The second sex

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4

LAST MALE SPERM PRECEDENCE IS MODULATED BY FEMALE MATING RATE IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

Within the reproductive tract of a multiply-mated female, sperm from different males compete for a chance at fertilization. In many species, a common outcome of this interaction is last male sperm precedence (LMSP): a strong bias in paternity in favour of the last mate. Most of what we know about LMSP comes from studies utilizing the fruit fly, *Drosophila melanogaster,* employing a paradigm where a female mates with two different males about 3 days apart. However, *D. melanogaster* females naturally mate with more than two males and at shorter intervals, which might lead to different patterns of paternity than predicted from this two-male paradigm. We therefore investigated the degree of LMSP when females mated with 2 or 3 males and in quick succession. We first generated transgenic males that produce green, red or blue fluorescently labeled sperm. We then mated females to 2 or 3 of these males, sequentially and at varying intervals, and assessed paternity of the resulting male progeny based on the colour of the son's fluorescent sperm. We found a breakdown in LMSP in thrice-mated females compared to twice-mated, but only when females mated in quick succession. This indicates that the strength of LMSP is influenced not only by the number of female copulations but also the intervals between those copulations. Therefore, female mating rate, a combination of the number of partners and intervals between copulations, is an important factor in the modulation of LMSP, demonstrating that females are active determinants of post-copulatory sexual selection.

INTRODUCTION

Polyandry, a common feature of many mating systems, has considerable consequences for sexual selection. When females mate with multiple males, the sperm are held simultaneously within her reproductive tract (Manier et al., 2010). Therefore, sperm of various origins interact with each other as well as with the female bio-chemical environment and compete for fertilization. The outcome of these interactions can be observed in the patterns of offspring paternity. In the majority of insect species (Chaudhary et al., 2016; Kehl et al., 2013; Kock and Sauer, 2007; Parker, 1970; Scolari et al., 2014) as well as a number of vertebrate species (Birkhead and Møller, 1998; Santiago Moreno et al., 2014), offspring tend to be sired by the females' most previous mate, a phenomenon known as last male sperm precedence (LMSP; Clark et al., 1995). In species where females store sperm, this pattern of paternity is achieved via a process of sperm displacement, in which the sperm from the last male enters the sperm storage organs (SSOs) within the female reproductive tract and takes the place of the resident sperm (Manier et al., 2010), profoundly affecting the genetic composition of the offspring produced. As such, knowledge of the mechanisms underlying LMSP is crucial to understand the process of post-copulatory sexual selection.

Due to the inherent complication of observing events concealed inside the female reproductive tract, the mechanisms underlying sperm competition remain poorly understood. In response to the obstacles, researchers have made use of the model organism *Drosophila melanogaster*. Most investigations have traditionally employed a two-male paradigm that involves mating a virgin female, and then remating her to a phenotypically distinct male between 1 and 5 days later (see Appendix for a survey of variation in this assay in the literature). The progeny produced by this female are then genotyped to determine patterns of paternity and is expressed as either the proportion of offspring sired by the first male (P1) or the second (P2; Boorman and Parker, 1976; Manier et al., 2010). Although many aspects of this protocol vary between experiments, such as the social context in which the mating takes place, number of days between matings, and distinguishing characteristics of the mates used to genotype offspring (see Appendix), typically studies report ~ 80% of the offspring were sired by the second male.

Most attempts to identify the mechanism(s) underlying this phenomenon have focused mainly on the male contribution to P1 and P2. A range of male-specific variables were found to influence LMSP, including experience-dependent factors

such as sperm depletion through multiple mating (Chaudhary et al., 2016; Kehl et al., 2013; Kock and Sauer, 2007; Lefevre and Jonsson, 1962; Parker, 1970; Scolari et al., 2014), diet (Birkhead and Møller, 1998; Clark et al., 2012; Santiago Moreno et al., 2014), and the presence of rival males (Bretman et al., 2009; Clark et al., 1995); as well as genetic factors including natural genetic variation (Clark et al., 1995; Fiumera et al., 2007; Hughes, 1997; Lüpold et al., 2012; Manier et al., 2010) and specific genetic mutations (Prout and Bundgaard, 1977). LMSP also correlates with specific aspects of ejaculate composition such as sperm size (Manier et al., 2010), length and velocity (Lüpold et al., 2012), as well as specific molecular components of the seminal fluid transferred with the sperm (Begun et al., 2000; Chow et al., 2010; Civetta and Finn, 2014; Clark et al., 1995; Fiumera et al., 2007; Gilbert and Richmond, 1981; Gilchrist and Partridge, 2000; Harshman and Prout, 1994; Kalb et al., 1993; Price et al., 1999; Reinhart et al., 2015).

The female contribution to LMSP, on the other hand, has received comparatively little attention. The outcomes of sperm competition have for a long time been viewed as a matter resolved among males. However, over the last 20 years, the perspective on females' involvement in post-copulatory sexual selection has transformed (Gowaty, 1994), and new theories place females in roles such as umpires or architects who shape the rules to which the contest between sperm is played and therefore the criterion of the victors (Gowaty, 2012). This oversight of viewing females as the passive arena to sperm competition has been rectified with investigations into the female factors that influence patterns of paternity. Indeed, the female genome (Chow et al., 2010; 2012; Civetta and Clark, 2000; Clark and Begun, 1998; Clark et al., 1999; Reinhart et al., 2015; Wilson et al., 1997), reproductive tract morphology (Bangham et al., 2003; reviewed by Pitnick et al., 2009), age (Mack et al., 2003) and developmental condition (Amitin and Pitnick, 2007) have all been found to contribute to the outcome of sperm competition. Moreover, several studies have also highlighted involvement of the female central nervous system (Adams and Wolfner, 2007; Avila and Wolfner, 2009; Chow et al., 2012; Schnakenberg et al., 2011) suggesting that females can actively modulate LMSP. One instrument of modulation may be through female sexual receptivity, as mating rate and sperm competition are closely linked.

Polyandry is of course a prerequisite for sperm competition and applies selective pressure on the ejaculates to maximize male reproductive success in a rival-rich context (Moatt et al., 2014). In other arthropods, aspects of female remating behaviour such as remating latency and number of sexual partners modulate LMSP (Drnevich, 2003; Zeh and Zeh, 1994). However, the role of variation in number of mates on patterns of paternity in *Drosophila* remains unclear. Most investigations of

the relationship in this species have focused on either propensity to re-mate (yes or no) or interval length (24, 48, or 72 hrs) and failed to find a relationship (Chow et al., 2012; Giardina et al., 2011; Lüpold et al., 2013; Miller and Pitnick, 2003), meaning groups of females that were reluctant to re-mate or did so at longer intervals produced similar P1 and P2 values. Interestingly, when flies were observed in groups for 8 hours, which allowed for continuous interaction between females and males in between copulations, researchers found that females mated multiple times and produced very different patterns of paternity. Specifically, they found that females who mated with at least three males produced much less offspring sired by the last male than expected as LMSP was diluted from 80% to 40% (Billeter et al., 2012). This provides evidence that female mating rate may contribute to paternity patterns. Although little is known about the mean remating latency of *Drosophila* in nature, it is clear that females re-mate often as they typically hold the sperm of 4-5 males on average (Harshman and Clark, 1998; Imhof et al., 1998; Milkmann and Zeitler, 1974; Morrow et al., 2005; Ochando et al., 1996). Taken together, these results posit a need to understand whether LMSP scenario from mating with two males scales to scenarios when females mate more than twice.

The aim of this study was to investigate the effect of female mating rate on LMSP. We tested the effect of mating a female either twice or thrice in either 12 or 24 hours on pattern of paternity. We found that when females mate with three males within a 12-hour period, the proportion of offspring sired by the last mate was significantly reduced compared to when the female mated twice. We determined that number of copulations and the time interval between the last and second last mating significantly predicted the outcome of sperm competition with regard to LSP suggesting that female mating rate is a determinant of LMSP.

MATERIAL AND METHODS

Fluorescent marker donor vectors

*Nde*I restriction sites were introduced at the 5'- and 3'-ends of fluorescent markers eGFP, mTurquoise and mCherry by amplification with primers *Nde*GFP ('5-tgcatatgcaaggtgagcaagggcgaggagctgttcacc-3') and pKC26fB2,0.Fp-rev (5'-gcccatatgttacttgtacagctcgtccatgc-3') for eGFP and mTurquoise and primers *Nde*mCherry (5'-tgcatatgcaaggtgagcaagggcgcggaggataacat-3') and pKC26fB2,0.Fp-rev for mCherry. Fragments were ligated into pJET1.2 vector (Thermo Scientific)

by blunt-end ligation and cloned XL1-blue colonies were screened by restriction analyses.

Protamine B::fluorescent marker CDS

In order to create a CDS for Protamine B fusion protein with integrated eGFP, mTurquoise of mCherry, fluorescent markers were excised from the subcloned pJET1.2 plasmids using *Nde*I and ligated into the pBS/ProtB4.2KP*Nde*I (Manier et al., 2010) vector. Fragments are integrated in frame with the CDS on position 45 of exon 3 of the ProtB ORF via an introduced *Nde*I restriction site. Orientation of the fluorescent fragments (3' to 5') in the pBS/ProtB4.2KP-NdeI vector were determined by restriction analysis using *Dpn*I.

Fluorescent protamine B cloning and expression vector

To create vectors for the cloning of *Drosophila* to express protamine B::fluorescent marker fusion proteins, 4.8Kb *Xba*I fragments of the different pBS/ProtB4.2KP constructs containing the introduced florescent markers were subcloned into the *Xba*I side of plasmid pUAST-attB13. The vector used in this study contains an inactivated 5x UAS-hsp70 site, obtained after digestion with *Pst*I and religation of the 8.2Kb fragment. Orientation of the inserted protamineB::fluorescent marker CDS was determined by restriction analysis with BamHI. Sequence analysis of the reading frame of fluorescent markers and conformation of the direction of the protamineB::fluorescent marker CDS in pUAST-attB-ProtB::eGFP and pUAST-attB-ProtB::mTurquoise was performed using the primers Seq-ProtB-for (5'-ggacctgtcactaacaac-3'), Seq-GFCITU-rev (5'-gatgttgtggcggatctt-3') and SV40poly(A).1-rev (5'caccacagaagtaaggttcct) and primers Seq-ProtB-for, Seq-ProtB-rev (5'-gcgctattccaacatccta-3') and SV40poly(A).1-rev for pUAST-attB-ProtB::mCherry.

Generation of transgenic Drosophila

To generate transgenic *Drosophila* with eGFP, mTurquoise or mCherry expressing sperm heads, the different pUAST-attB vectors with protamine B::fluorescent marker CDS were used to inject PhiC31-containing attP docking site embryos of *Drosophila melanogaster* with genotype y[1] M{vas-int.Dm}ZH-2A w[*]; M{3xP3-RFP.attP}ZH-102D (BDSC stock number 24488). Embryo injection and generation of stable transformants was performed at BestGene Inc., U.S.A.

Drosophila stocks

Flies were reared on medium containing agar (10g/L), glucose (167mM), sucrose (44mM), yeast (35g/L), cornmeal (15g/L), wheat germ (10g/L), soya flour (10 g/L), molasses (30 g/L), propionic acid and Tegosept. Flies were raised in a 12:12 hour light/dark cycle (LD 12:12) at 25°C. Virgins were collected using CO_2 anesthesia and were aged in same-sex groups of 20 in vials for 5-7 days prior to testing. All females used in this study were from the wild-type strain *Canton-S*. All males used in this study were transgenic carrying the Protamine B fusion protein with one of three fluorescent markers, described above. Males carrying eGFP, mCherry, or mTurquoise are referred to as green fluorescent protein (GFP), red fluorescent protein (RFP), or blue fluorescent protein (BFP), respectively.

Mating paradigm

The mating dish was a 10 x 8 mm Petri dish with a layer of medium on the bottom containing 3 times the amount of yeast compared to rearing medium. All flies were transferred to assay chambers using a mouth pipette. All experiments began at Zeitgeber Time 0 (ZT0, 9 am). A single female was first transferred to the mating dish, followed by 3 males with the same fluorescent marker: GFP, RFP, or BFP. All dishes were visually monitored and the time of copulation was recorded. Any female that failed to mate was discarded. To determine differences between lines, we generated once-mated females. Immediately following the end of mating with one of the three males, males were discarded and females were transferred with an aspirator to a vial containing rearing medium.

To compare patterns of offspring production and paternity of twice- and thrice-mated females, females were first paired with three virgin GFP males. Immediately following the end of mating, males were discarded and three new virgin RFP males were added to the same dish containing the recently mated female. Any female that failed to mate with either the first or second male were discarded. A proportion of the twice-mated females were transferred with an aspirator to a vial containing rearing medium and referred to as "twice-mated isolated"

In order to produce thrice-mated females, the remaining proportion of twice-mated females continued in the same dish and three new virgin BFP males were added. Immediately following the end of mating, females were transferred with an aspirator to a vial containing rearing medium and referred to as "thrice-mated isolated".

To extend the observation period, the remaining twice-mated females that were paired with the BFP males were left overnight (ZT12-ZT0). To monitor mating behaviour, photos of the assay dish were taken at 2-minute intervals using Logitech 910C webcams controlled by the Security Monitor Pro software (Deskshare Inc). The images were collected and scored for both the number and time of mating events. Females that did not mate but were exposed to BFP males are referred to as "twice-mated exposed" and females that mated are referred to as "thrice-mated exposed". Females that mated more than once during the night observation time were discarded.

Progeny production

After mating, females were placed into a fresh vial containing rearing medium. Females were transferred at least 3 times: 48 hours (day 2), 150 hours (day 6), and 216 hours (day 9) after the start of the experiment (ZT0 on day 0). All offspring were counted. Male offspring were placed into a small vial, flash frozen with liquid nitrogen, and placed in -20ºC freezer until they could be genotyped. Females that did not produce offspring from all observed copulations were removed from the main data set. However, these females were considered for an additional data set (see section "Selective paternity may be connected to cryptic female choice").

Male offspring genotyping

Males were genotyped using a MZ10F stereomicroscope equipped with filters for the different fluorescent proteins. The reproductive tracts from all male offspring were removed and testes were inspected for the expression of either GFP, RFP, or BFP.

Statistical analysis

The majority of the statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., USA) with the exception of the standard regression model, which was performed in SPSS. All data was first analysed with a Kolmogorov-Smirnov test (with Dallal-Wilkinson-Lillie for P value) for normality. All data were normally distributed unless specifically noted. A One-way ANOVA was used to analyse differences in progeny produced by single-mated females (mated to males expressing either GFP, RFP, or BFP on the heads of their sperm), differences in remating latency between first and second mating for all 4 groups of polyandrous females (twice-mated, twice-mated exposed, thrice-mated, and thrice-mated exposed), and the patterns of paternity over time in twice and thrice mated females. If test indicated significant differences among groups, Tukey's post hoc tests were performed to assess differences between specific pairs of groups. In the

comparison of P3 in of thrice-mated isolated females over three different times bins, data was not normally distributed. Kruskal-Wallis tests followed by Dunn's *post hoc* test was used to assess differences over time. A Two-way ANOVA was used to analyse differences in number of progeny produced by polyandrous females, and proportion of offspring sired by the last male in polyandrous females. All followed by Bonferroni post-hoc tests. A Pearson Correlation was used to determine the relationship between remating latency and sperm precedence in all 4 groups of polyandrous females. A Mann Whitney test was used to determine differences in remating latency between second and third mating in thrice-mated isolated females that failed to produce offspring from the second males and females from the same group that did as the data from these data sets were not normally distributed. Finally, we performed a standard multiple regression to determine if number of copulations, exposure of males after copulation, and last to second last remating latency significantly influenced last male sperm precedence. Preliminary analyses were conducted to ensure no violation of the assumptions were committed. All predictors had a moderate and significant correlation with the dependent variable; collinearity diagnostics was performed and both tolerance and Variance Inflation factor were in an acceptable range (greater than 0.1 and less than 10, respectively); and the distribution of the data was visually assessed with a scatterplot. No violations of the assumptions were found.

RESULTS

Fluorescently labeled sperm transgenic lines to study LMSP

The aim of this study was to investigate whether LMSP is influenced by female mating rate. Specifically, we investigated LMSP in relation to the number of mates (2 or 3) and the timing between female mating (remating latency). In order to investigate this, we generated transgenic flies that express one of three fluorescent proteins on the head of the sperm (Figure 1A). We transformed *D. melanogaster* to express Protamine B, a histone-like protein expressed specifically in the sperm head, labeled with one of 3 fluorescent proteins: green (GFP); the red fluorescing mCherry (RFP) or the blue fluorescing mTurquoise (BFP). Despite fluorescing at different wavelength, these proteins have minimal amino acid sequence differences (Hadjieconomou et al. 2010). These 3 transgenes were introduced into the same genetic background and the same genetic location using the PhiC31 integrase system (Bischof et al., 2007). One major benefit of using these males to assess patterns in reproduction, such as progeny production and paternity biasing, is that one can easily determine paternity while keeping the genetic background of the different males similar allowing to assess the direct contribution of the female. To

check equivalence of the three lines and to ensure that any unapparent differences between these lines did not influence the males' ability to sire offspring, we mated single males from each of the lines to single females and determined the number of offspring sired. As the transgenic lines did not significantly differ in fecundity (One-way ANOVA F (2, 48) = 1.77, p = 0.69; Figure 1B), we conclude that these three transgenic males are indeed equivalent. Using confocal microscopy, we were able to directly visualize sperm expressing the different fluorescent proteins both within the reproductive tract of males (Figure 1C), and wild-type females mated to one of the three transgenic males (Figure 1D). We have thus produced three transgenic lines that allow us to determine the influence of female mating rate on LMSP.

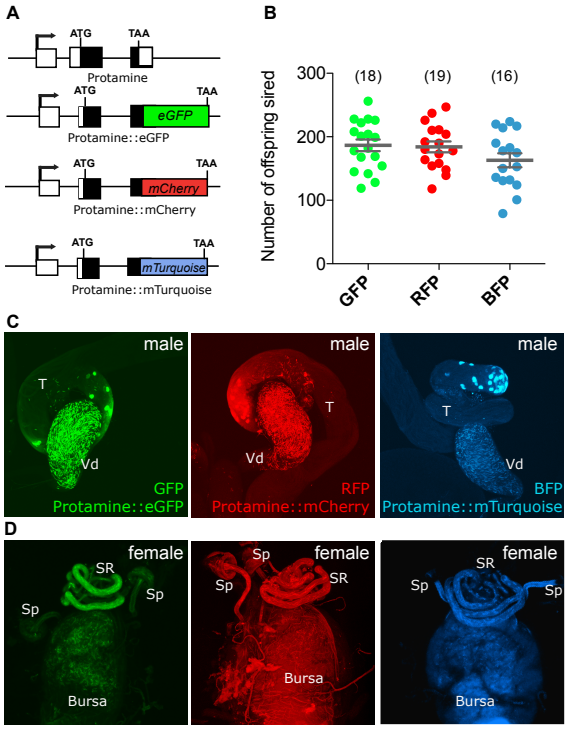

Figure 1. Transgenic males express fluorescent protein labeled sperm heads. (a) Representation of genetic constructs derived from the *ProtamineB* gene. Arrow indicates the start site of transcription; ATG and TAA the Open Reading frame; boxes indicate exons. **(b)** Mean number of offspring produced by once-mated females mated to one of the three transgenic males expressing fluorescent protein labeled sperm; green fluorescent protein (GFP; specifically eGFP), red fluorescent protein (RFP, specifically mCherry), blue fluorescent protein (BFP, specifically mTurquoise). Differences between groups were assessed with a One-way ANOVA (F (2, 48) = 1.767, p = 0.18). For full statistical analysis see Supplementary Table 1. Error bars indicate s.e.m. Number of replicates is between brackets. **(c)** Micrographs of male testes expressing fluorescent protein labeled sperm heads: GFP, RFP, and BFP, respectively. Location of the testes (T) and vas deferens (Vd), are indicated. Images are maximum projections of confocal stacks. **(d)** Micrograph of female sperm storage organs and anterior uterus of wild-type female mated to one of the three transgenic males: GFP, RFP, and BFP, respectively. Location of seminal receptacle (SR), paired spermathecae (Sp); and uterus/bursa (Bursa) are indicated.

LMSP breaks down in thrice mated females

To investigate the influence of number of female copulations on LMSP, we first mated females to GFP males, and immediately re-mated them to RFP males. Following this, females were either isolated or presented with BFP males for a third mating after which females were immediately isolated to lay eggs. This generated two groups of mated females: twice-mated isolated and thrice-mated isolated (Figure 2A). In addition to number of mates, we also investigated the influence of

time intervals between copulations (remating latency). We did this by extending the observation period of twice-mated females paired with three virgin BFP males for an additional 12 hours. This extension also allowed us to investigate the influence of exposure to males after the female has mated during the time of sperm storage. As females exchange resident sperm from within their sperm storage organs with newly acquired sperm in their uterus within the first few hours after remating (Manier et al., 2010), the presence of males in the environment may be perceived by females as a potential mate triggering cryptic female choice that may influence the fate of sperm from the first male and contribute to paternity patterns. Continuous monitoring allowed us to determine whether a third copulation occurred in these twice-mated females, which happened in 45% of the groups. Immediately following the observation period, females were isolated to lay eggs. This resulted in 2 additional groups of mated females: twice-mated exposed and thrice-mated exposed (Figure 2A). Offspring produced by these 4 groups of females (Figure 2A) were counted and sons were genotyped using the colour of their sperm (green, red of blue) to determine paternity (see Material and Methods). Since the sex ratio of the offspring was 1:1.05, we concluded that genotyping males acted as a good proxy for the general pattern of offspring paternity.

We genotyped over 9000 males and calculated the mean percentage of offspring sired by the first GFP male (P1) and second RFP male (P2), as well as the percentage of offspring sired by the third BFP male (P3) for thrice-mated females (Figure 2B). A Two-way ANOVA on LMSP, as determined by the percentage of offspring sired by the last male (*i.e.* P2 in twice-mated and P3 in thrice-mated females), with number of copulations and exposure to males as factors, revealed that the number of copulations (Two-way ANOVA, F (1, 92), $p = 0.005$) and exposure to males (Two-way ANOVA, F (1, 92), $p < 0.001$) both significantly affected LMSP.

Post hoc Bonferroni analyses comparing LMSP in all four groups revealed that thrice-mated isolated females produced significantly lower LMSP (P3) compared to thrice-mated exposed females (P3) as well as both groups of twice-mated females (P2; Figure 2B). In isolated thrice-mated females, LMSP fell to 46% of offspring sired compared to LMSP in the range of 70% in the other three groups. Paternity was also more mixed in the thrice-mated isolated females with the three males sharing paternity more equally (ie. 22%, 31%, 47% for P1, P2 and P3 respectively), than in exposed thrice-mated and both types of twice-mated females where the difference in paternity between last male and the other males is about 40% (Figure 2A). We conclude that the number of copulations performed by females can influence LMSP, leading to a dilution of LMSP in females with high mating rate.

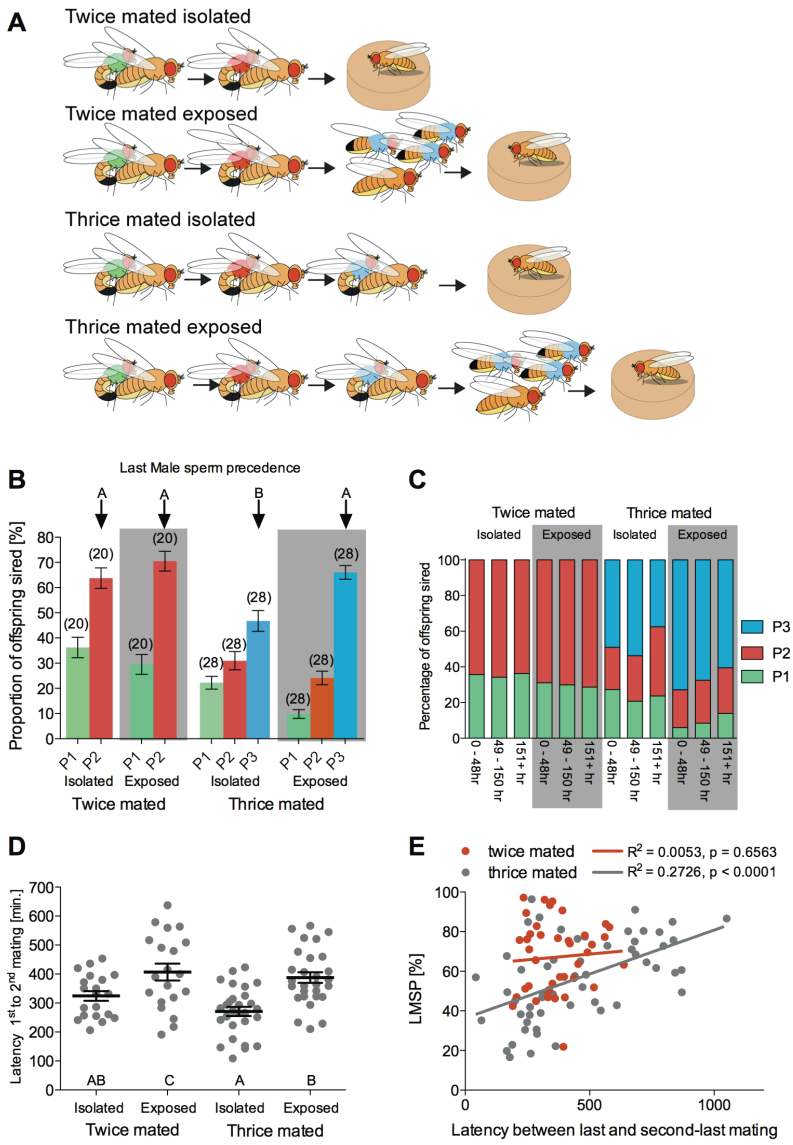

Figure 2. Female mating behaviour affects LMSP. **(a)** Schematic of the experimental procedure. Males with thorax of different colours indicate transgenic males with different coloured sperm. Arrows represent the removal of males after a successful copulation and their replacement with a new group of males. The fly on a brown disc indicates an isolated female on an egg laying patch. **(b** and **c)** Mean proportion of offspring sired by GFP males (P1 indicated with green bar), RFP males (P2 indicated with red bar), or BFP males (P3 indicated with blue bar) in thrice-mated females. Gray box indicates females that were exposed, post-mating, to BFP males. Differences between groups (b) were assessed with a Two-way ANOVA followed by Bonferroni post hoc test. Different letters indicate significant differences. Error bars indicate s.e.m. Number of replicates is between brackets Differences over time within groups (c) was assessed with four separate One-way ANOVAs or Kruskal-Wallis tests followed by Tukey's or Dunn's *post hoc* test respectively. **(d)** Mean remating latency between GFP and RFP male mating for four different groups of females. Error bars indicate s.e.m. Differences between groups were assessed with a One-way ANOVA followed by Tukey's post hoc test. Different letters represent groups that are significantly different from each other. **(e)** Relationship between last male sperm precedence (LMSP) and remating latency between last and second last mating (GFP and RFP males for twice-mated females represented in red; RFP and BFP for thrice-mated females represented in grey). Strength and statistical significance of the relationship was assessed with a Pearson Correlation. For full statistical analysis see Supplementary Table 1.

D. melanogaster females store sperm in two different SSOs: seminal receptacle stores sperm for immediate use; and the paired spermathecae store sperm for long-term storage. Interestingly, these SSOs also differ in the degree of sperm exchange after remating with much more exchange occurring in the seminal receptacle (Manier et al., 2010). It is of interest to determine if the differences we see in paternity are due to differences in storage location as it may contribute to our understanding of female influence over paternity biasing. To indirectly assess SSO-specific storage patterns, we explored how paternity ratios change over time in our 4 groups of females. We grouped offspring production into three different time periods in terms of hours after the start of mating (ASM): 0-48, 49-150, and 151+ (Figure 2C) and analyzed differences in LMSP within our groups of females over the three time intervals. Although there was no change in the proportion of offspring sired by the last male in thrice-mated exposed and both groups of twice-mated females (Supplementary Table 1), thrice-mated isolated females showed a significant change in P3 over the three different time periods ($KW = 6.70$, $p = 0.035$). Post hoc tests revealed that females produced significantly less offspring from the last male during the last time period (151+ hours ASM) compared to the second (49-150 hours ASM; Dunn's Multiple Comparison test, difference in rank sum = 16.39, $p < 0.05$). Since sperm used for fertilization at this last time point is most likely stored in the spermatheca, this patterns suggests that thrice-mated isolated females may experience less sperm exchange after mating with the third male specifically in this SSO. This predicts that thrice-mated isolated females may exclude the third male's sperm from the spermatheca, which may contribute to the overall decrease observed in P3.

Interpreting the significance of female exposure to males on LMSP is confounded by the fact that exposed females that mated a third time had longer remating latencies than isolated thrice-mated females (see Methods). Two factors may, therefore, influence LMSP: mating intervals and exposure to males. To disentangle these two factors, we first analyzed mating latency between the first (GFP) and second (RFP male) copulation for all four groups (Figure 2D). Both twice- and thrice-mated exposed females performed their second copulation roughly an hour later than twice- and thrice-mated isolated females (Figure 2D; Supplementary Table 1). Although it is clear that both groups of exposed females had a significantly longer mating interval between GFP and RFP males compared to their isolated equivalent, exposed twice-mated females did not show significantly different patterns of paternity compared to isolated twice-mated females (Figure 2B and C). Therefore, the differences we see in patterns of paternity between thrice-mated isolated and thrice-mated exposed females cannot be due to differences seen in GFP/RFP remating latency (Figure 2D). We then focused on the relationship

between LMSP and last to second last re-mating latency: for thrice-mated females this is the mating interval between RFP and BFP males; for twice-mated females this is the mating interval between GFP and RFP males. To investigate the relative influence of number of female copulations, exposure to males after copulation, and last to second last remating latency on LMSP, we performed a standard multiple regression with all three factors as explanatory variables. The total variance explained by the model as a whole was 24.1% (F (3, 92) = 9.742, $p < 0.001$). Only two of these three factors were statistically significant: number of copulations and remating latency. Number of copulations recorded a higher beta value (beta = -0.324, $p = 0.001$) than remating latency (beta = 0.297, $p = 0.015$). Exposure was not a significant predictor (beta = 0.165, $p = 0.164$). Therefore, the number of female copulations and females' latency between last to penultimate remating, and not exposure to males, are the main female factors that influence last male sperm precedence in our experiments.

Finally, we performed a Pearson Correlation to explore how the relationship between last to second last re-mating latency and LMSP changes depending on number of female copulations. We found that re-mating latency between last and second last mating was significantly correlated to the proportion of offspring sired by last male, but only in thrice-mated females (Figure 2E). This indicates that females that are faster to re-mate also tend to produce a smaller fraction of offspring from the last male (Figure 2E). This suggests when females mate with at least 3 males, the proportion of offspring sired by the last male can by modified depending on the timing of the third mating in relation to the second. We therefore conclude that the number and the time between copulations performed by a female, in other words her mating rate, significantly affect LMSP.

Mating rate does not correlate with fecundity

A higher female mating rate may result in higher fecundity due to higher amount of sperm and/or seminal fluids. If true, an increased mating rate would not only benefit the female by increasing the quantity and genetic diversity (due to having more sires) of offspring, but also might have little to no cost to males as the dilution in fraction of offspring sired would be compensated, or at least mitigated, by the overall greater number of offspring. In other words, males may indeed sire a smaller fraction of total offspring when mating with females with high mating rates but the absolute number of offspring would not differ, and thus female remating would bare little cost to the male. To test the influence of mating rate on offspring production, we performed a Two-way ANOVA on female fecundity (number of offspring produced) with number of copulations and exposure to males as factors.

This analysis revealed that the number of copulations did not significantly affect offspring number (Two-way ANOVA, F (1, 92), p = 0.61) but that exposure to males did (Two-way ANOVA, F (1, 92), p < 0.001; Figure 3A). Post hoc Bonferroni tests revealed that twice-mated females that were exposed had significantly less offspring than twice-mated isolated females (Figure 3A). Breaking down the number of offspring produced by the four groups of females in three different time intervals (0-48 hours, 49-150 hours, and 151+ hours ASM) consistently showed that females exposed to males after their final copulation event had less offspring than those females isolated immediately after copulation (Figure 3B).

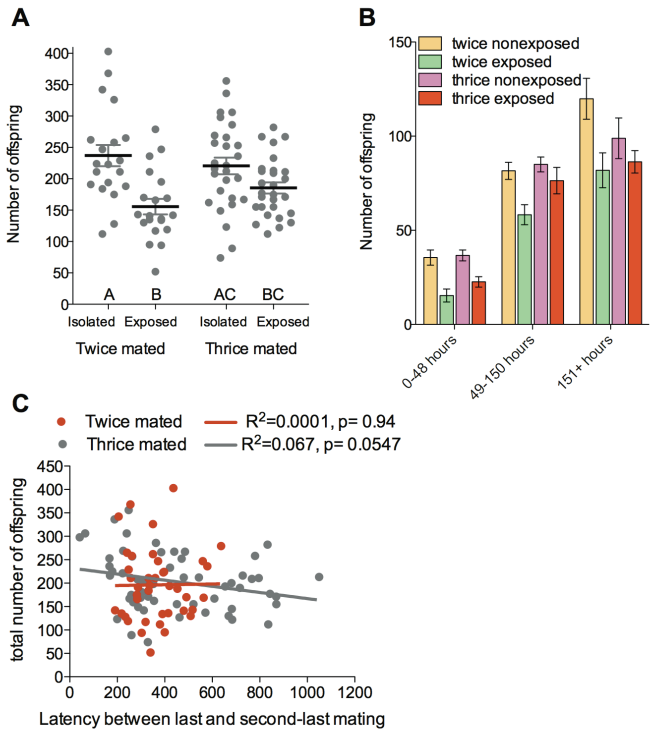

Figure 3. Number of offspring is not influenced by number of mates. (a) Mean number of offspring produced by four different groups of females. Differences between groups were assessed with a One-way ANOVA followed by Tukey's *post hoc* test. Different letters represent groups that are significantly different from each other. Error bars indicate s.e.m. For full statistical analysis see Supplementary Table 1. **(b)** Mean number of offspring sired by four different groups of females over three time periods as indicated from hours after the start of mating. Error bars indicate s.e.m. **(c)** Relationship between total number of offspring produced and remating latency between last and second last mating (GFP and RFP males for twice-mated females represented in red; RFP and BFP for thrice-mated females represented in grey). Strength statistical significance of the relationship was assessed with a Pearson Correlation. For full statistical analysis see Supplementary Table 1.

Given our conclusion that it is not exposure but latency between last and penultimate mating that affect LMSP, we plotted offspring production in twice and thrice mated females in correlation with the female mating latency (Figure 3C). No significant correlation between remating latency and offspring production was found for either twice or thrice- mated females (Figure 3C), suggesting again that mating rate does not significantly influence fecundity. Together, these findings suggest that males suffer a cost through reduced LMSP when mating with a female with a high mating rate that is not mitigated by higher female fecundity.

Selective paternity may be connected to cryptic female choice

We found that females that mated three times in quick succession, producing offspring from all three males, show a dilution of LMSP. However, females' ability to manipulate paternity may not be limited to the modulation of paternity ratios. We found a striking number of females that had foregone progeny production from specific individuals with whom they had mated. Although selective paternity production occurred in all four groups, an unusual percentage of thrice-mated isolated females failed to produce offspring from the second male (RFP; Figure 4A). One hypothesis is that the second male in the series failed to transfer an ejaculate to the female during copulation. However, since all males were virgin, and thus not sperm deprived, and RFP males were as fecund as GFP and BFP males with once-mated females (Figure 1B), a failure of ejaculate transfer is highly unlikely. Alternatively, females may have failed to successfully receive the ejaculate. We investigated this by comparing the remating latency between the second and third mating of thrice-mated isolated females who produced offspring from all males, and those that failed to produce RFP sons (second male offspring). If females failed to successfully receive a sufficient ejaculate, they should also have a quick remating time (Lefevre and Jonsson, 1962). Indeed, we found that females that did not produce offspring from the second male went on to re-mate with the third male faster than those females that produced second male offspring (Mann Whitney U = 99.5, $p = 0.0024$; Figure 4B).

This finding suggests that females failed to accept/retain the ejaculate and went on to quickly mate with a third male. Given that thrice-mated females also show diluted LMSP, we conclude that when twice-mated females are given a third mating opportunity, they may be able to control the fate of sperm within their reproductive tract to bias the genetic composition of their offspring: a form of cryptic female choice. Although the mechanism in which females achieve selective paternity is unknown, it is clear that females can both modulate and forego offspring production from males with which they have mated.

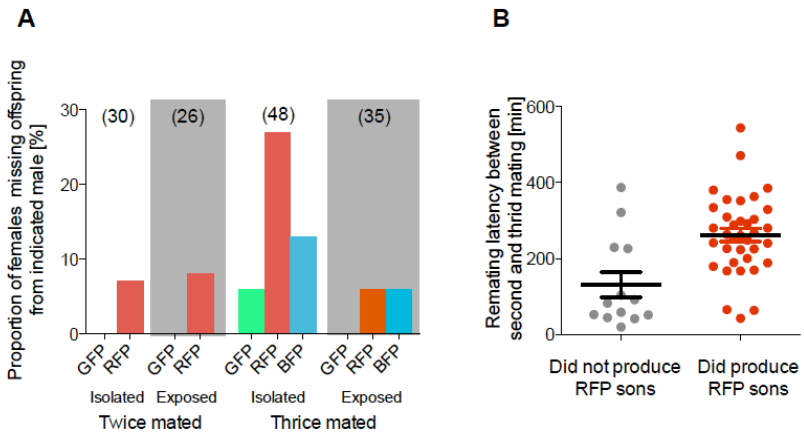

Figure 4. Increased mating rate is associated with selective paternity. **(a)** Proportion of females in the different groups that failed to produce offspring from a mate (GFP shown with green bar, RFP shown with red bar, and BFP shown with blue bar). Grey box indicates females that were exposed to third group of males. Number of replicates is between brackets. **(b)** Mean remating latency between second (RFP male) and third (BFP male) mating for thrice-mated females that did not produce offspring from second male (black) and those that did (red). Error bars indicate s.e.m. Differences between groups were assessed with a Mann Whitney U test. For full statistical analysis see supplementary Table 1.

DISCUSSION

Most research on LMSP has focused on a scenario were females mate with only two males and with long intervals between matings. Since *D. melanogaster* females are documented to mate with several males in the wild, we investigated whether lessons learnt from the two-male scenario extend to perhaps a more natural situation when females mate with more males, more frequently. We were also driven by the question of the contribution of females to LMSP and whether differences in her mating rate may affect patterns of paternity. Following in the footsteps of Manier et al. (2010), who generated males with green and red fluorescently labeled sperm in order to visualize the fate of sperm as they battle for fertilization in the female reproductive tract, we generated new transgenic males that express one of three different fluorescent proteins. This allowed us to determine the influence of the number of female copulations on patterns of paternity in the resulting offspring. Furthermore, as genetic variation in males affects variation in LMSP (Clark et al., 1995; Fiumera et al., 2007; Hughes, 1997; Lüpold et al., 2012; Prout and Bundgaard, 1977), we have ensured that these three male lines are virtually identical except for the few amino acid changes between the transgenic fluorescent proteins by inserting the new transgene at the exact same location in the genome of those males. In order to test LMSP in multiple-mating

scenarios and varying mating rate, we developed a novel mating paradigm, which allowed for multiple copulations within a 24-hour period. This paradigm coupled with new transgenic lines allowed us to test whether variation in number of mates and remating latency could contribute to variation in paternity ratios. Indeed, here we show that females that mated three times within 12 hours displayed a significant reduction in LMSP compare to females that either mated three times within 24 hours or females that mated twice. This finding provides evidence that female mating rate is a determinant of LMSP.

One supposed benefit of polyandry is to increase genetic diversity of offspring (Parker and Birkhead, 2013). However, the occurrence of LMSP, which reduces the offspring genetic diversity offered by mating with several sires by favouring the last male, brings that benefit of polyandry into question. As males gain reproductive success by hijacking paternity at the cost of the female, males and females are in conflict in regards to the optimal paternity patterns of the offspring of a multiply-mated female. The theory of sexual conflict predicts that over evolutionary time females who have developed counter-adaptations to male tactics that reduce her reproductive success should be selected for (Chapman et al., 2003a). We therefore hypothesized that since offspring genetic diversity is beneficial to females, over evolutionary time females should develop mechanisms to reduce males' ability to manipulate genetic composition of offspring. In other words, traits expressed by the male to increase his representation in the progeny should be met with female resistance. Here, we showed that females who remate in quick succession with three males counteracted LMSP, indicating that mating rate is a phenotype that may act as a counter-adaptation to male traits that produce LMSP.

Previously, some studies in *D. melanogaster* also observed paternity patterns in females that mated females with more than two males and they did not report a breakdown in sperm precedence. However, a consideration of their methods suggests possible sources of this inconsistency. First, the number of matings was not controlled for as mixed-sex groups were left unsupervised in for 24 hours (Morrow et al., 2005; Prout and Bundgaard, 1977). Second, researchers determined the paternity of offspring produced within the first 18 hours after remating (Morrow et al., 2005), which underestimates the number of offspring given that females can lay eggs for 5 days from a single mating and our finding that females who show a breakdown in sperm precedence also show significantly different patterns of paternity over time. Consistent with the findings from Billeter et al. (2012) who also observed each mating and genotyped all offspring, we conclude that mating rate can dilute LMSP.

What governs remating in this species?

The average remating latency stated in the literature, however, in *D. melanogaster* is ~4 days, which is inconsistent with our findings that females mate as often as 3 times within 12 hours. The previous remating latency suggested that female receptivity only returns at this time due to sperm depletion within the sperm storage organs, known as the "sperm effect" (Manning, 1967). This effect was later linked to the binding of sex peptide followed by its gradual release from the stored sperm, which chemically reduces sexual receptivity in mated females, allowing for this delayed or long-term depression in remating behaviour (Peng et al., 2005a). However, amount of sperm in storage is not always a determinant of sexual receptivity as females with longer seminal receptacles, who also consequently store more sperm, are also found to re-mate more rapidly (Amitin and Pitnick, 2007; Miller and Pitnick, 2003). Together with previous findings that females held in groups re-mate long before the cited 4 day latency (Billeter et al., 2012; Gorter et al., 2016; Laturney and Billeter, 2016), we conclude that our understanding of female mating rate in this species may have been limited by the nature of laboratory assays.

Interestingly, what little we do know about female mating rate suggests that it is an extremely plastic trait in *D. melanogaster* females as it has been found not only to be influenced by food availability (Gorter et al., 2016; Harshman et al., 1988), current nutritional status (Fricke et al., 2010), and developmental conditions (Amitin and Pitnick, 2007), but it has also been linked to natural genetic diversity (Clark and Begun, 1998; Giardina et al., 2011). Mating rate has also been found to increase with group size (Gorter et al., 2016; Laturney and Billeter, 2016; Chapter 3), and group diversity (Billeter et al., 2012; Krupp et al., 2008) suggesting that females can detect variation in males in her social group. Together, this shows that females may be able to adjust remating behaviour, possibly in order to balance the context-dependent benefits and costs of reproductive behaviour.

Modulation of paternity: adjustment to omission

In this present study we not only found that females that mated three times in quick succession diluted LMSP, but we also showed that a large number had foregone progeny production from specific individuals with whom they had mated with. One hypothesis is that mating with a third male immediately followed by isolation blocks the female's usage of second male sperm, proposing that our manipulation of exposure influenced paternity selection. However, we also found

that thrice-mated females that failed to produce offspring from the second mating also displayed significantly shorter mating latency between second and third male compared to thrice-mated females that produced offspring from all mates. This casts doubt on this hypothesis because this quick remating occurred before we isolated the females. Therefore, it is unlikely that our isolation blocked sperm usage in the fly and much more likely that the females failed to receive/maintain the ejaculate. This failure likely resulted in a short remating latency, and due to our experimental procedure, we then selected this females for the "thrice-mated isolated" group. Alternatively, the blockage of the sperm use may be an interaction between the short remating latency and the isolation.

The short remating latency in this group suggests that females failed to successfully receive the ejaculate from the second male (Lefevre and Jonsson, 1962). However, since all males were virgins and the problems associated with progeny production were specific to this group, it is unlikely that males failed to transfer, and more likely that females failed to accept or retain. It is possible that these females removed the ejaculate quickly after receiving it via sperm ejection, which would account for the quick remating as females have been found to re-mate soon after ejection (Laturney and Billeter, 2016; Chapter 3). It would also account for the lack of offspring produced from the second male as short ejection latency (time between mating and ejection) has been shown to reduce offspring from the most previous mate in both *D. melanogaster* (Lee et al., 2015; Lüpold et al., 2013) as well as in feral fowl, *Gallus gallus domesticus* (Pizzari and Birkhead, 2000).

Different patterns in twice- and thrice-mated patterns of paternity may reflect changes in sperm storage location

Consistent with previous findings that investigated patterns of paternity in twice-mated females with at least 48 hours of isolation between copulations (Amitin and Pitnick, 2007), the other three groups of females produced similar ratios of paternity in the various days after mating. However, unlike the other groups females that mated with three males in quick succession produced significantly different offspring from the third male over time. Specifically, females produced fewer offspring from the third male around 6 days after mating. Of the two types of sperm storage organs that females possess, the spermatheca is regarded as the long term storage organ (Pitnick et al., 1999). Interestingly, it has also been found to experience fewer sperm displacement after remating, at least in twice-mated females (Manier et al., 2010). Given that the sperm stored in the spermathecae is

used later, and we found a drop in P3 over time, it is possible that thrice-mated females may specifically block sperm entry into the spermathecae allowing only third-male sperm to enter the seminal receptacle, contributing to the dilution in LMSP.

Spatial discrimination of sperm storage based on male mating order is observed in *D. simulans*, *D. melanogaster*'s evolutionary cousin. In *D. simulans*, females spatially discriminate first and second male sperm within their reproductive tract (Manier et al., 2013). As a result, females are able to bias paternity by preferentially accessing the specific SSO (Manier et al., 2013). Although it is unlikely *D. melanogaster* females are able to bias offspring in a similar fashion, our findings suggest that females may be able to restrict storage to the spermatheca, resulting in altered patterns of paternity. This prediction will be tested by imaging and counting sperm from all three males in thrice-mated females, which is made possible by the creation of the fluorescently labeled sperm lines described here.

CONCLUSION

Our results provide further support to a growing body of evidence demonstrating that females contribute to post-copulatory sexual selection. Similar to other arthropods, aspects of female remating behaviour such mating rate, a combination of remating latency and number of sexual partners, modulates LMSP (Drnevich, 2003; Zeh and Zeh, 1994) and therefore variation in polyandry results in differences in patterns of paternity in *Drosophila*. By modulating the paternity, females can maximize benefits of polyandry and increase genetic diversity of offspring. These findings also suggest that there may be active control over sperm storage process and hint that mechanisms of cryptic female choice are at play. A full understanding of active female sperm storage is therefore warranted in the future.

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AUTHOR CONTRIBUTIONS

M.L. and J.-C.B. designed and interpreted the study. R.v.E. made the genetic constructs. M.L. and R.v.E. performed all experiments. M.L. and J.-C.B. performed the statistical analysis. M.L. and J.-C.B. wrote the manuscript.

SUPPLEMENTAL INFORMATION

Figure 1B **Number of offspring sired by transgenic male line**

	GFP	RFP	BFP
KS distance	0.12, $p > 0.10$	0.14, $p > 0.10$	0.13, $p > 0.10$

One-way ANOVA $F(2, 48) = 1.767$, $p = 0.18$

Figure 2B **Analysis of LMSP in mated twice and thrice mated females**

		Twice iso	Twice exp	Thrice iso	Thrice exp
KS distance		0.14, $p > 0.10$	0.17, $p > 0.10$	0.13, $p > 0.10$	0.11, $p > 0.10$
Two-way ANOVA	Matings	$F(1, 92) = 8.09$, $p = 0.005$			
	Exposure	$F(1, 92) = 11.82$, $p < 0.001$			
	Interaction	$F(1, 92) = 2.74$, $p = 0.10$			
Bonferroni post-hoc test		2 matings context, exposed vs isolated t = 1.17, $p > 0.05$			
		3 matings context, exposed vs isolated t = 3.95, $p < 0.001$			

Figure 2C **Analysis of LMSP within groups of females over time**

Group	time	n	mean	KS	p	One-way ANOVA/Kruskal-Wallis
Twice iso	0-48	20	64.3	0.12	>0.1	$F(2, 57) = 0.044$, $p = 0.96$
	49-150	20	65.8	0.16	>0.1	
	151+	20	63.7	0.63	>0.1	
Twice exp	0-48	13	68.9	0.21	>0.1	$F(2, 50) = 0.057$, $p = 0.94$
	49-150	20	70.1	0.15	>0.1	
	151+	20	71.4	0.13	>0.1	
Thrice iso	0-48	27	49.1	0.12	>0.1	**$KW = 6.70$, $p = 0.035$**
	49-150	28	53.7	0.10	>0.1	
	151+	28	37.5	**0.19**	**0.01**	
Thrice exp	0-48	24	72.9	0.14	>0.1	$F(2, 75) = 2.54$, $p = 0.086$
	49-150	26	67.5	0.10	>0.1	
	151+	28	60.5	0.11	>0.1	

Figure 2D **Analysis of remating latency between first and second mating**

Group	n	mean	KS	p	One-way ANOVA/Kruskal-Wallis
Twice iso	20	324.4	0.15	>0.1	**$F(3, 92) = 10.19$, $p < 0.001$**
Twice exp	20	406.9	0.11	>0.1	
Thrice iso	28	271.0	0.10	>0.1	
Thrice exp	28	387.9	0.10	>0.1	

Figure 2E **Correlations: remating latency and LMSP**

	Twice mated	Thrice mated
n	40	56
mean ± S.D.	67.13 ± 17.94 S.D.	56.39 ± 20.76
KS distance	0.11 $p > 0.10$	0.077, $p > 0.10$
Remating Lat:	Pearson r = 0.073 $p = 0.66$ $R^2 = 0.0053$	Pearson r = 0.52 $p < 0.001$ $R^2 = 0.27$

Multiple regression

Model	R square = 0.241 F (3, 92) p < 0.001
Number of matings	beta = -0.324, p = 0.001
Exposure	beta = 0.165, p = 0.164
Remating latency	beta = 0.297, p = 0.015

Correlations of dependent variable to each of the predictors (preferably above 0.3)

Number of matings	r = -0.263, p = 0.005
Exposure	r = 0.348, p < 0.001
Remating latency	r = 0.332, p < 0.001

Correlations of predictor variables to each other (preferably below 0.7)

	Number of matings	Exposur	Remating latency
Number of matings		$r = 0, p = 0.5$	$r = 0.207, p = 0.021$
Exposure			$r = 0.618, p < 0.001$

Collinearity diagnostics

	Tolerance	VIF
Number of matings	0.931	1.075
Exposure	0.601	1.664
Remating latency	0.575	1.739

Figure 3A **Number of offspring sired by polyandrous females**

		Twice iso	Twice exp	Thrice iso	Thrice exp
KS distance		0.15, $p > 0.10$	0.19, $p = 0.59$	0.066, $p > 0.10$	0.11, $p > 0.10$
Two-way ANOVA	Matings	$F (1, 92) = 0.26, p = 0.61$			
	Exposure	$F (1, 92) = 20.29, p < 0.001$			
	Interaction	$F (1, 92) = 3.21, p = 0.076$			
Bonferroni post-hoc test		2 matings context, exposed vs isolated t = 4.12, $p < 0.001$			
		3 matings context, exposed vs isolated t = 2.10, $p > 0.05$			

Figure 3C **Correlations: remating latency and LMSP**

	Twice-mated	Thrice-mated
n	40	56
mean ± S.D.	196.3 ± 77.30	203.0 ± 61.83
KS distance	0.10 $p > 0.10$	0.077, $p > 0.10$
Remating Lat:	Pearson r = 0.01 $p = 0.94$ $R^2 = 0.0001$	Pearson r = -0.26 $p = 0.054$ $R^2 = 0.067$

Figure 4B **Comparison of remating latency**

	Females that failed to produce RFP sons	Female that did produce RFP sons
n	13	36
mean ± S.D.	131.1 ± 119.5	262.1 ± 103.8
KS distance	**0.29 p=0.005**	0.10 p >0.10
Mann Whitney	**$U = 99.50, p = 0.0024$**	

Supplementary Table 1. Summary of statistical tests to compare progeny production and paternity patterns with mating behaviour. All data sets were first assessed for normality with a Kolmogrov-Smirov test (with Dallel-Wilkinson-Lillifor P value), which renders a KS distance statistic. A *p*-value greater than 0.05 indicates data are normally distributed. In the cases where data are not normally distributed, non-parametric tests were performed. All comparisons that were significant are bolded. Twice-mated isolated females = Twice iso; twice-mated exposed females = Twice exp; thrice-mated isolated females = Thrice iso; thrice-mated exposed females = Thrice exp; S.D. = standard deviation. Collinearity diagnostics for the standard multiple regression: Tolerance: (indicator of how much of the variability of that particular independent variable is not explained by other independent variables, preferably not less than 0.1); VIF: (Variance inflation factor) preferably values below 10 as it is simply the inverse of tolerance.