012, 2013). Therefore, we could reasonably expect enough genetic variation to detect phenotypic differences in the reproductive traits measured here. All females used in the assays were derived from a single cross of two non-GFP isolines to minimize female effects (e.g., Lüpold et al. 2020).

For the sperm-depletion assay, larvae from each of the 17 isolines were reared on either a "high", "intermediate", or "low" nutrient diet to create adult males of high, intermediate, and low condition, respectively, within each isoline. The high diet consisted of 12.5 ml cornmeal medium (75 g glucose, 100 g fresh yeast, 55 g corn, 8 g agar, 10 g flour, 15 ml Nipagin antimicrobial agent per liter of food medium). For intermediate- and low-diet treatments, the high diet was diluted in water and agar to 33% and 11% of the high nutrient concentration, respectively, and supplemented with 12.3 or 14.1 g of agar per liter of media for a final concentration of 15 g/L each to maintain equal media consistency. This choice of treatments was based on a pilot experiment, in which the low-diet treatment generated visibly smaller flies and approximately 20% excess larval mortality (i.e., quantifiably adverse conditions), whereas the intermediate treatment substantially reduced the nutrient contents but without significantly elevated mortality. All larvae were collected during their first instar and reared at equal density of 40 larvae per vial, replicated across four vials for each diet × isoline combination. Females used as mating partners were reared in culture bottles at moderate density. All vials and bottles were maintained at 24°C, 60% humidity, and a 14:10 light:dark cycle.

These rearing conditions were later repeated in a follow-up experiment to assay the short-term fitness consequences of sperm depletion. This time, however, focal males were derived from the outbred source population of the GFP isogenic lines and reared under the high- and low-diet conditions (for justification, see "short-term fitness assay" below). Standard females and standard competitor males were each represented by two independent crosses between non-GFP isolines.

Throughout our study, for each line and diet combination, experimental flies were collected as virgins within 8 h of adult emergence and transferred in groups of 15 individuals to single-sex vials containing standard food medium, separated by treatment, isoline, and day of eclosion.

SPERM-DEPLETION ASSAY

In each of two equal blocks across two consecutive days, five 6-day-old males per diet and isoline combination were randomly selected from the vials containing flies of the same age, diet, and isoline (i.e., 5 males × 3 diets × 17 isolines × 2 blocks = 510 males in total). All experimental males were mated once to a standardized virgin female 24 h before the sperm-depletion assay (i.e., 5 days posteclosion) to avoid potential "virgin" effects on the first experimental mating (see Bjork et al. 2007). Males were observed until mating, then separated and housed individually until the sperm-depletion assay, and females were discarded. On the day of the experimental matings (i.e., 6 days posteclosion), males were given the opportunity to mate sequentially with five different females over an 8-h observation period. Each focal male was placed in a food vial with two virgin females standardized for age, diet (high nutrient concentration), and genetic background and was observed until mating commenced with one of the females. Immediately after mating initiation, the unmated female was removed by gentle aspiration without disturbing the mating pair. Immediately after copulation ended, the mated female was removed, and the focal male was provided with two new standardized virgin females. Two females were provided in each trial to mitigate potential female reluctance that would slow a male's mating rate. For each mating, the latency to start mating with one of the two females (i.e., since entering the mating vial for the first mating, and since the end of the previous mating for all subsequent matings) and mating durations were recorded.

Females were frozen individually within 15 min of the end of mating to avoid sperm ejection (i.e., to get an accurate count of the sperm transferred without a potential effect ofcryptic female choice; Manier et al. 2010; Lüpold et al. 2013), and males were frozen after achieving five matings or reaching the end of the 8-h mating window.

To quantify the number of sperm transferred by males, the female reproductive tract was removed from the abdomen and placed into a drop of water on a microscope slide, the seminal receptacle was uncoiled with a fine probe, and a coverslip was placed over the sample and sealed with rubber cement. All sperm within the bursa, spermathecae, and seminal receptacle were counted under an Olympus BX51 fluorescence microscope (Olympus America, Melville, USA) with a green-fluorescent filter, and summed to obtain the total number of sperm transferred. Finally, male thorax length was measured as a proxy of body size under a Leica MS5 stereomicroscope (Leica Microsystems, Heerbrugg, Switzerland) at 40× magnification.

All behavioral observations and sperm counts were done blind to diet and isoline.

SHORT-TERM FITNESS ASSAY

To quantify the fitness consequences of sperm depletion, a second assay was conducted, following the same general protocol as for the sperm-depletion assay, but using only the two extreme diet conditions (i.e., the high- and low-nutrient diets) and focal males from an outbred population instead of isogenic lines. This logistically simplified approach was chosen for three reasons. First, male body size (a proxy for male condition) did not differ significantly between males from the high- and intermediate-diet treatments in the sperm-depletion assay (see *Results*), thus limiting the benefit of using all three treatments. Second, in the sperm-depletion assay, the variance component of genotype (i.e., isoline effect) was small (see *Results*). Third, the fitness consequences (e.g., male-induced female refractory period on the total offspring produced by males across all females of a mating sequence) had been studied previously, including their genetic effects (Douglas et al. 2020). Thus, our goal was to quantify effects of multiple mating and diet on the number of offspring sired in competitive and non-competitive settings (i.e., before and after the female remated) and on the level of sperm replenishment.

Consistent with the sperm-depletion assay, each focal male ($N = 70$ and 60 males per high and low diet, respectively, all 3–5 days old) was presented with up to five sequential pairs of standardized virgin females (the same isoline cross as in the sperm-depletion assay), again removing the spare female after the start of each copulation. However, instead of being frozen immediately after copulating, mated females were transferred to fresh vials with standard fly food to oviposit. The following day, each female was allowed to remate with a standard, virgin, non-GFP competitor male to investigate potential effects of sperm depletion on female refractory period. Females not remating within 4 h were separated and given up to 5 consecutive days of additional 4-h remating opportunities until they remated (hereafter "female remating interval"). After remating, females were transferred to fresh individual food vials (post-remating vials) and allowed to lay eggs of mixed paternity for another 3 days. The offspring of all oviposition vials were reared and counted posteclosion to estimate male fitness before (hereafter "prior offspring") and after female remating, with the post-remating vials being used to quantify sperm defense of the focal (GFP) males as the proportion of first-male paternity ("P_1"; Boorman and Parker 1976).

To investigate the effect of frequent mating on the recovery from sperm depletion (i.e., sperm replenishment), each focal male was mated once more with a standardized virgin female on the day after the experimental mating sequence. These females were again frozen immediately to count the number of sperm transferred by males after a refractory period.

All measures of male fitness were done blind to male diet treatment.

STATISTICAL ANALYSES

All analyses were conducted in the statistical software package R version 4.0.3 (R Core Team 2020), using the package *lme4* (Bates et al. 2015) for linear and generalized linear mixed-effects models (LMMs and GLMMs, respectively) and *lmerTest* (Kuznetsova et al. 2017) to obtain *P*-values based on the Satterthwaite approximation for denominator degrees of freedom (Schaalje et al. 2002). The package *glmmTMB* (Brooks et al. 2017) was used to correct for GLMMs with zero-inflated Poisson distributions by applying a single zero-inflation parameter to all observations. Models with overdispersion were corrected by including an observation-level random effect (OLRE). The assumption of equal variance was assessed using the Levene's test implemented by the *car* package (Fox and Weisberg 2019) for LMMs and the *DHARMa* package (Hartig 2020) for GLMMs. Variance components were calculated using the *rptR* package (Stoffel et al. 2017).

All analyses consisted of models that included all potentially relevant covariates and random effects (see below). Apart from models of male thorax length and the total number of times males mated during the 8-h observation period (hereafter "M_{tot}"), all models included main effects of diet, mating order (i.e., position of a given mating among a male's sequential matings, hereafter "M_i"), a diet × M_i interaction, as well as M_{tot} and male thorax length (standardized using *z*-scores within the diet treatment to eliminate collinearity with the categorical effect of diet) as covariates. M_{tot} was included as a covariate as males varied substantially in the number of matings they achieved during the 8-h observation period, which could explain some of the variation in male mating behavior, sperm transfer, and fitness. All

models (apart from male thorax length and sperm replenishment) included individual ID as a random effect and all models from the sperm-depletion assay included the block of the mating assay as a random effect. All continuous and ordinal fixed effects were standardized using z-transformation, as this puts the variance explained by each model component in the same "context" of the total phenotypic variation and allows for more accurate calculation of variance components (Schielzeth and Nakagawa 2020).

In the sperm-depletion assay, treatment effects on male thorax length were analyzed using an LMM with diet as a fixed effect and diet × isoline as a random effect. The same factors, with the addition of male thorax length (standardized within diet), block, and an OLRE (individual ID) were included in a GLMM with a Poisson distribution to analyze treatment effects on M_{tot}.

The models testing for differences in male mating latency (square root-transformed to improve normality), mating duration, and the number of sperm transferred at mating were analyzed using LMMs with male ID, block, and diet × isoline as random effects. The fixed effects were diet, male thorax length (standardized within diet), M_i, and M_{tot}, as well as the diet × M_i interaction. Mating duration was included as a covariate in the model of sperm transfer. Initially, models were tested for linear and quadratic effects of M_i by including diet × M_i^2 and diet × mean-centered M_i (to avoid collinearity with M_i^2), but the quadratic term did not improve model fit (based on LRTs and a cutoff of $P > 0.1$) and was therefore removed from all models, whereas M_i remained in all models.

In the short-term fitness assay, the effects of diet and mating order on the number of days for the female to remate (i.e., "female remating interval"), the number of offspring produced by each female prior to remating (i.e., "prior offspring"), and the effect of male thorax length and diet on M_{tot} were tested using GLMMs with Poisson distributions. The analysis of prior offspring was corrected for zero-inflation using the *glmmTMB* package (see above). Focal-male sperm defense after each female remated with a standard competitor (i.e., proportion of first-male progeny, "P_1") was analyzed using a GLMM with a binomial error distribution and a log link function, and sperm transfer after a refractory period ("sperm replenishment") was examined using an LMM. All models included diet, thorax length (z-transformed), M_i, M_{tot}, and a diet × M_i interaction. An OLRE was also included in the P_1 analysis, and female remating interval was included as a fixed-effect covariate in both the prior and P_1 offspring analyses. All models with multiple observations per male (i.e., all models except the sperm replenishment model) included male ID as a random effect. As only three males from the high diet mated four times, these males were excluded from the analyses on female remating interval, prior offspring, and P_1 to include the diet × M_i interaction in the model; this did not affect any conclusions (see Table S1 for the analysis with these three males included).

The contribution of each random effect, including the diet × isoline random interaction, was calculated as the percent of variation explained out of the total phenotypic variation (see Schielzeth and Nakagawa 2020). Further, each figure in the main text depicts the least square means ± SE extracted from the corresponding model (described above) using the *effects* package (Fox 2003; Fox and Weisberg 2019), with M_i and diet as focal predictors. Plots with the unmodified data are available in the Supporting Information.

Results

SPERM-DEPLETION ASSAY

Diet significantly affected male thorax length ($\beta = -0.10 \pm 0.01$ (SE), $t_{406} = -11.24$, $P < 0.01$), with the low diet resulting in significantly smaller males compared to the intermediate and high diets. However, there was no difference in thorax length between males from the intermediate and high diets (Fig. S1A). The diet × isoline random interaction accounted for minimal variation (<1%) in male thorax length, but isoline accounted for 12.5% of total variation.

The total number of matings achieved during the 8-h observation period (M_{tot}) varied among individual males from zero to five matings, with 47 out of the 510 males never mating during that period (Fig. S2A). M_{tot} was not affected by diet ($\beta = 0.01 \pm 0.03$, $z_{415} = 0.31$, $P = 0.88$), but smaller males (thorax length standardized within diet) mated more often ($\beta = -0.10 \pm 0.03$, $z_{415} = -2.92$, $P < 0.01$). However, both isoline and the diet × isoline random interaction accounted for <1% of the total variation. Males that mated more times exhibited shorter mating latencies and mating durations, and there was a nonsignificant trend toward males that achieved more matings in total to also transfer more sperm per mating (Table 1).

Diet did not interact with mating sequence (M_i) to affect mating latency, mating duration, or the number of sperm transferred, but low-diet males transferred significantly fewer sperm overall (Table 1; Figs. 1 and S3). Further, although mating latency significantly increased with each successive mating, the number of sperm transferred significantly decreased (Table 1; Figs. 1 and S3). There was no significant change in mating duration with M_i nor a diet effect on mating latency or mating duration (Table 1; Figs. 1 and S3). Sperm transfer was not correlated with mating duration (Table 1; Fig. 1) and there was also no effect of male thorax length (standardized within diet) on mating latency, mating duration, or sperm transfer. The diet × isoline random interaction accounted for <1% of the variation in mating latency, mating duration, and the number of sperm transferred. Isoline alone

Table 1. **Main and interactive effects of the males' larval diet and the order of their consecutive matings (M_i), as well as covariates, on male mating latency, mating duration, and sperm transfer. Results with $P < 0.05$ are highlighted in bold. Negative effect sizes represent a decrease in the response variables with increasing M_i or M_{tot}, or with a decreasing nutrient concentration in the larval diet. "Na" represents covariates that were not applicable to certain models.**

	Mating latency ($N = 979$)			Mating duration ($N = 971$)			Sperm transfer ($N = 893$)		
	β	SE	P	β	SE	P	β	SE	P
Diet	0.02	0.11	0.85	0.49	0.37	0.19	**–54.76**	**16.01**	**<0.01**
Mating sequence (M_i)	**1.03**	**0.13**	**<0.01**	–0.13	0.27	0.62	**–295.25**	**13.73**	**<0.01**
Total matings (M_{tot})	**–1.64**	**0.13**	**<0.01**	–0.56	0.30	0.06	31.28	17.90	0.08
Mating duration	Na	Na	Na	Na	Na	Na	–5.23	17.88	0.77
Thorax length (standardized within diet)	–0.12	0.11	0.28	–0.04	0.29	0.88	**51.10**	**13.55**	**<0.01**
Diet × M_i	0.10	0.11	0.35	–0.24	0.24	0.33	–2.40	12.66	0.85

accounted for <1% of the variation in mating latency, and 3.70% and 5.75% of the variation in mating duration and the number of sperm transferred, respectively. Differences between individuals accounted for <1% of the variation in mating latency, and 7.09% and 15.16% of the variation in mating duration and the number of sperm transferred, respectively. The block of the mating assay accounted for <1% of the total variation for all models.

SHORT-TERM FITNESS ASSAY

As in the sperm-depletion assay, the low diet resulted in significantly smaller males in the short-term fitness assay ($\beta = -0.32 \pm 0.03$, $t_{110} = -9.63$, $P < 0.01$; Fig. S1B). Overall, males mated less often in the short-term fitness assay compared to the sperm-depletion assay, with only three males from the high diet reaching four consecutive matings during the 8-h observation period (Fig. S2B). As in the sperm-depletion assay, diet did not affect M_{tot} ($\beta = -0.05 \pm 0.07$, $z_{111} = -0.723$, $P = 0.47$), but contrary to the sperm-depletion assay, male thorax length had no effect on M_{tot} ($\beta = -0.05 \pm 0.07$, $z_{111} = -0.75$, $P = 0.45$).

Diet did not affect female remating interval, prior offspring, or P_1, nor was there a significant interaction of diet and male mating sequence (M_i) on any of these fitness traits (Table 2; Figs. 2 and S5). The number of prior offspring sired significantly decreased with increasing M_i after accounting for differences in female remating interval (Table 2; Figs. 2 and S5). P_1 increased with female remating interval but was unaffected by M_i (Table 2; Figs. 2 and S5). Additionally, the total number of times that males mated during the mating assay (M_{tot}) was not correlated with female remating interval, prior offspring, or P_1. However, males with higher M_{tot} transferred significantly ffewer sperm the following day, suggesting reduced ability to replenish their depleted sperm stores during the 24-h recovery period (Table 2; Figs. 3 and S6).

Male thorax length (standardized within diet) did not affect female remating interval, prior offspring number, P_1, or sperm replenishment, and differences between individuals accounted for <1% of the variation in M_{tot}, female remating interval, and P_1, and 8.20% of the variation in prior offspring number.

Discussion

Our results show that successive mating can be costly for some aspects of reproductive performance in male *D. melanogaster*, in that reproductive investment in, and fitness gains from, each mating opportunity declined if these opportunities arose in relatively rapid succession. Specifically, we found that males transferred fewer sperm with each successive mating, that the mating latency increased between these matings, and that females later in a male's mating sequence produced fewer offspring prior to remating with a competitor male. These results indicate that males become ejaculate depleted and require a longer refractory period to mate with additional females, and that the depletion of sperm or nonsperm components may reduce males' ability to sire offspring. Additionally, we also show that males reared on a lower nutrient diet transferred fewer sperm overall than those developing on a more nutritious diet. However, none of our measures of male reproductive performance were affected by the diet × mating order interaction, demonstrating that even a reduction in nutrient concentration by just under 90% does not mediate the rate of sperm depletion, nor changes in male mating behavior or fitness. Finally, the random interaction of diet and isoline accounted for <1% of the total variation in male mating behavior and sperm transfer, suggesting that the 17 genotypes used in this study exhibited very similar dietary effects on the traits measured. In fact, genotypic differences themselves contributed <6% to the total variation in reproductive traits.

The clear reduction in the number of sperm transferred with each successive mating, along with the incomplete sperm replenishment the following day, is consistent with previous theoretical and empirical research showing that males are unable to

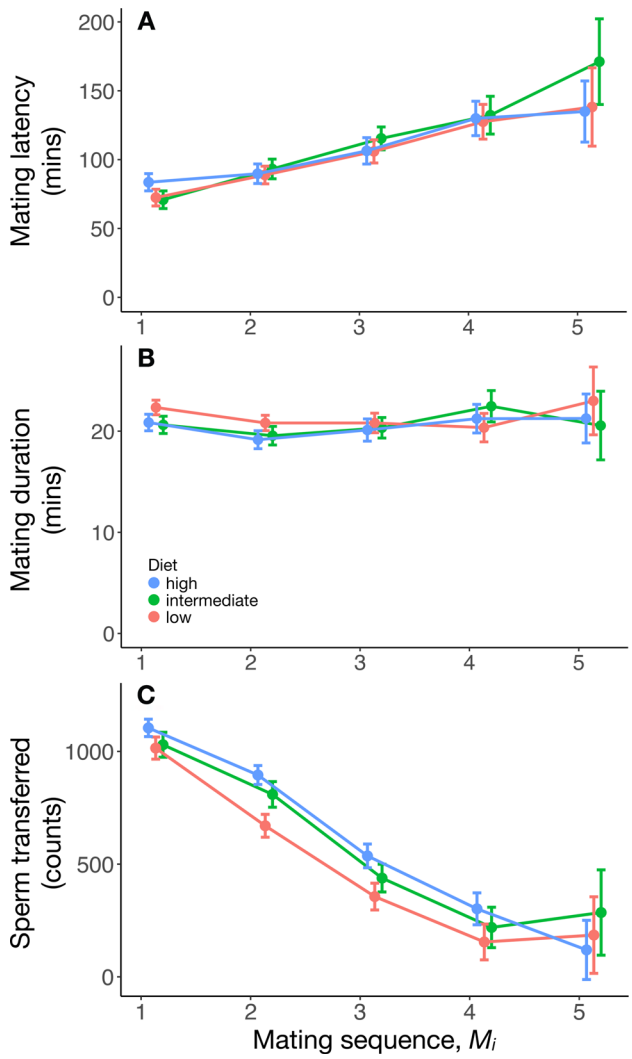

Figure 1. Effects of diet (blue = high diet, green = intermediate diet, red = low diet) and M_i (i.e., the order of consecutive matings) on (A) mating latency, (B) mating duration, and (C) sperm transfer. Diet and M_i did not interact to affect mating latency, mating duration, or the number of sperm transferred. Low-diet males transferred fewer sperm overall and mating latency significantly increased and the number of sperm transferred decreased with M_i. Plots represent the least squares means ± SE extracted from the corresponding mixed-effects models, with M_i and diet as focal predictors.

rapidly replenish depleted ejaculate stores (e.g., Markow et al. 1978; Dewsbury 1982; Hughes et al. 2000; Preston et al. 2001). These results clearly demonstrate that males do not have unlimited sperm supplies, and they are indicative of significant costs related to ejaculate production (Dewsbury 1982; Pitnick 1996; Olsson et al. 1997; Thomsen et al. 2006; Lüpold et al. 2016). That male sperm depletion was further accompanied by an increasing refractory period between successive matings could indicate that males may reduce their mating rate to conserve ejaculate stores and/or to replenish depleted stores to some degree (also see Macartney et al. 2020). However, this declining mating rate could also result from physical exhaustion or reduced motivation (Franklin et al. 2012), or from more time spent assessing females to maximize fitness benefits with the increasingly limited sperm reserves (Dewsbury 1982).

Females of several *Drosophila* species are unable to store all the sperm received by a male and thus eject a substantial proportion of them (e.g., Manier et al. 2013).It could therefore be argued that transferring more sperm does not necessarily result in higher fitness. However, while directly linking sperm transfer to progeny production is challenging as females need to be sacrificed for sperm quantification, females that receive more sperm also tend to store more in total, even if the proportion of sperm ejected increases (Fig. S7). Consequently, the number of sperm stored within the female is likely to be important, particularly because the number of stored sperm within the female can decline by an average of four to five sperm for every egg laid in *D. melanogaster* (Manier et al. 2013). This rapid decline of sperm within the female reproductive tract could greatly exacerbate a male's numerical disadvantage in sperm competition if he is unable to transfer a competitive number of sperm.

Our experiment further revealed that females later in a male's mating sequence produced fewer offspring before remating when controlling for the remating interval. In other words, these females not only produced fewer offspring per se but also did so at a lower daily rate, thereby reducing the male's marginal gains with each successive mating (also see Markow et al. 1978; Douglas et al. 2020). Because the decrease in progeny production was not paralleled by a shorter female remating interval (but see Douglas et al. 2020), the decline in prior offspring is more likely to reflect a more rapid depletion of ejaculate components that influence the female oviposition rate than of those inducing female refractoriness. Regardless of the underlying mechanism, our results add nuance to Bateman's (1948) classic study on sex-specific fitness gains via multiple mating. Although males clearly accumulated more offspring by mating repeatedly as predicted by Bateman (1948), our results suggest that male fitness may not simply be a linear function of mating success even under noncompetitive conditions (also see Douglas et al. 2020). This finding opens some interesting research avenues, including whether females prefer mates that are less likely to be ejaculate depleted or whether males themselves become choosier or allocate their ejaculates more prudently as they become increasingly ejaculate depleted (Bonduriansky 2001; Edward et al. 2011).

The lack of an effect of male mating sequence on P_1 could, in part, be explained by the fact that second-male sperm precedence in *D. melanogaster* is approximately 80% (Price et al. 1999), which can result in relatively low variation in first-male sperm

Table 2. Main and interactive effects of the males' larval diet and the order of their consecutive matings (M_i), as well as covariates, on female remating interval, prior offspring number, P_1, and sperm replenishment. Results with $P < 0.05$ are highlighted in bold. Negative effect sizes represent a decrease in the response variables with increasing M_i or M_{tot}, or with a decreasing nutrient concentration in the larval diet. "Na" represents covariates that were not applicable to certain models. Note that the three high-diet males with an M_{tot} of four have been removed to allow for the inclusion of the diet × M_i interaction.

	Female remating interval ($N = 129$)			Prior offspring ($N = 129$)			P_1 ($N = 127$)			Sperm replenishment ($N = 92$)		
	β	SE	P	β	SE	P	β	SE	P	β	SE	P
Diet	0.04	0.06	0.51	0.03	0.07	0.67	0.20	0.40	0.60	–35.77	45.60	0.43
Mating sequence (M_i)	–0.09	0.07	0.21	**–0.30**	**0.06**	**<0.01**	0.02	0.45	0.96	Na	Na	Na
Total matings (M_{tot})	–0.04	0.08	0.56	0.08	0.08	0.30	–0.48	0.51	0.34	**–112.99**	**41.50**	**0.01**
Mating duration	<0.01	0.06	0.98	0.07	0.06	0.23	–0.03	0.33	0.92	Na	Na	Na
Thorax length (standardized within diet)	–0.01	0.05	0.86	–0.05	0.07	0.48	0.30	0.37	0.42	20.49	49.98	0.68
Female remating interval	Na	Na	Na	**0.44**	**0.07**	**<0.01**	**1.41**	**0.39**	**<0.01**	Na	Na	Na
Diet × M_i	–0.04	0.06	0.50	–0.03	0.06	0.64	0.45	0.36	0.22	Na	Na	Na
Diet × M_{tot}	Na	Na	Na	Na	Na	Na	Na	Na	Na	–10.41	41.43	0.80

defense. It would thus be interesting to repeat our experiment focusing on second-male sperm offense (P_2) instead of P_1. However, as mentioned previously, without fast-remating mutant females it might be difficult to reliably mate second males in rapid succession and compare their sperm competitiveness across their matings sequence. This is particularly true on the first few days after their first mating when large numbers of first-male sperm are still in storage to generate sufficiently intense sperm competition and thus variation in P_2. Additionally, varying numbers of first-male sperm residing in storage at remating could greatly confound ejaculate investments by focal second males (Lüpold et al. 2012, 2020).

Although males of all treatments showed a decline in sperm numbers and progeny production, it was the low-diet males that consistently transferred fewer sperm than males of the other treatments. These males were also smaller and may thus have incurred higher costs per sperm produced (e.g., see Lüpold et al. 2016). These findings are consistent with condition dependence and life-history theories predicting that resource-limited males are more constrained in their investment in costly sexual traits, including postcopulatory traits such as ejaculates (see Macartney et al. 2019 for a review and meta-analysis). The combination of these two results therefore provides further evidence that ejaculate production and transfer is costly for male *D. melanogaster*, and that males reared on a low-nutrient diet are less able to invest in ejaculates (also see McGraw et al. 2007).

The lack of an interactive effect of diet and male mating sequence on any of our measures of male reproductive performance contrasted with our predictions. This result suggests that low-diet males did not suffer a steeper rate of ejaculate depletion across matings, for example, due to disproportionate investments in earlier matings (e.g., see De Nardo et al. 2021). Instead, males from all conditions invested a similar proportion of sperm relative to their total sperm stores. This conclusion is further supported by the lack of a dietary effect on the number of sperm transferred the day after the sequential matings in the short-term fitness assay, which indicates no differential latent costs of reproductive investment between diet treatments. Rather, low-diet males appeared to be equally able to replenish their sperm reserves as high-diet males over a 24-h recovery period. However, perpetual ejaculate donation combined with incomplete replenishment might still cause low-diet males to experience higher fitness costs in the longer term compared to males of superior condition (e.g., see Lüpold et al. 2016).

Isogenic lines from the same source population that our experimental lines were derived had previously shown significant genetic variation in reproductive traits, including several ejaculate parameters such as sperm length and sperm velocity, as well as the number of sperm stored by the female (Lüpold et al. 2012, 2013). In the present study, however, even the total genetic variation in male mating behavior and sperm transfer was relatively small (<6%), and the random interaction of diet and isoline accounted for <1% of the total variation in these traits. Therefore, based on our 17 independent isolines, neither genotypic variation nor its interaction with diet appeared to be very important for determining male mating behavior or sperm transfer in *D. melanogaster*. This finding contrasts with theoretical and empirical studies showing that male genotype can mediate sexual trait responses to environmental conditions (Rowe and Houle 1996; Hunt et al. 2004a; Gienapp and Merilä 2010; Ingleby et al. 2010; Evans et al. 2015). One possible explanation for the minimal effects of isoline on the traits measured here is that there might be

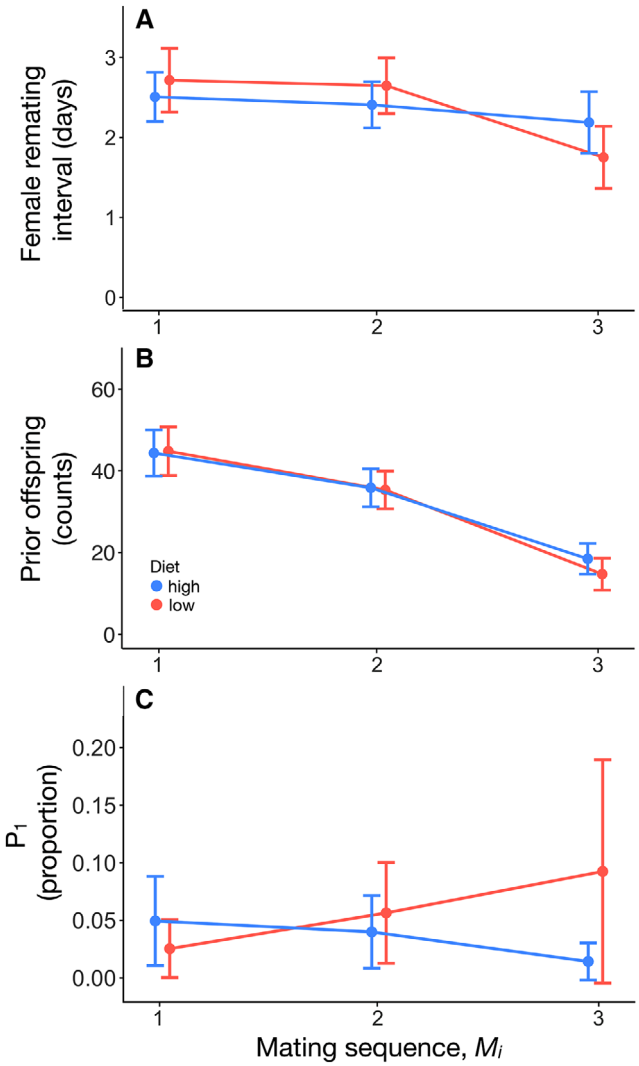

Figure 2. Effects of diet (blue = high diet, red = low diet) and M_i (i.e., the order of consecutive matings) on (A) female remating interval [days], (B) prior offspring, and (C) P_1. Diet and M_i did not interact to affect female remating interval, prior offspring, or P_1. Diet did not affect female remating interval, prior offspring, or P_1, and prior offspring was the only measure of male fitness that decreased with M_i. Plots represent the least squares means ± SE extracted from the corresponding mixed-effects models, with M_i and diet as focal predictors.

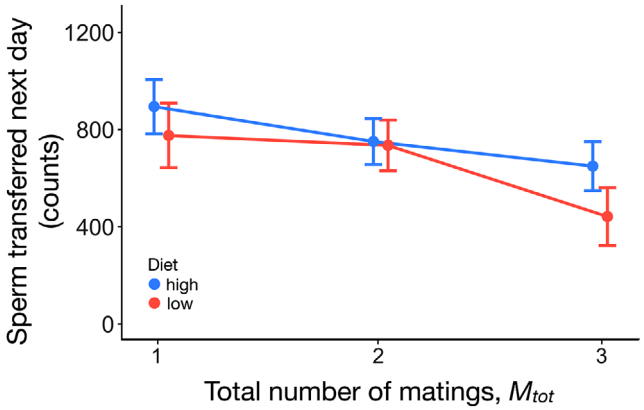

Figure 3. Effects of diet (blue = high diet, red = low diet) and M_{tot} (i.e., the total number of times males mated during the 8-h observation period) on the number of sperm transferred after a 24-h recovery period. Diet and M_{tot} did not interact to affect sperm replenishment, diet alone did not have any effect on the number of transferred, but the sperm replenishment decreased with M_{tot}. Plots represent the least squares means ± SE extracted from the sperm replenishment mixed-effects model, with M_{tot} and diet as focal predictors.

genetic canalization, meaning that phenotypic variation in male mating behavior and sperm transfer is "robust" to genetic differences due to the importance of such traits for male fitness (Waddington 1942; Wagner et al. 1997). However, it is possible that 17 isolines were simply too few to detect a potentially weak diet × isoline effect or that the differences between these lines were small, perhaps because the variance between crosses was inadvertently reduced by pairing isogenic lines with high and low trait values.

The positive correlation between female remating interval and P_1 hints toward some males transferring ejaculates of higher quality that both delay female remating and increase their competitiveness in a sperm defense assay. However, this effect could also be due to innate variation in female remating interval despite using standardized females and second males. Lastly, in both assays, diet did not affect male mating rate (M_{tot}), which was negatively correlated with both mating latency and mating duration. These correlations could reflect circular artifacts, as individuals with shorter mating latency and mating duration will have increased chances to reach higher M_{tot} within a time-bounded mating assay.

In conclusion, the combined results of the sperm depletion and short-term fitness assays point toward the limits of the male reproductive potential (Lüpold et al. 2016; Douglas et al. 2020). The relative cost of producing seminal fluid versus sperm remains unknown, but some studies suggest that seminal-fluid synthesis may be costlier than sperm production, and that seminal fluid can be depleted faster (Rogers et al. 2005; Linklater et al. 2007; Reinhardt et al. 2011). Regardless of these relative costs, the fact that both ejaculate components can be rapidly exhausted and that there are clear costs to fitness with successive mating indicates likely costs to females that mate with successful males if these become increasingly ejaculate depleted relative to other males (Preston et al. 2001). However, important unanswered questions remain about the typical mating rate under natural conditions. Males might, on average, spend more time between mating opportunities competing against other males and courting females,

given that non-virgin females may be commonly encountered and reluctant to mate. This could lead to an increased replenishment period for males, reducing their mating rate and allowing for greater investment in ejaculate transfer and fitness (although we found that males can remain sperm limited after a 24-h refractory period). The results presented here also pose interesting questions regarding mate choice mediated by male ejaculate depletion (e.g., Markow et al. 1978; Härdling et al. 2008; Scarponi et al. 2015). Overall, our results show that sperm transfer declines rapidly with successive matings and that this decline affects male reproductive performance.

AUTHOR CONTRIBUTIONS

ELM and RB conceived the research. ELM, VZ, and SL planned the experiment. ELM, VZ, AM, ANDN, and SL collected the data. ELM and VZ analyzed the data. ELM, VZ, and SL wrote this article. RB provided valuable feedback. All authors edited and approved the final version.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Unmodified dataset for the short-term fitness assay, including the three high-diet males with $M_{tot} = 4$.

Figure S1. Effects of diet on male thorax length for the sperm-depletion assay (A) and the short-term fitness assay (B).

Figure S2. Number of males that mated 0, 1, 2, 3, 4, or 5 times (i.e., M_{tot}) during the 8-h mating period grouped by diet during the sperm-depletion assays (left) and short-term fitness assay (right).

Figure S3. Raw data. Effects of diet (blue = high diet, green = intermediate diet, red = low diet) and M_i (i.e., the order of consecutive matings) on (A) mating latency, (B) mating duration, and (C) sperm transfer. Diet and M_i did not interact to affect mating latency, mating duration or the number of sperm transferred, but low-diet males transferred fewer sperm overall.

Figure S5. Raw data. Effects of diet (blue = high diet, red = low diet) and M_i (i.e., the order of consecutive matings) on (A) female remating interval [days], (B) prior offspring, and (C) P_1.

Figure S6. Raw data. Effects of diet (blue = high diet, red = low diet) and M_{tot} (i.e., the total number of times males mated during the 8-hour observation period) on the number of sperm transferred after a night rest.

Figure S7. Raw data showing the relationships of the number of sperm transferred with the number of sperm ejected (red) or stored (blue) after a single mating, with 95% CIs.