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Limits to male reproductive potential across mating bouts in *Drosophila melanogaster*

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Understanding the sources of variation in reproductive fitness is a central goal of sexual selection research. Research investigating factors limiting male reproductive potential typically focus on limited mate availability or mate access. This focus often minimizes the potential relevance of physiological or other limitations on male reproductive potential, in contrast to the emphasis on studying such limitations in females. This gap in knowledge leaves open questions about how variation in male reproductive success emerges across successive mating bouts. Here, we contribute to bridging this gap by examining male reproductive potential across successive matings and across time. To reveal limits to male reproductive potential, and sources of variation in these limits, we measured mating rate and offspring production in *Drosophila melanogaster* males under conditions in which mate limitation was abrogated and food was abundant. Even under these ideal conditions, we discovered distinct limits to male reproductive potential after just a few mating bouts. After males mated two to five times on a given day, additional matings often resulted in zero progeny. Furthermore, we found nonlinear relationships between the number of females a male mated with and the number of progeny he sired; and these relationships depended on the male's genotype, early life social environment and recent mating experience. These findings suggest that males who obtain more mates do not always sire the most offspring and that males who are highly successful in obtaining mates during one time period may not be able to continue this success on subsequent mating bouts and days. Together, these findings suggest trade-offs between current and future reproduction for males. More broadly, these results highlight how sexual selection studies may be expanded across individuals' lifetimes to develop a fuller picture of how sexual selection shapes variation.
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Understanding variation in reproductive fitness is the central goal of sexual selection. Under classical sexual selection theory, female reproductive fitness is thought to be limited mostly by resource availability and mate quality, while male reproductive fitness is thought to be limited by the number of females they can persuade (or coerce) to mate with them (Bateman, 1948; Futuyma & Kirkpatrick, 2017; Jones, Arguello, & Arnold, 2002; Kokko, Jennions, & Brooks, 2006). These ideas have enormous consequences for sexual selection theory, including what parameters should be measured in an experiment aimed at improving our understanding of sexual selection. First, female limitations on remating are expected to lead to relatively low variance in female fitness, allowing even low-quality females at least some reproductive opportunities. In addition, the number of females that a male mates with should be a key indicator of the male's reproductive fitness.

In recent years, many aspects of this framework have been challenged both theoretically and empirically. The resulting collapse of classical ideas about 'sex roles' has led to exciting research on processes of sexual selection that were previously neglected. For example, we now know that females often benefit from mating with many males (Gowaty, Kim, Rawlings, & Anderson, 2010; Tang-Martínez, 2016) and may not always be choosy (Boulton, Zuk, & Shuker, 2018). Similarly, males, too, are often choosy, both in whom they mate with (Bonduriansky, 2001; Byrne & Rice, 2006; Edward & Chapman, 2011) and in the quantity and quality of energetically costly benefits (e.g. ejaculate, nuptial gifts) they allocate to a particular mating ('crypic' male mate choice, reviewed in Bonduriansky, 2001).

This progress has highlighted gaps in our knowledge of how sexual selection acts on males (Fitzpatrick & Servedio, 2018), and in

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particular, how factors beyond attractiveness may limit male reproductive fitness. The existence of male mate choice suggests that male mating is associated with costs; but for most species, we still know very little about when and how these costs are incurred. For example, male mate choice could reflect opportunity costs incurred under high variation in female fitness (Byrne & Rice, 2006; Edward & Chapman, 2011; Reading & Backwell, 2007)—i.e. males exert preferences to avoid opportunity costs of mating with a lower-quality female. At the same time, the finding that males allocate different ejaculate components to different types of females may point to physiological constraints—i.e. males are physically incapable of mating more than *n* times.

Of course, it must be true that, at some point, males have mated so many times that they can no longer produce more offspring (for some refractory period). However, surprisingly little is known about such limits to male reproductive potential, including whether they might be adjusted through experience. For example, in *Drosophila*, mating behaviour has been studied for many decades, but surprisingly little is known about how many times males can mate on any timescale. The relative dearth of research on this topic suggests that researchers may implicitly assume that the limit on male remating potential is so high that it is irrelevant, i.e. *n* is usually assumed to exceed the number of females a male could ever hope to mate with. Under this assumption, any limits to reproductive potential are usually unimportant to sexual selection on males. In other words, although there is extensive knowledge about male mating strategies for a male's first mating bout, little is known about the limits of these strategies across multiple reproductive bouts.

Here, we aim to contribute to filling this gap in knowledge by investigating fundamental questions about limits to male reproductive potential: how many times can males mate? After mating some number of times, does fertility decline? If so, how much, and after how many mating bouts? And, how do genetic differences and prior experience modulate these parameters?

To answer these questions, we provided males with a series of virgin females over the course of 3 consecutive days in an environment free of male–male competition and nutrient limitation. We measured how many females each male could mate with, and how many offspring each mate then produced (experiment 1) as well as how quickly females chose to remate (experiment 2). By experimentally removing the factors already known as typical limitations to male reproductive potential, i.e. nutrition and mate availability, these conditions allowed us to directly answer the questions posed above. We emphasize that our experiment was not designed to mimic nature, but rather, to experimentally abrogate the limitations on reproductive potential that have already been studied in order to illuminate new ones. Furthermore, by providing males with an optimal environment for reproduction, we could establish a lower bound on the limitations that males might face in nature—i.e. could limitations to reproductive potential occur even under these ideal conditions?

Specifically, our design allowed us to test several predictions. First, we tested whether limitations to male remating potential are irrelevant until males have mated with an improbably high number of females. Of course, the number of mates that is 'improbably high' will differ among species, populations, seasons, etc.; see Methods for details about our study system.

If male reproductive potential is indeed limited, then males should make strategic decisions about how to allocate limited reproductive investment across matings (Edward & Chapman, 2011; Ingleby, Lewis, & Wedell, 2010; Wigby et al., 2009). We predicted that these strategic decisions, if any, should be based on

To investigate limits to male reproduction, and whether they are modified by experience, we provided males with either no social experience, with access to cues from females (providing information about female abundance), or with access to cues from males (providing information about potential competition), before assaying their remating limits as described above. We predicted that males exposed to cues from potential rivals prior to mating would 'assume' that rivals are abundant in their local population. Thus, we predicted that these 'male-experienced' males would invest more into early reproductive bouts, but at a cost to future reproductive effort. These males should thus show a sharp drop-off in fertility and/or mating behaviour after a relatively small number of matings. Similarly, males who experienced cues from females should 'assume' that females are abundant and/or that male rivals are rare, and therefore tailor their strategy to invest less in each individual female and invest more towards mating with as many females as possible. We therefore predicted that these 'female-experienced' males would show a more gradual decline in fertility and/or mating behaviour across subsequent matings, relative to male-experienced males. Isolated males were included to mimic conditions used in typical experiments.

Our experiments revealed surprising limits to male reproductive potential that compounded across days and depended on the male's genotype and early life exposure to cues from conspecifics. These findings highlight deficiencies in our understanding of variation in reproductive fitness and raise new questions about how sexual selection acts on males.

METHODS

Study System

Drosophila melanogaster is a classically important system for studying sexual selection. Males spend much of their adulthood looking for food and mates (Powell, 1997). Flies form aggregations on rotting fruits with rich sources of microorganisms, flies' food. These aggregations are semi-stable, with individuals frequently moving among food patches (Wertheim et al., 2002, 2003, 2006). Thus, males encounter variable numbers of both receptive mates and prospective rivals as a natural consequence of their ecology. For example, the sex ratio on breeding sites in the wild tends to be female biased (Markow, 1988).

In *D. melanogaster*, wild females produce broods sired by at least four to six males (which is probably an underestimate of how many times the female mated; Imhof, Harr, Brem, & Schlötterer, 1998); in the laboratory, females are capable of mating up to six times in a 24 h period (Billeter, Jagadeesh, Stepek, Azanchi, & Levine, 2012; Krupp et al., 2008). These findings suggest the potential for high remating rates in this species: on average, flies may mate several times per day, and particularly attractive flies may mate much more (Mery et al., 2009). Therefore, limits to male reproductive potential can be safely ignored if they typically only occur after many more than four to six matings per day (approximately).

Variation in the social environment in nature suggests that not all males have equal access to females, both in the days after eclosion and later on. Therefore, males should benefit from being able to adjust their remating strategy. Indeed, studies of single mating events in *D. melanogaster* suggest that males can alter their investment in a particular mating bout. As in many other species, when *D. melanogaster* males are exposed to a rival male prior to mating, they respond by increasing both sperm production (Moatt, Dytham, & Thom, 2014) and mating duration (Bretman, Fricke, &

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competition (Wigby et al., 2009). Additionally, males adjust their reproductive strategies based on female mating status and quality. For example, they transfer more sperm to mated, large or young females (Lüpold, Manier, Ala-Honkola, Belote, & Pitnick, 2011). Furthermore, males adjust the composition of seminal fluid proteins in the ejaculate in response to female mating status, withholding fecundity-stimulating proteins from mated females who most likely received those proteins from a prior mate (Sirot et al., 2011).

Genotypes

Multiple natural genotypes were tested to identify genetic variation in reproductive limits, and to ensure that our results were not particular to any one genotype.

All flies used in this study were F1 heterozygous progeny of wild-derived inbred lines originally collected from a single population in Raleigh, North Carolina, U.S.A. (Mackay et al., 2012). To control for maternal effects, maternal and paternal designation were kept constant. For example, flies of genotype A/B are the offspring of females of inbred line A crossed to males of inbred line B. In experiment 1, we used five focal male genotypes (208/712, 304/862, 306/391, 360/335 and 732/774) and one stimulus genotype (303/313) that also was used for the female mating partner genotype (These numbers (e.g. 208). are simply the 'names' of each genotype and do not have any other meaning.) In experiment 2, to test female remating latency, we used a second stimulus genotype (437/324) for the male mating partner genotype.

Rearing and Social Experience Manipulation

Flies were reared on approximately 10 ml of standard fly food under conditions that minimize variability and larval densities; each vial began with 10 males and 10 virgin females. They were maintained under a 12:12 h light:dark cycle with lights turning on at 0900 hours. Adults were collected as virgins using CO_2 anaesthesia within the first 3 h of lights on.

Experience treatments (males only)

After collection, focal males were aged for 5 days before the start of the mating trials. To manipulate male social experience, males were housed in three different social environments during these 5 days ('experience treatments'). Males were housed in standard food vials with a central subchamber filled with three virgin females, three virgin males, or left empty. A perforated 1000 μl pipette tip was used as the central subchamber to prevent physical contact between the stimulus males or females and focal males but still allow for the transmission of other informative cues, through for example sight and smell. (For a photograph of this set-up, see Appendix, Fig. A1.)

Female rearing

Virgin 303/313 females were aged in single-sex vials of 10–20 individuals to be offered as mating partners during the trial. Female age was kept constant across the three trial days: males were always offered 5-day posteclosion females each day.

Ethical Note

Experiment 1: Changes in Mating Behaviour and Offspring Production Across Bouts

To measure limits to male reproductive potential, we measured the number of females a male could mate with during a 4 h period across each of 3 days. We counted the progeny resulting from each successful mating.

Measuring how many times males could mate

Mating trials were conducted during the first 4 h after lights on. On day 1, individual males were removed from their experience vial and transferred (by gentle aspiration) to an unoccupied food vial. They were each provided one virgin female at the start of the 4 h mating period. Once the male and female had mated, the female was removed (by gentle aspiration) and another virgin female was added. This process continued for 4 h.

On day 2 and day 3, after resting in isolation overnight, the male was again offered a series of virgin females during a 4 h mating period. We discarded data from males that did not complete all three mating periods due to death or escape. To our knowledge, no males died due to mating-related injuries or exhaustion.

Copulation duration

To measure how long each mating event lasted, we recorded the start and end times of each mating.

Courtship effort

To estimate the courtship effort of each male for each mating, we conducted visual presence/absence scans every 1 min. We calculated 'courtship effort' as the proportion of scans during which males were seen courting before the start of copulation.

Offspring production

Each mated female was transferred to an unoccupied food vial and allowed to lay eggs for 1 week. Emerging adults were counted 1 week and 2 weeks later. Offspring from females that died or were lost before the end of the first week were not counted.

Replication

We repeated each focal male genotype–experience treatment combination five times for a total of 75 trials. In total, we observed 75 males mate with 1116 females to produce 39 768 offspring.

Experiment 2: Female Remating Latency

Through mating, males influence diverse traits in females, not just offspring production (Wolfner, 1997). One of the most important of these traits, from the perspective of sexual selection and mating systems, is female remating behaviour. Thus, we tested how variation among males—in experience treatment, mate number so far that day, and genotype—influenced the remating behaviour of their female mates.

Manipulating male characteristics

We repeated the first day of the mating trials from experiment 1. Males differed in genotype and experience treatment as described above. Females who mated with the same male may nevertheless have different mating experiences, if something about males (e.g. male quality) changes across mating bouts. Therefore, we recorded whether each female was the first, second, third, etc. to mate with a

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Measuring remating

After isolating the mated females overnight, we provided them a virgin male partner of genotype 437/324 (by gentle aspiration) for a 1 h mating period. We recorded whether the female remated with this 'tester' male. After 1 h, any unmated males were removed from the vials and isolated in an unoccupied food vial. If no mating occurred, the male and female remained isolated overnight and then were reunited for another hour the next morning. (While it is possible that, in some cases, remating failed because of some problem with the male, these hypothetical rare 'duds' would be random with respect to characteristics of the female's first mate). One-hour mating periods were repeated each day across a 4-day span.

Replication

We repeated each focal male genotype–experience treatment combination two times for a total of 30 trials.

Statistical Analysis

Overall approach

To understand variation in male and female mating behaviour and the resulting offspring production, we took a mixed model approach. If residuals from initial models were normally distributed, as indicated by nonsignificant Shapiro–Wilk tests, we used a Gaussian error distribution in a linear mixed model (LMM) framework. If residuals from initial models were not normally distributed, we used alternative distributions in a generalized linear mixed model (GLMM) framework as described below.

Fixed effect predictors included the day of the experiment, the focal male's experience treatment (i.e. solo, male-experienced, female-experienced), and additional predictors described below. To determine which interaction terms to include, if any, we used Akaike's information criterion (AIC), where the model including the lowest AIC was chosen (Burnham & Anderson, 2004). In all cases, the model that was the best fit to the data, considering the number of parameters, was unambiguous (i.e. delta AIC>2).

Except where indicated, we included random effects of genotype and male identity (ID). The latter accounted for nonindependence of measurements of the same male, as well as any overdispersion present in the model (Elston, Moss, Boulinier, Arrowsmith, & Lambin, 2001). Models were implemented in the 'lme4' package (for LMMs; Bates, Mächler, Bolker, & Walker, 2015) and the 'glmmADMB' package (for one zero-inflated Poisson model; Fournier et al., 2012). For the best-fit models as indicated by AIC, we tested the significance of fixed effects using type III tests of deviance (similar to *F* tests) implemented in the 'car' package (Fox & Weisberg, 2011). To extract least-squares means, we used the package 'lsmeans' (Lenth, 2016). We tested the significance of random effects using likelihood ratio tests, implemented with the 'ranova' function in 'lmerTest' (Kuznetsova, Brockhoff, & Christensen, 2017), or, for 'glmmADMB models', using the 'anova' function in base R.

Details and R code for this analysis can be found in the Supplementary Material.

Model details

How many times did males mate (and why)? To identify factors influencing variation in the number of times males mated (mate number), we fitted linear mixed models where the response variable was the total number of times each male mated each day. Residuals from initial models were normally distributed as indi- interaction between these factors did not improve model fit (delta AIC between main-effects-only model and interaction model = 6.1).

We next tested whether changes in mating rate were mirrored by changes in courtship effort. Males were observed courting in about one-fourth of our scan samples (mean = 0.25, range 0–1). To model this variation, we square-root transformed courtship effort to ensure normality of residuals, as indicated by a nonsignificant Shapiro–Wilk test. For this analysis, we excluded one observation in which the male courted in more than 100% of samples, presumed to be an error. We tested whether variation in courtship effort was associated with variation in the males' experience treatment, the day of the experiment and female rank. 'Female rank' refers to the order in which females mated, with the first female to mate with a particular male on a particular day having a rank of 1, the second female to mate with that male that day having a rank of 2, and so on. A significant effect of this parameter would indicate that male courtship effort changes across mating bouts within a given day. AIC analysis indicated that the best-fit model included only main effects, and no interaction terms (delta AIC between best and next-best models = 5.7). We included random effects of male ID and genotype.

How does mate number translate to offspring production? To identify how the number of mates acquired and experience treatment influenced offspring production, we started by considering the relationship between mate number and offspring sired across the entire 3-day experiment. We modelled the relationship between the total number of offspring sired (summed across the 3 days of the experiment) and the males' experience treatment and total number of mates (summed across the 3 days of the experiment). We also included a quadratic function of mate number. These terms tested the hypotheses that experience treatment influenced the relative benefit of remating, and that the relationship between mate number and offspring sired may be nonlinear, respectively. In this case, AIC analysis indicated that the best-fit model included an interaction between the linear effect of mate number and experience treatment (delta AIC between best and next-best models = 7.8). Residuals from initial models were normally distributed as indicated by a nonsignificant Shapiro–Wilk test, so a Gaussian error distribution was used. We included random effects of genotype; for this model, no random effect of male ID was needed, because we had only one measurement of 'total offspring sired across all 3 days' for each male.

To better understand how each mating event contributed to males' total offspring sired, we considered what factors might explain variation in the number of offspring produced by each female. As a reminder, females were identical in age, experience treatment and genotype; therefore, the only possible influences over reproductive output for females were the characteristics of their male mate, random differences among females and measurement error/noise.

Initial models showed deviation from a Gaussian error distribution as indicated by graphical analysis and significant Shapiro–Wilk tests. Furthermore, the number of offspring produced by each female was in the form of counts, and appeared to be zero-inflated. Therefore, we modelled variation in this response variable using generalized linear mixed models with a zero-inflated Poisson distribution. We tested whether variation in the number of offspring a single female produced from a single mating was influenced by the day of the experiment (for her male mate), her mates' experience treatment and her 'rank' (i.e. whether she was the first, second, third, etc., female to mate with the male on that day; see above). For this model, AIC analysis indicated that the best-

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Therefore, all possible two- and three-way interactions were included.

How does male experience influence female remating behaviour? In experiment 2, we measured how quickly females were willing to remate with a new male, and whether this behaviour depended on characteristics of her first mate. For each female we had measures of how many days she waited to remate; if she did not remate by the end of the fourth day, she was assigned a score of 5.

We modelled the relationship between how many days each female waited to remate and the characteristics of her first male mate. We included female rank and male experience treatment as fixed effects, and male ID and male genotype as random effects. Initial models showed normality of residuals as indicated by nonsignificant Shapiro–Wilk tests, and AIC analysis revealed that including an interaction between female rank and experience treatment did not improve the fit of the model (delta AIC between main-effects-only model and interaction model: 7.9).

RESULTS

Experiment 1

Male mate number declines across days and varies among genotypes

Males mated an average of 4.96 times per day (range 1–11). We found no support for an effect of experience treatment on mate number (χ^2_2=4.6, P =0.10). In contrast, variation in male mate number was significantly influenced by the day of the experiment (parameter estimate = -0.74, χ^2_1=54.5, P <0.0001). The negative parameter estimate indicates that male mate number declined across days. Furthermore, we found genetic variation in mate number (likelihood ratio = 8.9, df = 1, P=0.0028).

Courtship effort declines over time but is not fully concordant with mate number

We next tested whether courtship effort declined in concert with male mate number. We found that courtship effort varied across days (parameter estimate = -0.054, χ^2_1=37.2, P <0.0001), across subsequent matings within a day (female 'rank'; parameter estimate = -0.061, χ^2_1=250.7, P <0.0001) and among males with different experience treatments (χ^2_2=8.22, P = 0.016). The negative parameter estimates for both day and female rank indicated that male courtship effort declined over time, similar to mate number. Unlike mate number, we found differences between males from different experience treatments; examination of least-squares means indicated that female-experienced males courted the most (least-squares mean, detransformed to original scale of the data: 0.44) and solo males courted the least (least-squares mean: 0.31) with male-experienced males showing intermediate levels of courtship effort (least-squares mean: 0.40).

Also in contrast to the results for mate number, we found no support for genetic variation in male courtship effort (likelihood ratio = 0.24, df = 1, P = 0.6).

Males who mate with the most females do not necessarily sire the most offspring

Across the 3 days, males sired 530 offspring on average (range 114–871). This variation was influenced by male mate number but not by male experience treatment (χ^2_2=0.50, P = 0.78). Specifically, we found both linear and quadratic effects of male mate number on male offspring sired (linear: parameter estimate = 56.6, χ^2_1 = 17.9, P expected. The negative parameter value for the quadratic effect indicates that the benefits of remating, in terms of number of offspring sired, diminished across subsequent remating bouts. Another way of saying this is that males who mated with the most females did not necessarily sire the most offspring. We also found evidence for genetic variation in offspring sired (likelihood ratio = 11.6, df = 1, P = 0.0007; Fig. 2).

Complex interplay among male mate characteristics influence female fecundity

Each female produced, on average, 35.6 offspring from their single mating (range 0–126). Surprisingly, 192 females out of 1116 tested produced no offspring, even though we directly observed them mating. This phenomenon explained the need for the zero-inflated model.

One possible explanation for the absence of offspring is that matings were not long enough for successful sperm transfer. In *D. melanogaster*, sperm transfer takes about 8 min (Gilchrist & Partridge, 2000). Of the 1116 matings we observed, only two matings were shorter than 8 min; both of these matings lasted 7 min. One of these 7 min matings indeed yielded no offspring, but the other yielded 48 offspring. Moreover, mating duration was slightly negatively correlated with fecundity (Kendall's rank correlation: τ = -0.1, P<0.0001), indicating that matings that took longer typically produced slightly fewer progeny than shorter matings in our sample. These findings suggest that matings in our experiment were of sufficient duration to transfer sperm, but some of these matings still failed to produce (many) progeny.

Our next step was to consider how other dynamic male characteristics influenced female offspring production, as described above. In the best-fit model, we found a significant three-way interaction between the day of the experiment (for the male), the male's experience treatment and female rank (χ^2_2 = 33.9, P < 0.0001) as well as two-way interactions between all three fixed effects (day*experience treatment: χ^2_2 = 12.6, P = 0.002; day*-female rank: χ^2_1 = 44.7, P < 0.0001; experience treatment*female rank: χ^2_1 = 59.6, P < 0.0001). We also found significant main effects of each fixed effect (day: χ^2_1 = 9.0, P < 0.0001; experience treatment: χ^2_2 =19.9, P < 0.0001; female rank: χ^2_1= 73.1, P < 0.0001). We also found a significant effect of the male's genotype on his mate's offspring production (likelihood ratio = 6.52, df = 1, P = 0.011).

Graphical analysis (Fig. 1a,b) indicated that the three-way interaction between day, experience treatment and female rank arose because the shape of the relationship between female rank and female offspring production differed across experience treatments, but those differences between experience treatments diminished over days.

Experiment 2

Females later in the mating order remate rapidly

In experiment 2, we found substantial variation in how rapidly females remated. The analysis showed that this variation was influenced by the female's rank on the day of her first mating (parameter estimate = -0.55, χ^2_1 = 161.3, P < 0.0001). The negative parameter estimate indicates that the first females to mate with a male on a given day—i.e. females who were first or second in 'rank'—were slower to remate than females whose first mate had already mated with many other females that day (Fig. 3)

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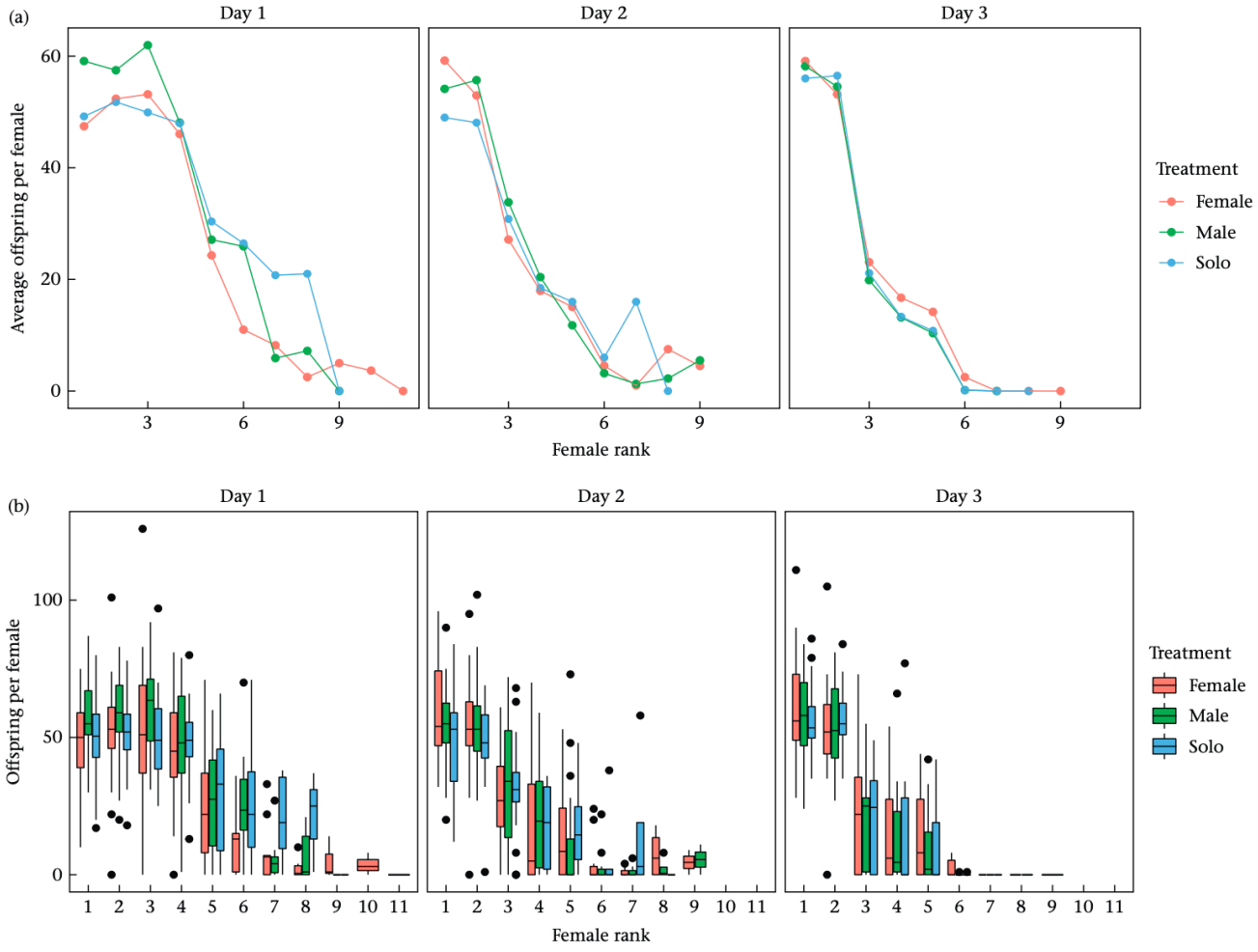

Figure 1. (a) Curves illustrating the relationship between the average number of offspring produced by one female (*Y* axis; dots with connected lines) based on characteristics of her mate. The *X* axis represents female 'rank' (i.e. whether she was the first, second, third, etc., female to mate with the male on that day). Colours represent the experience treatments of the male mates, and each panel depicts data for 1 day of the experiment. (b) The same data as in (a), but plotted as a box plot to display variation among individual females. As in (a), the *Y* axis represents the number of offspring produced by each female, the *X* axis is female rank, and the colours represent the experience treatments of the males. Panels represent the 3 days of the experiment. Central lines represent median values.

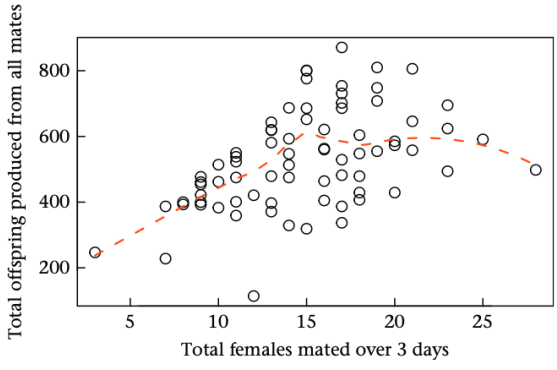

Figure 2. Each dot represents a single male. The *X* axis is the total number of females the male mated across the 3-day experiment, and the *Y* axis is the total offspring that the male's mates produced. Red dotted line is a polynomial regression with loess

DISCUSSION

In species in which individuals can reproduce more than once, lifetime reproductive fitness emerges from the accumulation of reproductive success across potential mating bouts. Dynamic changes in reproductive potential across bouts are important to life-history theory and inherent to ideas about reproductive trade-offs; but changes in mating behaviour and reproductive output across mating bouts have been surprisingly understudied in males. Here, we examined the dynamics of male reproductive potential across successive matings and across time, and in response to different information during early life. We find that males are capable of many more matings than are generally examined. While male total reproduction does tend to increase with mate number, this relationship can be dramatically reshaped by the social environment during early life, by mating experience, and over time. Specifically, our most important results are that (1) male fertility

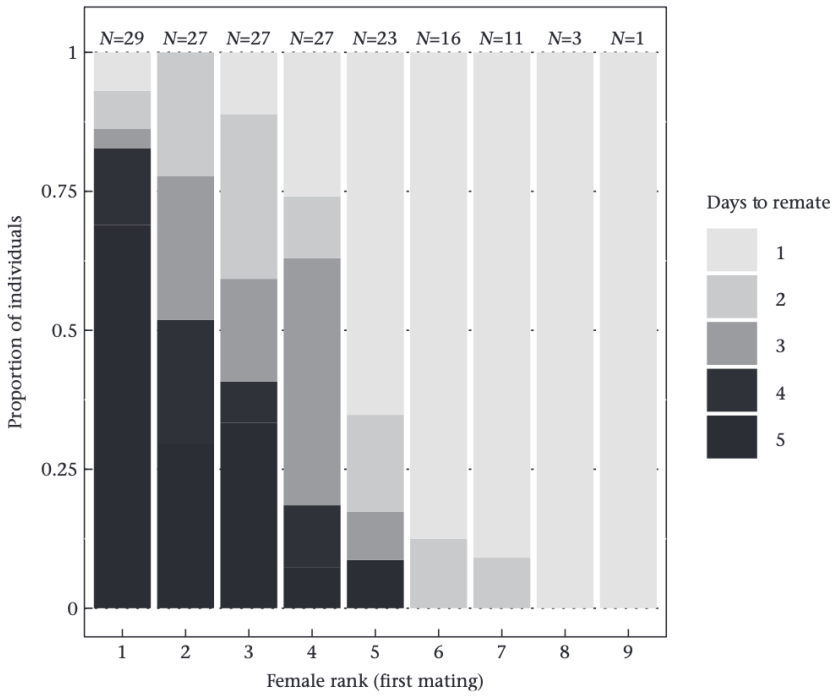

Figure 3. Data from experiment 2. The *X* axis is female rank and the *Y* axis is the proportion of individuals who mated after *N* days, with *N* ranging from 1 to 5+ as indicated by greyscale. Sample sizes are displayed at top and vary across female rank because males differed in how many times they mated on the first day. Females who did not remate for all 4 days of the experiment were given a score of 5.

experiences during early life; and (4) females who received low-quality partners, i.e. males who already mated several times that day, were able to quickly compensate by remating. Together, these findings suggest that males who are particularly successful in attracting mates at one time step (e.g. one morning) will have relatively low reproductive potential during the next few bouts or even days. These profound changes over time in the relative and absolute fitness benefits of mating further suggest that previous studies examining only one to two mating bouts may not provide a full picture of sexual selection.

Our approach, by design, is expected to represent a 'lower bound' on the limits to reproductive potential that males would face in nature. Our data show that male fertility declines sharply after two to five matings, depending on the male's genotype, experience treatment and the day of the experiment (i.e. recent mating experience). The best estimates of mating rates in nature suggest that the opportunity to mate two to five times per day is possible or even likely, particularly for attractive males, although further research is needed on sexual selection dynamics in wild populations. Furthermore, in nature, males may face reproductive limits from other sources, such as nutrient stress. Therefore, the fact that male fertility declines precipitously after two to five matings under ideal conditions suggests that limits to male reproductive potential may be even more severe in the wild.

As expected, we found an overall positive relationship between total mate number and total offspring produced, highlighting that males who mate with more females usually sire more offspring, compared to males who mate with fewer females. At the same time, we found that male reproductive success was not a simple linear function of the number of acquired mates; instead, we found a pattern of diminishing returns, in which mating more did not support for our prediction that male-experienced males would invest more in early matings, compared to males from other experience treatments (Fig. 1b, 'Day 1' panel). The early reproductive output enjoyed by male-experienced males was counteracted by declines in reproductive output later, resulting in no overall difference among experience treatments in the total number of offspring sired. Similarly, female-experienced males showed more courtship behaviour than males from other experience treatments, which supports our prediction that female-experienced males should strategically invest in mating with as many females as possible. However, this adjustment in behaviour did not have the expected effects on number of offspring sired, which was similar for all experience treatments.

These data broadly support our expectation that social cues during early life should modify how males allocate their limited reproductive investment, but highlight ongoing deficiencies in our understanding of the relationships between physiological limits to male reproduction, plasticity in behaviour and the resulting patterns of offspring sired over time.

As males gained more experience over days, effects of the experience treatments diminished, and for all males, courtship effort declined across successive matings and days. These patterns suggest that males adjust their investment in reproduction dynamically based on both early life experiences (i.e. experience treatment) and experience during recent mating bouts.

Our experiment did not evaluate the functional mechanisms underlying limits to male reproductive potential, but previous work can provide some hypotheses. Three previous studies have suggested that, after five matings, *D. melanogaster* males run out of ejaculate (Linklater, Wertheim, Wigby, & Chapman, 2007; Loyau, Blanchet, Van Laere, Clobert, & Danchin, 2012; Sirot, Buehner,

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that we observed was due to ejaculate depletion is also consistent with our results from experiment 2, where we found that females remated quickly if their first mate had already mated several times that day (Fig. 3). For example, in experiment 2, 96% of the females that were fifth or later in the mating order remated within 4 days, and over 80% of those females remated on the first day. Based on the results of experiment 1, these females would be expected to produce few offspring or even no offspring. By contrast, only 30% of the females that were first in the mating order—i.e. females expected to produce abundant offspring—remated after 4 days (Fig. 3). If males are semen limited, then females late in the mating order would be expected to receive low levels of accessory gland proteins during mating. These proteins include those, such as sex peptide, that inhibit remating (Aigaki, Fleischmann, Chen, & Kubli, 1991; Avila, Ram, Qazi, & Wolfner, 2010; Ram & Wolfner, 2009; Wigby & Chapman, 2005). Thus, the presence or abundance of sex peptide may serve as a cue to females about the quality of a particular mating bout. This plasticity in female remating behaviour is consistent with findings from other multiply mating species, such as the tephritid fly *Anastrepha obliqua*, in which female likelihood to remate increases with mating order for successively mated males (Perez-Staples, Aluja, Macías-Ordóñez, & Sivinski, 2008). Similarly, in species where mating failures are common, postcopulatory selection to remate is thought to be stronger than precopulatory selection to avoid 'failed' matings (Greenway, Balfour, & Shuker, 2017; Tyler & Tregenza, 2013).

Our results suggest the opportunity for a positive feedback between male and female mating rates: the more males mate, the more they should become low-quality partners, inducing females to rapidly remate to compensate. This potential for feedbacks may explain the dramatic variation in mating rates in this species across different experiments (Billeter et al., 2012; Krupp et al., 2008). One mechanism that may break this feedback is the reduced courtship rates we observed in males who had mated several times already. This reduced courtship rate may allow females to discern among males with different recent mating experiences. Complicating this picture, the deleterious effects of remating for females may be mitigated if they mate with experienced, rather than virgin, males (Castrezana, Faircloth, Bridges, & Gowaty, 2017). Further investigations of the dynamics of mating costs and benefits across the life span, and how these vary with partner experience and other characteristics, is needed to better understand the evolution of mating systems.

Costs and limits to male mating affect attractive males, i.e. those that are able to gain mates at all. In flies and many other species, the short-term mating opportunities available to an attractive male are expected to be amplified by mate choice copying (Mery et al., 2009). Among these males, the reproductive limits we identified would be expected to place an upper limit on the reproductive fitness of any one male. Disproportionate costs of reproduction paid by the most attractive or highest-quality individuals have also been found in females (Long, Pischedda, Stewart, & Rice, 2009) and provide a mechanism that reduces fitness differences among individuals. Further investigating limits to male reproduction in different environments and mating systems, and for different types of males, may help explain the persistence of intrapopulation genetic and phenotypic variation among males despite seemingly strong selection (Gillespie, 2004; Hall, Lailvaux, Blows, & Brooks, 2010; Radwan, 2008).

Overall, our findings highlight deficiencies that still remain in our understanding of sexual selection on males. We found unexpected, dramatic limits to male reproductive fitness across mating female partner, depended heavily on how many prior partners the male acquired. And, these limits produced complex patterns of relationships between mating success and offspring production, calling into question the common assumption that males that attract more mates sire more offspring. Our findings suggest trade-offs between current and future reproduction for males, and indicate that a complete picture of sexual selection in males will require an understanding of how each aspect of mating and reproduction may change with time and experience.

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Supplementary Material

Supplementary associated with this article is available, in the online version, at https://doi.org/10.1016/j.anbehav.2019.11.009.

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Appendix

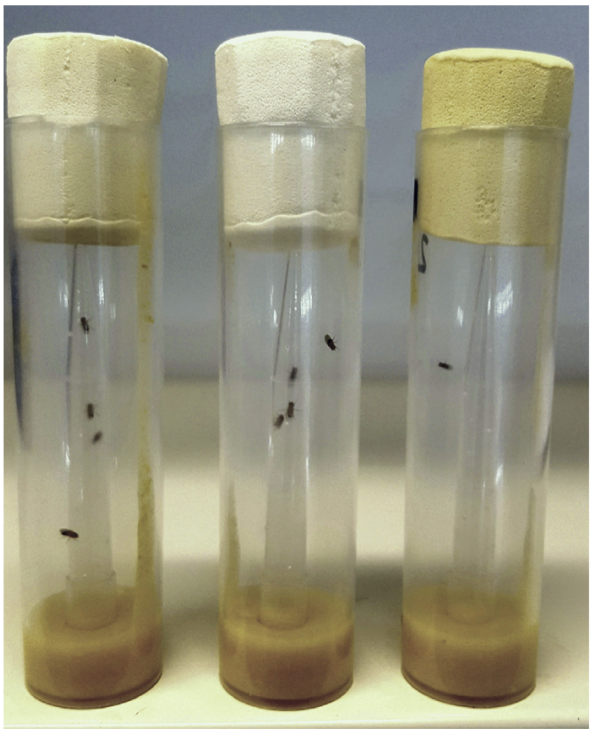

Figure A1. Image shows focal males in rearing environments with conspecific stimulus flies in central subchambers. On the left, three male stimulus flies, in the middle subchamber, three female flies, and on the right, an empty subchamber.

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