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Multiple mechanisms of cryptic female choice act on intraspecific male variation in *Drosophila simulans*

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Abstract

Postcopulatory sexual selection can arise when females mate with multiple males and is usually mediated by an interaction between the sexes. Cryptic female choice (CFC) is one form of postcopulatory sexual selection that occurs when female morphology, physiology, or behavior generates a bias in fertilization success. However, its importance in nonrandom reproductive success is poorly resolved due to challenges distinguishing the roles of females and males in generating patterns of fertilization bias. Nevertheless, two CFC mechanisms have recently been documented and characterized in *Drosophila simulans* within the context of gametic isolation in competitive hybrid matings with *D. mauritiana*: sperm ejection and nonrandom use of sperm storage organs for fertilization. Here, we explore if and how female *D. simulans* employ these two mechanisms of CFC in response to intraspecific male size variation. We used transgenic males expressing green (GFP) or red fluorescent protein (RFP) in sperm heads to document postcopulatory processes, in conjunction with a probabilistic analytical model. We unexpectedly found that differential reproductive success was also a function of male population (GFP or RFP), suggesting that females use different CFC mechanisms to select for different male traits. Moreover, concordance of selection at the precopulatory (as measured by mating latency) and postcopulatory stages depends on both the male trait and the CFC mechanism examined. Larger males were more successful both before and after mating, but we unexpectedly found that females also mated more quickly with males with GFP-labeled sperm, while fertilization bias favored RFP-labeled sperm.

Significance Statement (131 words)

Females often mate with multiple males and store their sperm for extended periods in specialized sperm storage organs, leading to sexual selection on both male traits for competitive fertilization success and female traits that mediate sperm choice. In *Drosophila simulans*, females switch use of sperm for fertilization between two types of sperm storage organ, a pair of spermathecae and a seminal receptacle (SR), during sperm competition between a conspecific and heterospecific male to bias sperm use in favor of the conspecific male. Moreover, the timing of when females eject excess sperm after mating significantly influences paternity success. Here, we found that after competitive matings with two conspecific males varying in size, females adjust ejection time in response to male size but unexpectedly exhibit fertilization bias in response to male population identity.

Keywords Precopulatory sexual selection, postcopulatory sexual selection, sperm competition, female ejection, female preference, fertilization bias

Introduction

Females of most species mate with multiple males, generating the potential for postcopulatory sexual selection on traits of both sexes that influence competitive fertilization success via sperm competition and/or cryptic female choice (CFC; Parker 1970; Eberhard 1996). Non-random fertilization success favoring one male over another has historically been most readily attributed to sperm competition and male- or ejaculate-mediated traits, since sperm competition alone can cause paternity bias without invoking CFC (Partridge & Halliday 1984). However, paternity success is often a function of male × female interactions, such that P_2 (proportion of progeny sired by the second male) depends on the identities of both sexes (Lewis and Austad 1990; Clark et al. 1999; Nilsson et al. 2003; Evans and Marshall 2005; Urbach et al. 2005; Rosengrave et al. 2008; Evans et al. 2013). These interactions alone are evidence of both CFC and sperm competition in postcopulatory sexual selection (Pitnick and Brown 2000). Indeed, studies in diverse taxa have convincingly shown that females are not simply passive arenas in which sperm compete to fertilize eggs but can actively influence paternity (e.g., Sakaluk and Eggert 1996; Clark and Begun 1998; Pizzari and Birkhead 2000; Pilastro et al. 2004; Brennan et al. 2007; Dean et al. 2011; Lüpold et al. 2013; Manier et al. 2013a, b, c). This is not to say that evidence for CFC (or female choice in general) suggests no role for male-mediated processes, since it is difficult, if not impossible, to experimentally separate the two in a biologically relevant manner. Thus, any discussion of evidence for female choice, cryptic or otherwise, does not necessarily exclude male-mediated processes that may also and simultaneously influence patterns of non-random mating success or paternity.

Despite widespread evidence for male × female interactions, mechanisms of postcopulatory sexual selection due to sperm competition and/or CFC are generally difficult to resolve due to challenges in differentiating sperm from different males and determining their fate within the female reproductive tract, particularly regarding fertilization. Nevertheless, we know more about mechanisms of sperm competition than CFC, because the latter is often mediated by female behavioral, morphological, physiological, or biochemical processes (Thornhill 1983; Eberhard 1996; Pitnick and Brown 2000) that are difficult to empirically observe. Complexity of the female reproductive tract is considered to provide females with greater opportunity for control over fertilization (Eberhard 1985, 1996; Birkhead et al. 1993; Hellriegel and Ward 1998; Hellriegel and Bernasconi 2000). For example, female ducks have evolved complex morphological adaptations to thwart insemination through forced copulation by males, including a vagina that corkscrews in the opposite direction of the male's penis and false dead-end ducts that prevent sperm from reaching the ova (Brennan et al. 2007). Moreover, female reproductive tract complexity often coevolves with morphology of male genital or ejaculate traits (Dybas and Dybas 1981; Presgraves et al. 1999; Pitnick et al. 1999, 2003; Morrow and Arnqvist 2003; Miller and Pitnick 2002; Minder et al. 2005; Anderson et al. 2006; Brennan et al. 2007; Rugman-Jones and Eady 2008; Rönn et al. 2011; Higginson et al. 2012), potentially leading to post-mating pre-zygotic reproductive isolation between closely-related species (Rugman-Jones and Eady 2007; Manier et al. 2013b).

When considering CFC in species that store sperm, it is useful to discriminate between two stages at which postcopulatory sexual selection can act: (1) formation of the "fertilization set" (the population of sperm potentially competing to fertilize eggs; sensu Parker et al. 1990)

and (2) use of sperm in the fertilization set for fertilization. In Stage 1, males and females likely interact to determine how sperm are stored to create the fertilization set through influences on copulation duration, sperm transfer, sperm displacement, sperm storage, and sperm ejection (e.g., Siva-Jothy 1987; Simmons et al. 1999; Engqvist et al. 2007; Manier et al. 2010, 2013a, b; Dean et al. 2011). Sperm ejection, in particular, is a Stage 1 mechanism of CFC in which excess sperm from the second male's ejaculate, as well as any first-male sperm that may have been displaced from the storage organs, are forcefully ejected from the female reproductive tract (Pizarri and Birkhead 2000; Manier et al. 2010, 2013a, b; Dean et al. 2011). It should be noted that previous studies have used the term "ejection" to refer to loss of sperm from storage (e.g., Snook and Hosken 2001), a different process from what we are describing here.

In *Drosophila*, sperm ejection terminates the displacement process and establishes the fertilization set for Stage 2 of postcopulatory sexual selection. In *D. melanogaster*, the timing of ejection has a strong genetic component and signature of female mediation, with an estimated heritability of 0.36 (Lüpold et al. 2013). In *D. simulans*, *D. mauritiana*, and *D. melanogaster*, the proportion of second-male sperm in the sperm storage organs (S_2) is significantly correlated with paternity success (P_2; Manier et al. 2010; Lüpold et al. 2012, 2013; Manier et al. 2013c), and in *D. melanogaster*, longer ejection times result in higher S_2 (Lüpold et al. 2013). Although we did not find evidence for a relationship in *D. simulans* between ejection time and S_2 in conspecific matings (Manier et al. 2013a), competitive hybrid matings between *D. simulans* and *D. mauritiana* males with a *D. simulans* female had much shorter ejection times as a mechanism of conspecific sperm precedence (Manier et al. 2013b).

Stage 2 CFC determines how sperm are used from the female sperm storage organs for fertilization and may also be driven by male × female interactions (Manier et al. 2013b, 2013c). During this stage, the fertilization set (sensu Parker et al. 1990) represents the population of sperm potentially competing to fertilize eggs, often within specialized sperm storage organs within the female reproductive tract. Patterns of paternity success may deviate nonrandomly from the null expectation that sperm are used in direct proportion to their numerical representation in the fertilization set, indicative of fertilization bias (Manier et al. 2013c). In other words, fertilization bias occurs when one male's sperm are disproportionately used over another's above and beyond their relative abundance in the fertilization set. Fertilization bias should not be confused with sperm precedence, which describes the proportion of progeny sired by a male depending on mating order. A pattern of second-male sperm precedence, for example, reveals nothing about fertilization bias, because displacement or other processes may establish a fertilization set that is numerically dominated by the second male's sperm. In this case, an unbiased pattern of sperm use will produce second-male sperm precedence in the absence of a fertilization bias. Note that Stage 1 and Stage 2 CFC apply to a mating of any order (first, second, third, etc.), because they describe sperm storage and use in a general sense, regardless of the number of male ejaculates that comprise the fertilization set.

The greater opportunity for differential sperm storage of different ejaculates generated by increased female reproductive tract complexity may especially influence the potential for Stage 2 CFC. In *Drosophila*, females have evolved increased reproductive tract complexity through utilization of two types of sperm storage organ: a pair of spermathecae and a long coiled seminal receptacle (SR). Morphology of these structures and the degree to which they are used in sperm

storage are evolutionarily labile across the *Drosophila* lineage, but they are always both present (Pitnick et al. 1999). Although relatively complex reproductive tracts can provide females with an opportunity to engage in CFC, they may not always evolve mechanisms to do so. Using a probabilistic analytical model (Manier et al. 2013c), we estimated three parameters of fertilization bias between sperm from two males: 1) bias in first- or second-male sperm used from the spermathecae, 2) bias in first- or second-male sperm used from the SR, and 3) an "organ use bias" that described which (if either) of the two sperm storage organ types are used preferentially. This approach was applied in *D. melanogaster*, *D. simulans*, and *D. mauritiana* and revealed different modes of fertilization bias in all three species. In *D. melanogaster*, the SR was favored over the spermathecae, while *D. simulans* exhibited second-male bias from the spermathecae but first-male bias from the SR. We found no fertilization bias at all in *D. mauritiana* (Manier et al. 2013c). In a follow-up study, we showed that the unusual pattern of fertilization bias in *D. simulans* is another important mechanism of conspecific sperm precedence in competitive hybrid matings with *D. mauritiana*. Females switch which sperm storage organ type is used to favor the preferred conspecific male, depending on whether he is first or second to mate. For example, when her first mate is the heterospecific male, and her second mate is the conspecific male, sperm are used primarily from the spermathecae, which are second-male biased (Manier et al. 2013b). Thus, female *D. simulans* employ both Stage 1 (early ejection) and Stage 2 (bias in sperm storage organ use) CFC as mechanisms of conspecific sperm precedence.

Here, we examine the degree to which CFC at both stages is employed when male quality (body size) is systematically varied in conspecific matings. We also evaluate any associations

among precopulatory female choice (mating latency) and both stages of CFC. Evidence for consistent selection across precopulatory and postcopulatory stages of sexual selection is mixed, but more studies have found that attractive males enjoy a competitive advantage during sperm competition (Lewis and Austad 1994; Edvardsson and Arnqvist 2000; Bangham et al. 2002; Evans et al. 2003; Pilastro et al. 2004; Locatello et al. 2006; Hosken et al. 2008; Bretman et al. 2009; Fricke et al. 2010) than have not (Droge-Young et al. 2012; Pischedda and Rice 2012). Consistent with this pattern, Hosken et al. (2008) found that in *D. simulans*, attractive males (those that mated faster) sire more offspring, with a significant correlation between second-male mating latency and second-male paternity success (P_2). Part of this study's elegance lies in allowing experimental females to define male attractiveness rather than the researchers, but other studies found that female *D. simulans* mate more frequently with larger males both in natural (Markow and Ricker 1992) and laboratory populations (Taylor et al. 2008). We manipulated male size in a fully factorial double mating design, in which wild type females were randomly assigned to one of four mating treatments: two large males (LL), two small males (SS), a large male followed by a small male (LS), and a small male followed by a large male (SL). We previously found evidence that *D. simulans* females use their first mate as a basis for evaluating their second mate in competitive heterospecific and conspecific matings, such that females ejected heterospecific sperm much sooner if the heterospecific male was her second mate than if he was her first mate (Manier et al. 2013b). We thus predicted that females exposed to a second male that was much larger (SL) or much smaller (LS) than her first mate would yield the strongest evidence for both precopulatory and postcopulatory female choice.

Materials and methods

For all four treatment groups (LL, SS, SL, LS) across three concurrent experiments described below, we quantified the following parameters for both first and second matings: mating latency and copulation duration (first copulation from Experiments 1-3, second copulation from Experiments 2-3), number of sperm transferred (first copulation from Experiment 1, second copulation from Experiment 2), and from Experiment 3, we measured number of progeny produced until remating and three days after remating, timing of sperm ejection by females after remating, number of progeny sired by each male, and the number of each male's sperm remaining in the female's reproductive tract three days after remating.

Stocks and experimental males

For all matings, we used *D. simulans* males from lines with GFP- or RFP-labelled sperm heads (henceforth "GFP" or "RFP"; Manier et al 2013a). GFP lines also carried a fluorescent GFP eye marker that is physically linked to the sperm label, which allowed paternity assignment of offspring sired by GFP males. All females were derived from the same wild type population into which the transgenic populations were backcrossed for five generations. All stocks were maintained at ambient room temperature (23–25°C) and light regime in half-pint milk bottles on standard corn meal–agar–yeast–molasses medium sprinkled with live yeast grains.

206 Males of two distinct body size classes were generated by transferring first-instar larvae
207 to vials at densities of 300 individuals with 0.25 cm^3 medium and 50 individuals with 1.5 cm^3
208 medium. Larval density has previously been shown to influence larval development and adult
209 body size through competition for nutrients in *D. melanogaster* (e.g., Pitnick and García-
210 González 2002; Byrne and Rice 2005; Amitin and Pitnick 2007; Lüpold et al 2011). Using
211 thorax length as a reliable measure of adult body size (Robertson and Reeve 1952), low-density
212 males (L) were found to be significantly larger (mean ± SD, N: 71.3 ± 3.7, 277) than high-
213 density males (S; 55.5 ± 4.6, 235; t_{766} = -53.1, $p < 0.001$; Electronic Supplementary Material
214 (ESM)). We generated L and S males from both GFP and RFP lines to allow sperm competition
215 between an L or S GFP male against an L or S RFP male in a fully factorial mating design (Table
216 S1). Large and small males differed in thorax length (all Tukey p-values < 0.001 in pairwise
217 comparisons of L and S males) and L-GFP males were a little bit larger than L-RFP males, while
218 S-GFP and S-RFP males did not differ in thorax length (see ESM and Fig. S1 for further details).
219 Experimental males and females were collected as virgins within 6 h of emergence under
220 CO_2 anesthesia and maintained in plastic vials (10 flies per vial) with 1.5 cm^3 medium
221 supplemented with live yeast. Females were mated at 2-3 days, and males at 3-7 days post-
222 eclosion. All individuals were virgins when initially mated, and all matings occurred in food
223 vials sprinkled with live yeast grains, with a single pair per vial. Flies were aspirated into mating
224 vials without anesthesia.
225
226 **Experiment 1: First-male sperm transfer**
227

To quantify number of sperm transferred to virgin females, we paired twenty individual males of each type (L-GFP, S-GFP, L-RFP, S-RFP; total N = 80) with a wild type female and recorded the time until mating (mating latency) and copulation duration. Immediately after mating, pairs were frozen in their vials and maintained at -70°C until data collection. Thorax length was recorded for both males and females, and female reproductive tracts were dissected into 1X phosphate buffered saline (PBS) and mounted with a cover slip. RFP and GFP sperm in the reproductive tract were counted at 400X on an Olympus BX60 compound microscope with a mercury fluorescent lamp and multiband GFP-DsRed-A filter set (Semrock, Rochester, NY).

Experiment 2: Second-male sperm transfer

In order to quantify number of sperm transferred to previously mated females, a second group of virgin females were haphazardly assigned to 8 remating treatments that varied male size, male line (GFP or RFP), and mating order (for LL, SS, SL, N = 50 for each treatment, for LS N = 70; Table S1). Sample size for LS matings was higher than in the other treatments, because we expected females to be less willing to remate with a male that was smaller than her first mate. Females were provided a 4-hr opportunity to remate with a second male 2, 3, and 4 days after their first mating. For first and second matings, we recorded mating latency and copulation duration. For second matings, mating latency was measured in number of minutes of interaction, accumulated over days, if applicable. For example, a female that remated after one hour on the second day of remating showed a mating latency of 300 minutes (5 hrs), which also accounts for the previous day's 4-hr opportunity to remate. Immediately after remating, females were frozen,

dissected, and sperm in the reproductive tract counted as described above. All males were frozen after mating and thoraxes measured for body size. RFP and GFP sperm were counted at 400X or 630X on a Nikon Eclipse Ni-U compound microscope. For all experiments, sperm counts were performed blind to treatment and male mating order.

Experiment 3: Female sperm ejection, paternity share, and fertilization bias

In order to quantify how sperm are used for fertilization, we used the same experimental design as for the second-male sperm transfer experiment (for LL, SS, SL, N = 50 for each treatment, for LS N = 70; Table S1) and mated a third group of flies as described above. We quantified the timing of ejection by gently aspirating females immediately after copulation into individual wells of glass 3-well spot plates (Pyrex) and covered with glass coverslips secured with spots of clay. Females were monitored for ejection under a stereoscope every 5–15 min, until either an ejected mass (slightly smaller than an egg) was observed or a 4-hour time limit was reached.

After ejection, females were transferred without anesthesia into fresh food vials every day for three days, after which females were frozen, dissected, and sperm counted in all regions of the reproductive tract. Proportion of progeny sired by the second male (P_2) was estimated from offspring produced in the first three days after remating. This time period corresponds with an average remating time for *D. simulans* of 2.7 days (Manier et al. 2013a). The GFP line was not completely fixed for the fluorescent sperm label and eye marker. Thus, the stock was selected for GFP eye marker for three generations prior to the experiment, and only males with the eye marker were used in the experiment. Because the sperm and eye markers are dominant,

all sperm are labeled in heterozygous individuals (Manier et al. 2010, 2013a); sperm counts are not affected, but paternity assignment may be inaccurate. GFP males that mated with females in the paternity treatment were subsequently test-crossed with wild type females to assess heterozygosity. Males were then frozen for thorax measurements. Progeny of homozygous GFP males were assigned to a sire based on the fluorescent eye marker. Progeny of heterozygous GFP males were assigned to a sire based on male line detected by dissecting a single testis from each son (daughters were excluded from the paternity analysis). Sons lacking an eye marker had testes with either RFP labeled sperm (RFP sire) or unlabeled sperm (GFP sire).

We applied the paternity and sperm count data to estimate fertilization bias using an analytical model that calculates first- or second-male bias from the spermathecae (x) and the SR (y), as well as bias in sperm use from the different types of sperm storage organ (z; Manier et al. 2013c):

$$P_2 = \frac{X2(x)(1-z)+Y2(y)(z)}{(1-z)[X1(1-x)+X2(x)]+z[Y1(1-y)+Y2(y)]},$$

where $X1$, $X2$, $Y1$, and $Y2$ are the numbers of first- and second-male sperm in the spermathecae and SR, respectively. Parameter estimates and standard errors were obtained using non-linear mixed model regression implemented using SAS PROC NLMIXED (SAS Institute 2008) with the model statement "Model P2 ~ B(N1 + N2, P2);" where B represents binomial, N1 is the number of progeny sired by the first male, and N2 is the number of progeny sired by the second male, and P2 is the proportion of progeny sired by the second male. Parameters x, y, and z are all bounded by 0 and 1; when $z = 0.5$, sperm are equally likely to be selected from either storage organ, X or Y. x or $y = 0.5$ represents equal probabilities for first- and second-male sperm to be

used for fertilization (i.e., no fertilization bias, or sperm from competing males are used in proportion to their relative abundance in storage).

It is important to note that this model estimates bias in how sperm are used for fertilizations above and beyond the relative proportions of first-male and second-male sperm in storage. The null hypothesis for this test is that there is no fertilization bias; that is, sperm are used from the first and second male in direct proportion to their relative abundance in storage. Thus, even if the second male's sperm outnumber first-male sperm in storage, fertilization bias may still favor the first male's sperm. This bias may not result in an overall P_2 less than 0.5 (the first male's sperm "wins" overall), but it would yield a lower P_2 than under no fertilization bias.

Statistical analysis

For all statistical analyses, we used R 3.0.2 (R Development Core Team 2013). We used t-test to detect the difference between large and small males, and general linear models (lm) with male type (L-GFP, S-GFP, L-RFP, S-RFP) as a factor to detect size differences among male larval density treatments in the separate experiments. To detect size differences among male treatments, multiple comparisons were performed with Tukey's tests using the "multcomp" package in R (Hothorn et al. 2008). We used log-linear Poisson generalized linear models (GLM) to test whether male line or size affects female mating or remating, because these data can be cross-classified into three dimensional contingency tables: two levels of mating status (mated, not mated), two levels of male line (GFP, RFP), and two levels of male size (L, S; Crawley 2007).

315 We used lms to analyse the effect of our size treatments on sperm transfer, number of
316 progeny produced before and after remating, mating latency in the first and second mating
317 (log_{10}-transformed), copulation duration in the first and second mating, and ejection latency
318 (log_{10}- transformed). Full models for mating latency and copulation duration in the first mating
319 included the first-male size × male line interaction and the experiment (first-male transfer,
320 second male transfer, fertilization bias) as factors. The full model for first-male sperm transfer
321 included the first-male size × male line interaction and female thorax length as factors. Female
322 body size may affect sperm storage, and males may transfer more sperm to larger females, as has
323 been documented in *D. melanogaster* (Lüpold et al. 2011). Full models for remating latency and
324 copulation duration in the second mating included the first-male size × second-male size
325 interaction, second-male line, and experiment (second-male transfer, fertilization bias) as factors.
326 The full models explaining the number of progeny produced after remating and ejection time
327 included the first-male size × second-male size interaction, second-male line, GFP genotype
328 (homozygous, heterozygous) of the GFP male and female thorax length as factor. The full model
329 for the number of progeny produced before remating also included remating latency as a
330 covariate, because the longer the remating latency, the more time there is for females to produce
331 progeny before remating.
332 We used GLMs with a negative binomial distribution and logarithmic link function
333 (glm.nb in MASS package in R, Venables and Ripley 2002) to analyse stored sperm data. The
334 full models included first-male size × second-male size interaction, second-male line, genotype
335 of the GFP male, and female thorax length. The one exception was the full model for the number
336 of first-male sperm in storage at the end of second copulation, which did not include the

337 genotype of the GFP male, because these data were from Experiment 2 for which we did not
338 have that information.
339 We applied model selection using the AIC (Akaike 1973) for lms and GLMs with a
340 negative binomial distribution. We applied a forward selection algorithm using R's AIC statistic
341 via the "step" function following Zuur et al (2013). We specified the minimal model to be the
342 model that only has an intercept and the maximal model to be the full model. Each term of the
343 full model was added in turn to the minimal model, and the model with the lowest AIC values
344 was chosen for the next round of model selection (see Table S4 for model selection process).
345 Proportional data, i.e., P_2 and S_2 in the SR and spermathecae, were analysed with GLMs
346 with quasibinomial error distribution (binomial models were overdispersed) and a logit link
347 function with sample sizes as weights. The full model included first-male size × second-male
348 size interaction, second-male line, GFP genotype, female thorax length, and the number of
349 progeny produced before remating. The number of progeny produced before remating can affect
350 P_2 values, because the more first-male sperm that are used for fertilization prior to remating, the
351 fewer that will be available for fertilization after remating, potentially increasing P_2 (see Table 2
352 in Ala-Honkola et al. 2010). Here we did not perform model selection, as AIC is not defined for
353 quasibinomial models.
354 To test the hypothesis that ejection time depends on the size difference of the two
355 consecutive males, we used planned orthogonal contrasts (Crawley 2007) to compare the LL *vs*
356 LS treatment and the SS *vs* SL treatment. All statistical models were validated by examining the
357 homogeneity and independence of errors.
358

Results

Heterozygosity for the GFP marker did not differ among treatments ($\chi^2 = 5.2$, df = 3, $p = 0.16$). Homozygous males tended to have a lower proportion of second male sperm in the SR (S_2 SR) than heterozygous males ($t_{82} = 2.0$, $p = 0.052$), but this difference did not result in significantly lower P_2 (Table 5).

Copulation duration

In matings with virgin females, small males ($t_{485} = 4.2$, $p = 0.002$) copulated longer than large males (Table 1, Table 3). In rematings, copulations with GFP males lasted on average 1.3 ± 0.7 minutes longer than with RFP males ($t_{326} = 1.9$, $p = 0.063$; Table 4), and small males copulated on average 1.2 ± 0.7 minutes longer than large males ($t_{326} = 1.8$, $p = 0.068$) but these differences were not significant at the $\alpha = 0.05$ level.

Mated females mate more quickly with larger males

Although virgin females were equally likely to mate with large or small males (deviance = -0.03, $p = 0.85$, df = 1; Table 1; Fig. 1a) and with no difference in mating latency (Tables 1, 3 & S4; Fig. 2a), mated females were more likely to remate with large males (deviance = -35.1, $p <$

0.001, df = 1; Fig. 1b, Table 2) and were quicker to do so (t_{325} = 3.5, p < 0.001; Fig. 2b; Table 2 & 4). First male size had no effect on remating latency (t_{325} = -0.4, p = 0.69; Table 4).

Females mate more quickly with GFP males

In addition to male size, females unexpectedly also mated more quickly with males according to line, with a shorter mating latency with GFP males. Virgin females were equally likely to mate with GFP or RFP males (deviance = -0.04, p = 0.84, df = 1; Fig 1a) but were quicker to mate with GFP males (t_{487} = 4.2, p < 0.001, Fig. 2a; Table 3). Mated females were both more likely to mate with GFP males (deviance = -12.3, p < 0.001, df =1; Fig. 1b) and had a shorter mating latency with them (t_{325} = 4.5, p = 0.027; Fig. 2b, Table 4). These results suggest that both virgin and mated females overall choose to mate more quickly with GFP males.

Paternity success depends on sizes of both the first and second male

Although females remated more often and faster with larger males, the outcome of sperm competition between two males depended on the interaction between their sizes (t_{123} = 2.2, p = 0.027; Table 5). P_2 was highest for LL, SS, and SL treatments, with small males faring the worst in sperm competition when in the offensive (second-male) role against large males (LS; Fig. 3, Table 2, Table 5).

There are a number of factors that could explain this low P_2 for the LS treatment. We found that small males transfer fewer sperm upon remating than large males (t_{108} = -6.4, p <

0.001; Fig. 4; Table 2, Table 4), with no effect of the first male's size on the number of sperm transferred by the second male (Table S4). We also found that all four male types (L-GFP, S-GFP, L-RFP, S-RFP) had no differences in number of sperm transferred to virgin females (Table 1, Table 3, Table S4). In *D. simulans* and related species, the number of sperm in an ejaculate is directly correlated with displacement of resident first-male sperm from storage (Manier et al. 2010, 2013b, 2013c), suggesting that the smaller ejaculate sizes of small males give them a competitive disadvantage in the offensive (second-male) role. However, we would then expect the SS treatment to also have a lower P_2, but it does not (Fig. 3).

There is some evidence that *D. simulans* females use their first mate as a basis by which to evaluate their second mate (Manier et al. 2013b), suggesting that cryptic female choice may play a role in treatments where a favorable male is followed by an unfavorable male (e.g., LS). In this case, timing of ejection as a cryptic female choice mechanism best explains the low P_2 of the LS treatment, because females ejected sperm sooner if their second mate was small (t_{117} = -2.2, p = 0.028; Table 2, Table 4), as well as if their first male was small (t_{117} = -2.5, p = 0.015; Table 2, Table 4). Ejection time is affected most by a decrease in quality (size) from the first male to the second (LS) than an increase in quality (SL). Using planned orthogonal contrasts, we found that females that mate first with a large male eject sperm of small males sooner than those that mate with a second large male (LS vs. LL; t = 2.23, p = 0.026). However, females whose first mate is small relative to their second mate have no difference in ejection time (SL vs. SS; t = 1.08, p = 0.28), suggesting that a step down in male quality has more of an effect on ejection time than a step up. Overall, females mate more quickly with larger males and eject sperm more quickly from smaller males, demonstrating a consistency between pre- and postcopulatory choice

with regards to male size. However, the analytical model for fertilization bias revealed no evidence that in the egg-laying stage, there is consistent sperm use bias based on male size (see below).

GFP males have higher P_2

Across all treatments, GFP males had higher paternity success than RFP males ($t_{123} = 7.8$, $p < 0.001$; Fig. 3; Table 5). At the same time, GFP males transferred more sperm to mated females than RFP males ($t_{108} = 6.3$, $p < 0.001$; Fig. 4, Table 4). Larger ejaculates of GFP males are predicted to more effectively displace first-male RFP sperm when in the offensive second-male role, allowing them to achieve a higher P_2. Furthermore, GFP first males had more sperm remaining in storage upon remating than RFP first males ($z_{109} = -4.5$, $p < 0.001$; Table S3), giving them a greater advantage in resisting displacement in the defensive role.

Interestingly, timing of female ejection (Stage 1 CFC) was not explained by male line (Table S4), suggesting that higher P_2 of GFP males is best explained by their superior sperm numbers rather than female ejection. In the egg-laying phase (Stage 2 CFC), we predicted that females would shift use of their sperm storage organs between the second-male biased spermathecae ($z < 0.5$) , and the first-male biased SR ($z > 0.5$) based on whether their preferred male was first or second. Here, we define fertilization bias favoring first-male or second-male sperm as disproportionate use of first-male or second-male sperm beyond relative proportions in the sperm storage organ(s). In other words, sperm that are heavily outnumbered in storage may still be used for fertilization in a biased manner if they are used disproportionately more often

than the numerically dominant sperm. In this experiment, we found persistent second-male bias in the spermathecae ($x > 0.5$; Fig. 5a, b), consistent with Manier et al. (2013b, c). On the other hand, the SR switched from first-male biased ($y < 0.5$) to second-male biased ($y > 0.5$), depending on the line of the second male, to favor RFP sperm (Fig. 5c, d; with the exception of SS with GFP as second male). When RFP was the second male, sperm storage organ use favored the second-male biased spermathecae ($z < 0.5$), with no clear pattern of organ bias when GFP males were second ($z = 0.5$; Fig. 5e, f). Despite this cryptic female preference for RFP sperm, GFP males have a higher P_2, likely due to their larger ejaculates. We therefore found evidence that females mate more quickly with GFP males but may select against them at the egg-laying phase. Nevertheless, GFP males have higher P_2 due to superior sperm numbers over RFP males.

Discussion

Previous studies of female choice have typically examined the effects of a single measure of male attractiveness, although mate choice is presumably often based on several cues that may or may not be interacting (see review by Candolin 2003; examples of studies on the use of multiple cues in mate choice in e.g. Lehtonen et al 2007; Simmons et al. 2013; Vortman et al 2013). Here, we unexpectedly have the opportunity to ask how female choice mechanisms differ for two sources of male traits (body size and male line) and to evaluate these differences for both pre- and postcopulatory stages of sexual selection. For male body size, we found evidence suggesting that in the precopulatory stage, females mate more quickly large males and select against small

males in the postcopulatory stage via the cryptic female choice mechanism of ejecting undesirable sperm sooner after mating. Previous work in *D. simulans* also found that females mate more quickly with larger males (Taylor et al. 2008) and that preferred males fared better in sperm competition (Hosken et al. 2008). In contrast, we did not find that larger males have greater paternity success across the board; rather, females seem to evaluate the quality of their mates relative to their previous encounters. Furthermore, males preferred at the pre-copulatory stage may or may not fare as well at the post-copulatory stage, depending on the male trait and the mechanism of cryptic female choice examined. Here, we found that paternity success of large males was dependent on the relative size of the first male, such that P_2 dropped significantly only when females mated with a large male followed by a small male (traded down).

At the same time, females mated more quickly with GFP-labeled males, and these males were also more successful in sperm competition against RFP males in both the offensive (second male) and defensive (first male) roles. As first males, more GFP sperm were retained in storage upon remating as well as at three days following remating, allowing greater resistance to displacement by RFP sperm. For rematings, GFP ejaculates contained more sperm, which resulted in proportionally more first-male sperm displaced from storage (Fig. S2, Fig. 3; concordant with Manier et al. 2010, 2013a, b; Lüpold et al. 2012), and best explains the higher P_2 of GFP males. Unlike with male size, the timing of female ejection was not affected by male line, but we found that fertilization bias actually tended favor RFP sperm. We thus found that the direction of precopulatory choice opposed that of Stage 2 postcopulatory choice for male sperm label.

Non-random fertilization success may be a function of both CFC and ejaculate traits simultaneously, just as non-random mating success may be influenced by both male traits and female choice for those traits. If ejaculate traits alone determined fertilization bias, we would expect consistent patterns of bias in both the spermathecae and SR, both of which tend to be used for fertilization when male quality (e.g., size) is not systematically varied (Manier et al. 2013c). Different patterns of bias in the spermathecae and SR documented here and elsewhere (Manier et al. 2013b, c) suggest that there is some female mediation of fertilization bias. It is unclear what ejaculate traits might also play a role in fertilization bias, but possibilities include sperm length, sperm velocity, and accessory gland proteins. This study provides the first documentation that females may exhibit different patterns of pre- and postcopulatory preferences for different male traits.

Most studies show that pre- and postcopulatory sexual selection act in concert, such that the same males are successful during both selection episodes. For example, in red flour beetles (*T. castaneum*), *Drosophila simulans*, and *D. bipectinata*, male precopulatory success is positively associated with sperm competition success (Lewis and Austad 1994; Hosken et al. 2008; Polak and Simmons 2009), while in guppies (*Poecilia reticulata)*, more ornamented (attractive) males sire more offspring when the sperm of two males are artificially inseminated in equal numbers (Evans et al. 2003). However, in water striders (*Gerris lacustris*), pre- and postcopulatory sexual selection cancel each other out as larger males are favored during precopulatory sexual selection and smaller males during the postcopulatory phase (Danielsson 2001). Moreover, Droge-Young et al. (2012) tracked fitness of isogenic populations across precopulatory, postcopulatory, and offspring viability episodes of selection and found no

511 correlates between precopulatory and postcopulatory selection but did find a relationship
512 between paternity success and offspring viability. Our results offer an explanation for these
513 conflicting findings: perhaps the relationship between pre- and postcopulatory selection depends
514 on the trait being examined. We found that male size was subject to similar precopulatory and
515 postcopulatory selection, but GFP line was favored by precopulatory selection (females remated
516 more quickly with GFP males) but not by postcopulatory selection (fertilization bias favored
517 RFP sperm). Moreover, Stage 1 CFC was employed with regards to male size, while Stage 2
518 selected for sperm label.

519 It is difficult to speculate on the adaptive significance of female preference for GFP
520 males without knowing what male trait females are selecting on. The GFP line contains a GFP-
521 protamine; GFP-3xP3 gene construct that allows visualization of sperm heads in conjunction
522 with a GFP eye marker. There is no evidence to suggest that females might select on sperm
523 protamine (a DNA packaging protein) or an eye marker that is only visible under fluorescence. It
524 is more likely that the transgenic construct is physically linked to a trait that females do select on,
525 such as a gene that influences cuticular hydrocarbon (CHC) profile (which has been shown to
526 affect mate choice in *D. simulans*; Sharma et al. 2012; Ingleby et al. 2013), courtship song
527 (Ritchie et al. 1999), wing interference pattern (Katayama et al. 2014), or genital morphology
528 (House et al. 2013). Whatever the underlying trait, fertilization bias favoring the GFP line gives
529 us a system for studying mechanisms of postcopulatory sexual selection in which male
530 phenotype is clearly defined, binary, and recognizable. Further studies are needed to determine
531 the genomic location of the transgene in GFP and RFP lines, how persistent the GFP preference
532 is across multiple generations, and what the fitness consequences are for males and females.

Regardless of its adaptive significance, female selection of an easily distinguishable male marker nevertheless provides a valuable opportunity to dissect precopulatory and postcopulatory female preference of multiple male traits with unprecedented resolution.

We found evidence that females adjust the timing of sperm ejection based on the comparison between the quality of their first and second mates. Females had shorter ejection times after remating when their second male was of lower quality than their first male (smaller), but no increase in ejection time when their second male was larger than their first male. In other words, ejection time only changed when the second male was perceived as a "step down". This result also reflects our previous finding that in hybrid competitive matings, female ejection time in *D. simulans* decreases to favor the conspecific male, but only when the heterospecific male is second to mate (again, a step down; Manier et al. 2013b). It is possible that the disparity in attractiveness based on male line is of a lesser degree than that based on male size, since females did not alter ejection time based on male line at all.

There are few studies that have examined how a female's first mate influences her remating behavior or preference for a second mate, but the available evidence shows no clear patterns (e.g., Byrne and Rice 2005). Pitnick (1991) tested several hypotheses explaining female remating pattern in *D. melanogaster*, including "Mate improvement", in which females remate more quickly with a higher quality (larger) male, and "Mate diversity", in which females will remate more quickly with a phenotypically different male. His results supported a "Courtship threshold hypothesis", in which females remate more quickly with larger males, presumably due to their more vigorous courtship (Partridge et al. 1987). Our results support an entirely different hypothesis that we call the "Poor male avoidance hypothesis", in which females adjust remating

behavior (ejection time) in response to a decrease, but not an increase (or no change), in male quality from her first mate to her second mate.

In summary, we found that different male traits are favored via different mechanisms at both the precopulatory and postcopulatory stages. Large males had a consistent advantage over both stages, with timing of sperm ejection the primary mechanism of cryptic female choice. In contrast, females mated more quickly with GFP males, but fertilization bias favored RFP males at the postcopulatory stage. Overall, we found that the superior sperm numbers of GFP males played a larger role in the competitive success of GFP sperm than fertilization bias favoring RFP sperm.

Compliance with Ethical Standards

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Conflict of interest: Authors Ala-Honkola and Manier declare they have no conflicts of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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Figure captions

Fig. 1 Mating rates for first (**a**) and second mating (**b**) for all treatments and GFP and RFP males (mean ± SE).

Fig. 2 **a** Mating latency and **b** remating latency for all treatments and GFP and RFP males (mean ± SE).

Fig. 3 P_2 for all treatments and GFP and RFP males (mean ± SE).

Fig. 4 Second-male ejaculate size for all treatments and GFP and RFP males (mean ± SE).

Fig. 5 Fertilization bias in the spermathecae (*x*) when the second male is GFP (**a**) and RFP (**b**), fertilization bias in the SR (*y*) when the second male is GFP (**c**) and RFP (**d**), and sperm storage organ use bias (*z*) when the second male is GFP (**e**) and RFP (**f**). Boxes (mean and 95% confidence intervals) overlapping with 0.5 indicate no significant bias; less than 0.5 indicates bias toward the first male (**a-d**) or spermathecae (**e-f**); and greater than 0.5 indicates bias toward the second male (**a-d**) or SR (**e-f**).

811 # Tables

Table 1 Percentages or mean ± SD, N for mating performance variables from the first mating by male type.

	L-GFP	S-GFP	L-RFP	S-RFP
Mating rate	94% (131/140)	97% (116/120)	96% (134/140)	93 % (112/120)
Mating latency (min)	53.2 ± 52.7, 130	58.6 ± 57.0, 115	69.7 ± 51.5, 133	65.5 ± 54.5, 112
Copulation duration (min)	24.3 ± 5.9, 130	26.5 ± 5.7, 115	25.0 ± 4.5, 133	28.6 ± 5.5, 112
First-male sperm transfer	1906 ± 527, 18	2216 ± 565, 17	2328 ± 412, 18	2035 ± 348, 17

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Table 2 Percentages or mean ± SD, N of reproductive and sperm storage traits measured in the second male transfer and /or the fertilization bias experiment.

	LL	SS	SL	LS
Remating rate	96% (94/98)	74% (71/96)	86% (84/98)	66% (82/125)
Remating latency (min)	152 ± 124, 94	202 ± 175, 71	164 ± 154, 84	263 ± 180, 80
Copulation duration in second mating (min)	26.9 ± 6.1, 94	28.2 ± 5.4, 71	27.3 ± 6.3, 84	28.5 ± 6.5, 80
Second-male sperm transfer	2032 ± 622, 34	1189 ± 661, 25	1869 ± 664, 29	1308 ± 716, 24
Ejection time (min)	114 ± 45.3, 32	86.8 ± 47.4, 25	92.6 ± 36.5, 35	93.7 ± 48.4, 29
Number of progeny produced before remating	52.7 ± 14.5, 38	61.9 ± 18.6, 25	64.0 ± 25.0, 38	66.3 ± 25.0, 33
Number of progeny produced after remating	105 ± 31.4, 38	103 ± 36.4, 25	116 ± 31.5, 38	105 ± 33.0, 33
P_2	0.85 ± 0.21, 38	0.83 ± 0.19, 25	0.85 ± 0.21, 38	0.71 ± 0.28, 33

S_2 SR	0.83 ± 0.32, 20	0.85 ± 0.19, 19	0.87 ± 0.24, 28	0.65 ± 0.36, 25
S_2 SPT	0.70 ± 0.43, 20	0.62 ± 0.34, 19	0.70 ± 0.34, 29	0.54 ± 0.38, 26

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Table 3. Optimal general linear models (based on AIC model selection) for factors explaining variance in traits measured at first mating (mating latency with virgin females, copulation duration and 1st male sperm transfer).

	Effect	Parameter estimate	SE	*t*-value	*p*	df residual
Mating latency (log_{10}-transformed)	Intercept (1st male GFP, sperm use bias experiment)	1.62	0.03	46.5	<0.001	487
	1st male RFP	0.16	0.04	4.2	**<0.001**	
	2nd male transfer experiment	-0.13	0.04	-3.2	**0.001**	
	1st male transfer experiment	-0.25	0.06	-4.4	**<0.001**	
Copulation duration (min)	Intercept (1st male L & GFP, sperm use bias experiment)	24.27	0.55	43.8	<0.001	485
	1st male S	2.17	0.69	3.1	**0.002**	
	1st male RFP	0.70	0.67	1.0	0.297	
	2nd male transfer experiment	-0.26	0.53	-0.5	0.626	
	1st male transfer experiment	1.26	0.74	1.7	0.088	
	1st male S×2nd male S	1.42	0.98	1.5	0.146	
1st male sperm transfer	Intercept	2121	58.5	36.3	<0.001	69

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Table 4. Optimal general linear models (based on AIC model selection) for factors explaining variance in remating latency, ejection time, 2nd male ejaculate size, number of progeny produced before remating and the number of progeny produced after remating during 3 days.

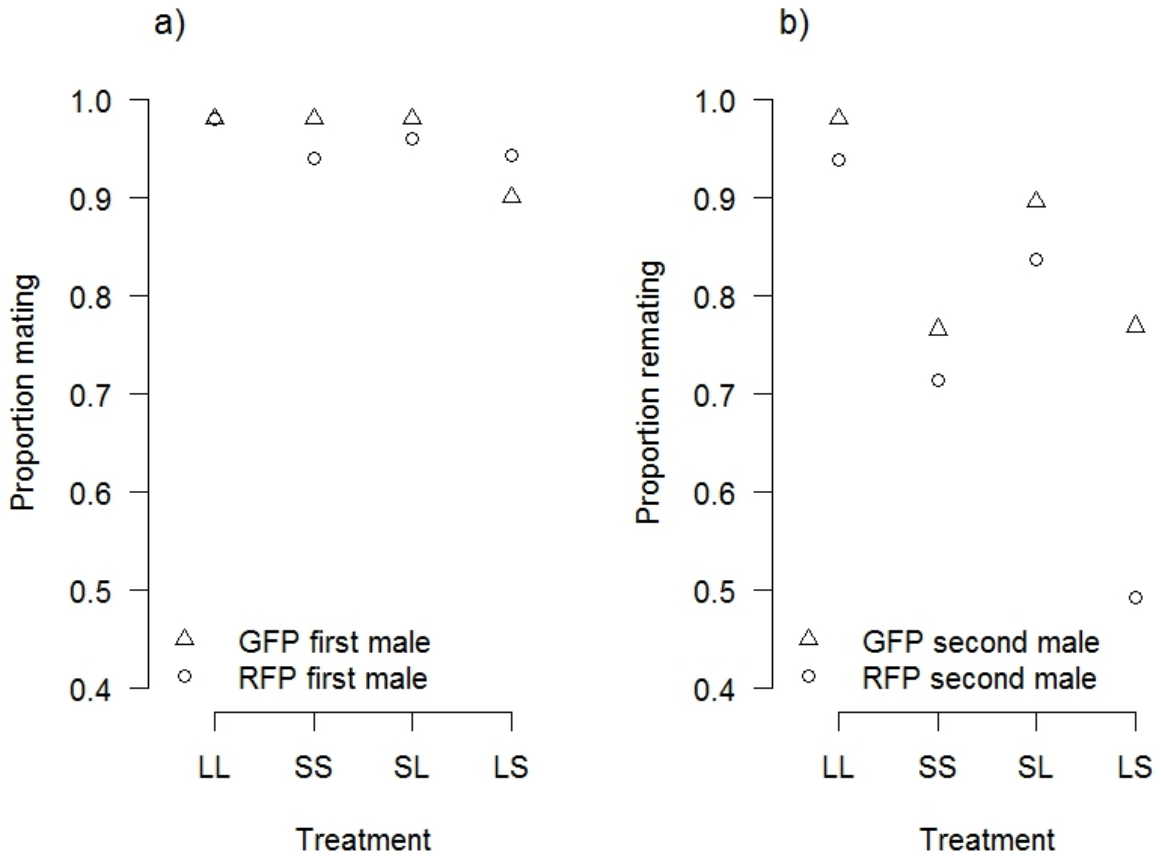
	Effect	Parameter estimate	SE	t-value	p	df residual
Remating latency (log10-transformed)	Intercept (1st male L, 2nd male L, 2nd male RFP)	2.09	0.05	39.0	<0.001	325
	1st male S	-0.03	0.07	-0.4	0.692	
	2nd male S	0.24	0.07	3.5	**<0.001**	
	2nd male line GFP	-0.11	0.05	-2.2	**0.027**	
	1st male S×2nd male S	-0.16	0.10	-1.6	0.119	
Copulation duration in 2nd mating (min)	Intercept (2nd male L, 2nd male RFP)	26.42	0.57	46.3	<0.001	326
	2nd male line GFP	1.26	0.67	1.9	0.063	
	2nd male S	1.23	0.67	1.8	0.068	
2nd male sperm transfer	Intercept (2nd male L, 2nd male RFP)	1594	93.5	17.1	<0.001	108
	2nd male S	-694	109	-6.4	**<0.001**	
	2nd male line GFP	679	108	6.3	**<0.001**	
Ejection latency after 2nd mating (log10-transformed)	Intercept (1st male L, 2nd male L)	2.02	0.03	78.9	<0.001	117
	1st male S	-0.07	0.03	-2.5	**0.015**	
	2nd male S	-0.07	0.03	-2.2	**0.028**	
Number of progeny produced before remating	Intercept	-45.43	53.9	-0.84	0.401	128
	Mating latency in remating	0.07	0.01	6.9	**<0.001**	
	Female thorax length	1.10	0.63	1.7	0.084	
Number of progeny produced after remating	Intercept	108.5	2.8	39.1	<0.001	130

Table 5. Full models (GLMs with quasibinomial error distribution) of factors explaining variance in P_2 (proportion of offspring sired by the second male to mate), S_2 SR (proportion of second male sperm in seminal receptacle) and S_2 SPT (proportion of second male sperm in spermathecae).

	Effect	Parameter estimate	SE	*t*-value	*p*	df residual
P_2	Intercept (1st male L, 2nd male L, 2nd male RFP, GFP male heterozygous)	-3.99	3.78	-1.1	0.293	123
	1st male S	-0.13	0.31	-0.4	0.672	
	2nd male S	-1.01	0.25	-3.3	**0.001**	
	2nd male line GFP	1.93	0.25	7.8	**<0.001**	
	Progeny produced before remating	0.001	0.01	0.21	0.835	
	Female thorax length	0.06	0.04	1.3	0.195	
	Homozygous GFP male	-0.02	0.25	-0.9	0.929	
	1st male S × 2nd male S	1.02	0.46	2.2	**0.027**	
S_2 SR	Intercept (2nd male L, 2nd male RFP, heterozygous GFP male)	1.53	5.56	0.3	0.784	82
	1st male S	0.11	0.46	-0.2	0.807	
	2nd male S	-1.31	0.54	-2.5	**0.016**	
	2nd male line GFP	2.40	0.33	7.2	**<0.001**	
	Progeny produced before remating	0.005	0.01	0.6	0.579	
	Female thorax length	-0.008	0.07	-0.1	0.903	
	Homozygous GFP male	-0.63	0.32	-2.0	0.052	
	1st male S × 2nd male S	0.90	0.71	1.3	0.208	
S_2 SPT	Intercept (1st male L, 2nd male L, 2nd male RFP, GFP male heterozygous)	-12.54	6.99	-1.8	0.076	84
	1st male S	-0.39	0.57	-0.7	0.491	
	2nd male S	-1.79	0.51	-3.5	**<0.001**	
	2nd male line GFP	1.36	0.36	3.7	**<0.001**	
	Progeny produced before remating	0.001	0.01	-0.1	0.912	
	Female thorax length	0.16	0.08	1.9	0.064	
	Homozygous GFP male	-0.02	0.36	-0.1	0.962	
	1st male S × 2nd male S	1.42	0.78	1.8	0.073	

Figures

Fig. 1



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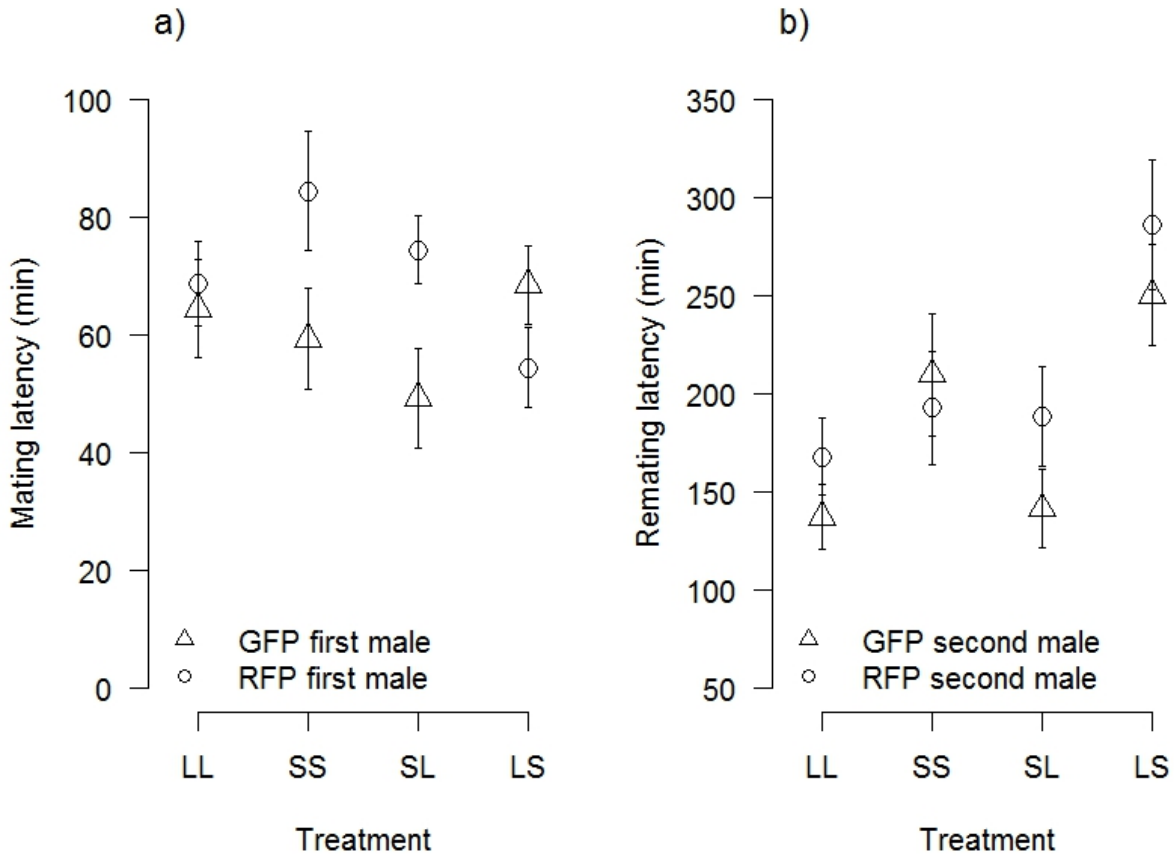

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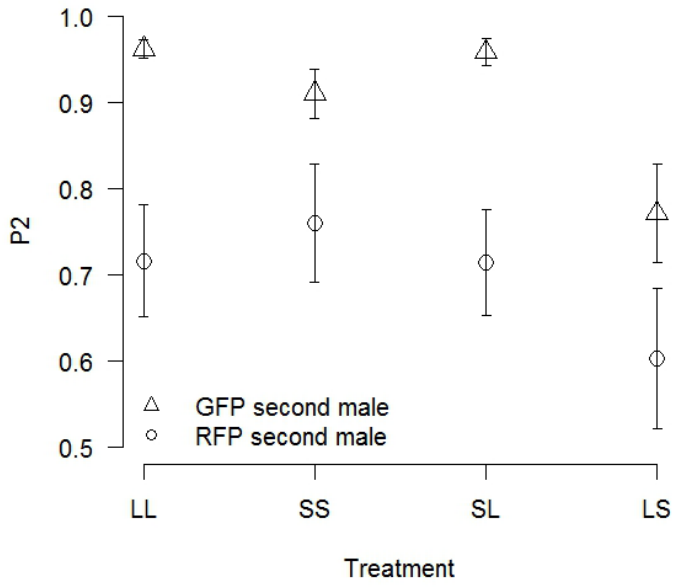
831 Fig. 3



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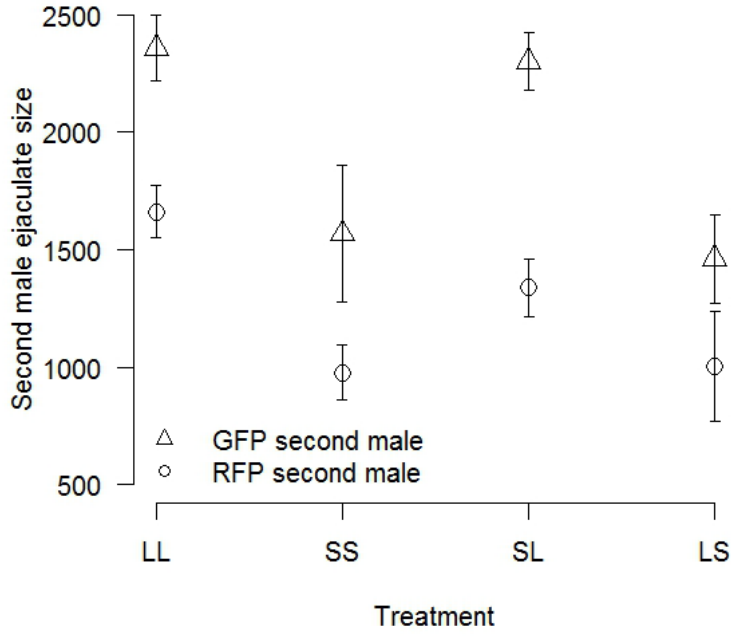

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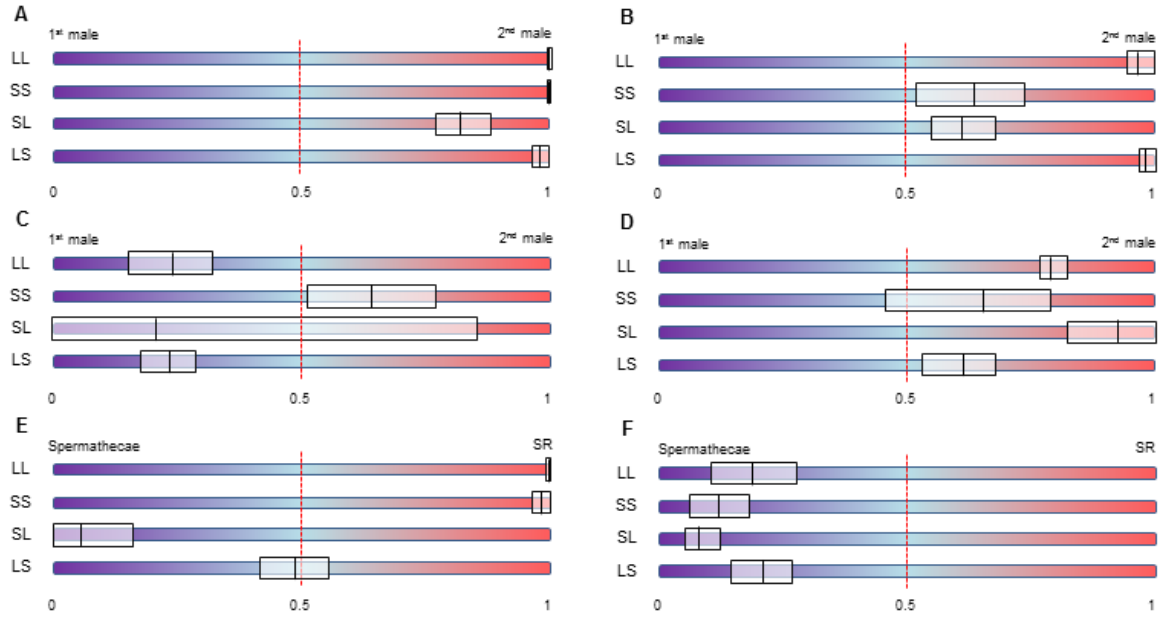
837 Fig. 5



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