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No evidence for external genital morphology affecting cryptic female choice and reproductive isolation in *Drosophila*

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Genitalia are one of the most rapidly diverging morphological features in animals. The evolution of genital morphology is proposed to be driven by sexual selection via cryptic female choice, whereby a female selectively uptakes and uses a particular male's sperm on the basis of male genital morphology. The resulting shifts in genital morphology within a species can lead to divergence in genitalia between species, and consequently to reproductive isolation and speciation. Although this conceptual framework is supported by correlative data, there is little direct empirical evidence. Here, we used a microdissection laser to alter the morphology of the external male genitalia in *Drosophila*, a widely used genetic model for both genital shape and cryptic female choice. We evaluate the effect of precision alterations to lobe morphology on both interspecific and intraspecific mating, and demonstrate experimentally that the male genital lobes do not affect copulation duration or cryptic female choice, contrary to long-standing assumptions regarding the role of the lobes in this model system. Rather, we demonstrate that the lobes are essential for copulation to occur. Moreover, slight alterations to the lobes significantly reduced copulatory success only in competitive environments, identifying precopulatory sexual selection as a potential contributing force behind genital diversification.

KEY WORDS: ***Drosophila mauritiana*, *Drosophila simulans*, genital coupling, posterior lobe, sexual selection.**

In species with females that mate with multiple males, selection on male genitalia may result from different postcopulatory mechanisms, including cryptic female choice (Thornhill 1983; Eberhard 1996; Kvarnemo and Simmons 2013), sperm competition (Parker 1970; Simmons 2001), and sexual conflict (Arnqvist and Rowe 2002; Tatarnic et al. 2014). Under the cryptic female choice hypothesis, a female may influence which male's sperm is used to fertilize her eggs on the basis of an evaluation of his genital morphology (De Wilde 1964; Robertson and Paterson 1982; Eberhard 1985, 1992; Robertson 1988). It has long been postulated that this mode of postcopulatory sexual selection can subsequently act as a reproductively isolating barrier between species, whereby females reject heterospecific males during copulation due to their divergent genital shape (Robertson 1983, 1988; Cobb et al. 1988; Eberhard 1992; Coyne 1993; Coyne and Orr 2004; Masly 2012).

One of the most widely studied groups for identifying the genetic basis of male genital divergence is the *Drosophila melanogaster* complex (Coyne et al. 1991; Liu et al. 1996; Macdonald and Goldstein 1999; Zeng et al. 2000; McNeil et al. 2011; House et al. 2013): *D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia* (Lachaise et al. 1988). Females and males of this species complex are very similar morphologically except for the shape of the flat, bilaterally projecting, and symmetrical posterior lobes of the male's external genital region (Fig. 1A, B; Coyne 1992), making these lobes a commonly used tool for distinguishing the species within the complex (Robertson 1983, 1988; Cobb et al. 1988; Coyne 1992; Liu et al. 1996). The lobes also make this species group an ideal model to evaluate the potential role of cryptic female choice on male genitalia. The lobes are external, cuticular, and are not used to transfer sperm, allowing for alterations of genital shape that are nonlethal and do

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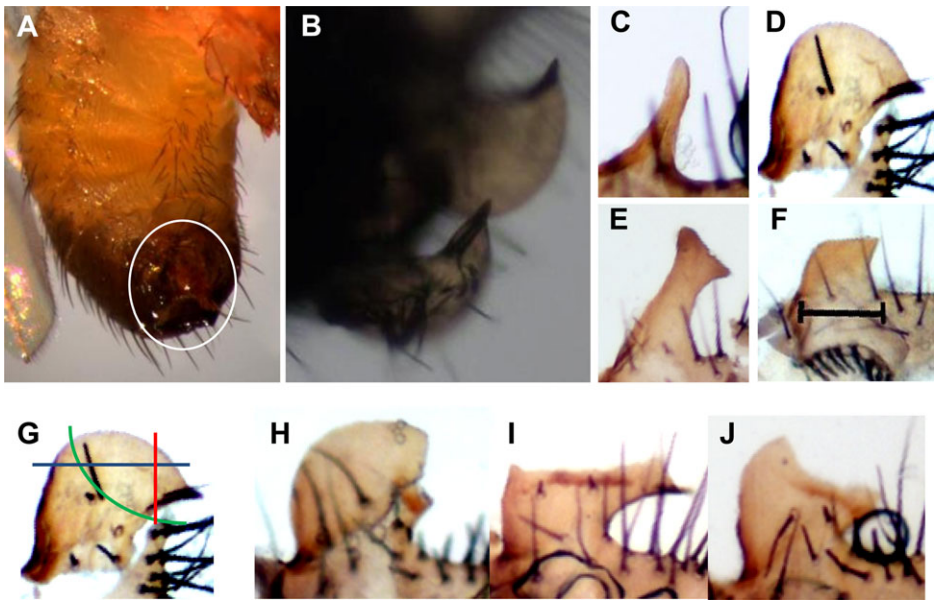

Figure 1. The *Drosophila* male posterior lobe. (A) The location of the *Drosophila* males' genital lobes, outlined in a white circle on the posterior male abdomen. (B) The two bilaterally symmetrical posterior lobes of *Drosophila simulans* males. (C–F) The species-specific lobe shapes in the *Drosophila melanogaster* species complex when dissected and laid flat: (C) *Drosophila mauritiana*, (D) *D. simulans*, (E) *Drosophila sechellia*, (F) *D. melanogaster*. (G–J) Alterations performed on both lobes of *D. simulans* males: (G) Unaltered lobe, with three colored lines indicating where the laser cut to remove lobe material, producing lobes similar to (H) tips (vertical red line), (I) crowns (horizontal blue line), and (J) severely altered (curved green line).

not induce sterility, thus by-passing the common historic barriers to studying the influence of cryptic female choice on genital shape (Arnqvist 1997; Arnqvist and Thornhill 1998). While they are not used as a conduit to transfer sperm, the lobes are inserted between the 8th and 9th tergite of the female and are thought to serve to stabilize copulation and assist in species recognition (Robertson 1988). As lobe shape is species-specific (Fig. 1C–F) and highly divergent between species (Robertson 1983, 1988; Cobb et al. 1988; Coyne 1992; Liu et al. 1996; Masly 2012), it is assumed that the lobes play a critical role in mating and have been under strong sexual selection, potentially through cryptic female choice (Coyne 1993; Coyne and Orr 2004; Masly 2012).

It has long been speculated that variation in lobe shape contributes to reproductive isolation among species within the *D. melanogaster* species complex. In particular, the species pair of *D. simulans* and *D. mauritiana* has been exemplified as a genetic model for both genital divergence (Coyne 1992, 1996; Liu et al. 1996; Zeng et al. 2000; Price et al. 2001) and cryptic female choice acting on male genital morphology (Coyne 1993; Coyne and Orr 2004; Masly 2012). *Drosophila mauritiana* males have a thin, stick-like lobe (Fig. 1C), while *D. simulans* males have a large helmet-shaped lobe (Fig. 1D). These species experience asymmetrical behavioral isolation: *D. mauritiana* females rarely mate with *D. simulans* males, while mating readily occurs in the reciprocal cross (Coyne 1989), but with a high frequency of violent female rejection-like behaviors immediately following the onset of copulation, such as kicking and bucking motions (Robertson 1983; Cobb et al. 1988; Coyne 1992). This *D. simulans* female rejection-like behavior is associated with (1) a reduced copulation duration of 5–8 min (Robertson 1983; Coyne 1993; Jagadeeshan and Singh 2006), which is markedly less than the length of either pure-species pairing (about 25 min, *D. simulans*; 15 min, *D. mauritiana*; Cobb et al. 1988), and (2) inadequate transfer of sperm (Coyne 1992, 1993; Manier et al. 2013a), presumably caused by insufficient copulation duration and/or incomplete external genital coupling (Jagadeeshan and Singh 2006; Manier et al. 2013b). Based on these observations, the *D. mauritiana* male's divergent lobe shape is thought to affect the duration of copulation once it has begun in this interspecies pairing (Cobb et al. 1988; Robertson 1988; Masly 2012). Here, we directly test the role of genital morphology for copulation and sexual selection both within and between species within the *D. melanogaster* complex. We experimentally manipulated the genital lobes using microscale laser surgery and tested the effect of surgical treatment on cryptic female choice, female rejection behaviors, copulation duration, copulation occurrence, and competitive mating success. The results demonstrate that the long-standing dogma regarding the functional role of the *Drosophila* genital lobes in interspecies mating and postcopulatory sexual selection appears to be incorrect for this model system: we found no evidence that the lobes

play a role in influencing either copulation duration or cryptic female choice. Our data support an alternative hypothesis, that the genital lobes play a role in precopulatory sexual selection.

Materials and Methods

STOCKS

Pure-species wild-type stocks of *D. simulans*, *D. sechellia* (obtained from the *Drosophila* Species Stock Center #14021–0251.199 and #14021–0248.28, respectively), *D. mauritiana* (Synthetic; SYN, obtained from J. Coyne), and newly caught isofemale lines of *D. melanogaster* from London Ontario (obtained from B. Sinclair), were maintained on standard cornmeal-agar-molasses medium. All flies were housed on a 14-h light:10-h dark cycle, 21–23°C, 70% relative humidity.

LASER ABLATION

Males from the four species in the *D. melanogaster* complex were collected as virgins and left to age 24 h. Alterations were performed by anaesthetizing males on ice, placing them on the microscope platform, and altering the lobe shape with a Zeiss Observer Z1 laser microscopy system using PalmRobo software (Zeiss, Heidelberg, Germany). A second group of males used in copulation duration tests were altered with pulsed laser light from a Vector 532–1000–20 laser (Coherent, Santa Clara, CA) and focused through a 20× objective lens of an Olympus IX71 inverted light microscope; during surgery, the fly was anesthetized with a light stream of humidified CO_2 in a Plexiglas chamber with a thin glass bottom (Polak and Rashed 2010). While the latter instrument was used to generate altered *D. simulans* males for tests of copulation duration, the former apparatus proved more efficient and effective and was thus used to repeat these assays and in all other experiments. After alteration, between five and 10 males of the same treatment and species were held in a vial together for two to five days, allowing them to reach sexual maturity (Markow 1996).

The large *D. simulans* lobe was altered in a variety of specific ways (Fig. 1G–J), with "slight alterations," involving removal of approximately 5–10% of the lobe; "moderate alterations," including any that involved removal of both tips (Fig. 1H) or alteration to both crowns (Fig. 1I); and "severe alterations," involving alterations to both the tip and the crown of the helmet structure (Fig. 1J). All other species' lobes were too small for this level of specificity, and "moderate alterations" involved the removal of approximately 15–25% of the lobe. For tests of copulation duration with *D. mauritiana* females, two treatments were performed as follows: (1) sham control males where males were handled the same as the altered males, and kept on the microscope platform for the same approximate length of time (approximately 2–3 min), except the laser was pulsed without altering the male; and (2) double-altered males had both posterior lobes altered or removed to produce a shape that was not species-specific. For tests of copulation duration in *D. simulans*, four treatments were performed as follows: (1) sham control males, where males were handled the same as the altered males, and kept on the microscope platform for the same approximate length of time (approximately 2–3 min), except the laser was pulsed without altering the male; (2) surgical control males where hair from the genital region were removed; (3) single-lobe altered males had one of the two posterior lobes altered or removed; and (4) double-altered males had both posterior lobes altered or removed to produce a shape that was not species-specific. After the courtship and copulation assays (see below) were completed, males with altered lobes were frozen at −20°C for later lobe visualization via dissection to confirm the alterations performed and their severity. Genital lobes were dissected in TE (Tris, EDTA) buffer and observed using an E100 Nikon compound microscope equipped with a 5 megapixel digital camera (Nikon Corporation, Tokyo, Japan). The Nikon software NIS-Elements 3.1 was then used to measure and photograph the alterations (Fig. 1 H–J).

SPERM STORAGE IN *D. SIMULANS*

To visualize sperm in the reproductive tract, we used *D. simulans* males harboring a transgene for protamine B linked to green fluorescent protein (GFP) (sim^{GFP}; genotype: *w+*; *pBac{3xP3-EGFP, ProtB-EGFP}11B*; provided by J. Belote and S. Pitnick, Syracuse University, NY); these males produce sperm with green fluorescent heads. Laser alterations were performed as outlined above: males were either altered by having the crown of the lobe removed (Fig. 1G) or sham controls. We did not use males with full lobe removal as copulation occurrence was too infrequent to allow for sufficient sample sizes. Virgin altered and sham control sim^{GFP} males aged four to seven days were individually paired with virgin *D. simulans* females aged four to seven days. For those pairs that copulated, copulation duration was recorded. Upon completion of copulations lasting at least 15 min, at which time sperm transfer is expected to be complete (Price et al. 2000), females were either immediately dissected (within 2–5 min; $N = 6$ altered, $N = 5$ unaltered), or were aspirated into a separate vial and stored for two days (46–49 h) prior to dissection ($N = 9$ altered, $N = 12$ unaltered). The female's reproductive tract was dissected on a glass slide into 30 μl of testis buffer (183 mM KCl, 47 mM NaCl, 10 mM Tris-HCl) using ultrafine tweezers (Roboz Dumont #5; Roboz Surgical Instrument Co., Gaithersburg, MD); the seminal receptacle (SR) was uncoiled to allow for better visualization, then the sample was gently squashed with a cover slip. Sperm were visualized on a Leica DMI6000 B inverted microscope (Leica Microsystems,Wetzlar, Germany); Z-stacking was used as needed to allow for complete visualization of sperm number. Sperm were

counted by two separate individuals using ImageJ software (version 1.47, National Institutes of Health, Bethesda, MD); these counts were highly correlated ($r = 0.995$), and so the average sperm number from the two counts was used.

COMPETITIVE FERTILIZATION SUCCESS IN *D. SIMULANS*

We employed a standard double-mating protocol (Simmons 2001), where randomly selected *D. simulans* females from a wild-type laboratory population were first mated to a virgin irradiated (IR) male and mated a second time to a virgin test male (either control or lobe-altered). The proportion of eggs laid after the second mating that hatched was attributed to the second male (i.e., P_2), and served as our metric of competitive fertilization success of test males (Simmons 2001; Manier et al. 2013a). The experiment was carried out as two time blocks in immediate succession.

At 12 h posteclosion, males were IR with a 150 Gy dose of gamma radiation from a ^{60}Co source (Polak and Simmons 2009). IR males are able to fertilize eggs, but the zygotes die and fail to hatch as a result of lethal mutations (Simmons 2001). In our experiment, hatch rate of eggs laid by females once mated to IR males was 0.089% (11 eggs hatched of a total of 12,664 eggs deposited), confirming that the 150 Gy dosage yielded a negligible hatch rate (Simmons 2001). Following irradiation, males were maintained in groups of 15–20 males in standard cornmeal-agar food vials until they were five days old. Males were then individually mated to 4-day-old virgin females. All matings in the experiment were conducted in food vials between 8 and 11:00 a.m. at 24.0–24.5°C and fluorescent lighting (lights were turned on at 7:50 a.m.).

Immediately after copulation with IR males, females were individually transferred to a fresh food vial, and all IR males preserved in alcohol for later thorax length measurement. After 48 h, all females were paired individually with test males in fresh food vials. Test males were from one of two experimental groups: sham control males or males with slight to moderate lobe alterations, matching the alterations performed in the sperm storage experiment. In cases where a female did not mate to a test male, the female and male were separated, and the same individuals paired again in a fresh food vial 48 h later. Females that again did not remate were separated, and held for an additional 48 h, and paired a third and final time with their test males. Females failing to mate in this third attempt were preserved in alcohol for later measurement. Thus, there were three time points (i.e., dates) separated by 48 h at which females were given the opportunity to remate. The variable pre-P_2 eggs for each female was determined as the total number of eggs deposited between her first and second matings. As expected, there was a highly significant positive effect of remating date on pre-P_2 eggs ($F_{2,89} = 23.72, P < 0.0001$); thus, pre-$P_2$ eggs and not mating date was entered into statistical models (described below) to avoid this collinearity. Copulation duration was ascertained for all matings as the time elapsed from the time the male achieved copulation to when the pair separated. We measured thorax length as an estimate of body size of all males and females. Lobe-altered males were dissected under a stereomicroscope in a drop of saline, and the severity of the alteration was categorized as mild (10–15%) or moderate (>15%) without knowledge of males' P_2 scores.

Females that remated were placed individually into food vials, and allowed to lay eggs. After 24 h, they were transferred to a fresh food vial and allowed to lay eggs for an additional 24 h. All eggs were counted immediately after females were removed from a vial. All larvae were counted after eggs were given sufficient time (>24 h) to hatch. P_2 was calculated as the number of eggs that hatched divided by the total number of eggs deposited by a given female (Boorman and Parker 1976; Simmons 2001). We obtained P_2 data on a total of 92 experimental males across the two blocks of the experiment; overall mean ± SE P_2 (arcsine square-root transformed) in our study was 0.804 ± 0.048, similar to a previous report for *D. simulans* (Manier et al. 2013a).

The second male in some cases ($n = 13$) failed to fertilize any eggs (P_2 values equaled 0; Boorman and Parker 1976). We therefore began our sequence of inferential steps by using multiple logistic regression to model the relationship between the probability of $P_2 = 0$ and the following terms: block, surgical treatment (sham or altered lobe), block × treatment interaction, copulation duration and thorax length of first (IR) male, copulation duration and thorax length of second (experimental) male, interaction between copulation durations of IR and second male, interaction between thorax lengths of IR and second male, pre-P_2 eggs, and female thorax length. Terms with $P > 0.1$ were sequentially eliminated from the model, beginning with the least significant term.

Next, we analyzed variation in P_2, excluding the 13 cases in which $P_2 = 0$ (there were two instances of $P_2 = 1$ in the dataset, which were not excluded). The data were first arcsine square-root transformed. An initial general linear model included the following terms expected to explain variation in P_2 (Polak and Simmons 2009): block, surgical treatment (sham or altered lobe), block × treatment interaction, copulation duration and thorax length of IR male, copulation duration and thorax length of experimental male, interaction between copulation durations of IR and second male, interaction between thorax lengths of IR and second male, and pre-P_2 eggs. We sequentially eliminated from the model terms with $P > 0.1$, beginning with the least significant term. The final reported model minimized the Akaike information criterion (AIC; Burnham and Anderson 2002), and consisted of one categorical term (treatment) and one covariate (pre-P_2 eggs). Although the treatment effect was associated with a P of 0.3, it was retained in the reported model because it was of central interest to the present study. In a follow-up analysis,

we reran the final model, but with the altered category expanded to include mild and moderate lobe alterations. Because P_2 data typically are overdispersed (e.g., Hunter and Birkhead 2002), as in the present study, for thoroughness a complementary generalized linear model was constructed (again maintaining mild and moderate alterations separated) using a binomial error distribution and logit link function, where the number of hatched eggs after second matings was the response variable and the total number of fertile eggs laid was the binomial denominator. The data were fitted with Firth's penalized maximum likelihood estimation and Pearson adjustment for overdispersion. Statistical analyses were conducted in JMP ver. 10.0 statistical software (SAS Institute, Cary, NC).

COURTSHIP AND COPULATION ASSAYS

Drosophila males from all treatments that survived for at least two days after the alterations, retained full locomotion abilities and appeared to have normal body condition (assessed qualitatively), were used in the behavior assays (approximately 5% of males were excluded). This was done to ensure that the males used in the experiment were viable and healthy mating options for virgin females of the same age.

Short-term courtship and copulation occurrence (within 1 h behavior assay) and copulation duration was scored in the first hour of "lights on" as this is when most copulation occurs (Coyne 1993). The flies were observed for 1 h in 3 dram (11 mL) vials that had been lightly misted with water to maintain humidity. Female rejection behavior was scored as being present if females displayed kicking or attempted to dislodge the male after a 1–2 min "settling in" period after copulation was initiated; in all observed instances, this behavior was pronounced when present.

Copulation occurrence over a longer period of time (long-term copulation occurrence) was assessed by larval presence for conspecific pairs of each species, as well as the interspecific pair of *D. simulans* with *D. mauritiana*. Virgin females were aged four to five days and vials were scored for larval presence to ensure no previous copulations had occurred. Two treatments were used as follows: (1) males with slight alterations to the lobes removing 5–10% of the lobe, and (2) males with both lobes removed. Males were then left individually with one virgin female for seven to 10 days. The presence of larvae was scored and the males' genital lobes were dissected. Larval presence was used as a proxy for successful reproduction, that is, for a male's ability to successfully court, copulate, and fertilize at least a portion of a female's clutch of eggs.

COMPETITIVE MATING ASSAYS IN *D. simulans*

For competitive mating assays, two treatments of *D. simulans* males were performed as follows: one with a single lobe with slight alterations and the other with both lobes with slight alterations. One of these two treatment males was individually placed into a vial with an unaltered control *D. simulans* male and a virgin *D. simulans* female (three flies total) and observed for 1 h. Only assays where both the treatment and control males were seen courting the female were scored. If copulation occurred during the assay, the unsuccessful male was removed to a separate vial and both lobes were dissected and scored to determine if the altered or unaltered male copulated.

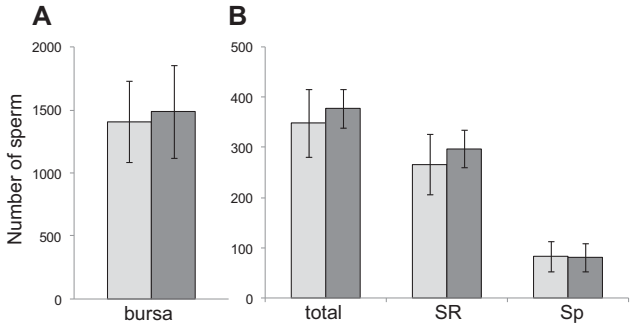

Figure 2. Male *Drosophila simulans* that were unaltered (light gray bars) or were slightly altered, with part of the crown of their posterior lobes removed (dark gray bars) were mated to females, and the number of sperm present in her bursa, seminal receptacle (SR), and the sum of the two spermathecae (Sp) were counted at 5 min (A) and two days (B). Sperm was only present in the bursa at 5 min and was only present in the SR and Sp at two days. Error bars are 95% CI.

Results

SPERM STORAGE IN *D. simulans*

To test whether divergence in lobe morphology affects differential sperm storage, we paired *D. simulans* females with altered or unaltered *D. simulans* males (with slight to moderate alterations), bearing a transgene causing the sperm head to fluoresce (Manier et al. 2013b), allowing us to count the sperm present in the female reproductive tract. Both altered and unaltered males transfer equal numbers of sperm (Fig. 2A), and alterations to the males' posterior lobes do not affect how many sperm are in the female sperm storage organs (Fig. 2B).

COMPETITIVE FERTILIZATION SUCCESS IN *D. simulans*

We then conducted a complementary experiment to test the effect of lobe alteration on male competitive fertilization success, measured as P_2 (Parker 1970; Simmons 2001). The surgical treatments were the same as those used in the sperm storage experiment, above. Logistic regression revealed no significant effect of surgical treatment on the probability of P_2 equaling zero (Table 1); for all covariates, the probability of P_2 equaling zero decreased over the range of data as indicated by the fact that all

Table 1. Results of logistic regression on the probability that the second male failed to fertilize any of a female's eggs in the competitive fertilization success assay (i.e., probability of $P_2 = 0$).

Predictor	χ^2	df	P	Log Odds Ratio
Block	1.926	1	0.165	.
Surgical treatment	0.869	1	0.351	.
Block × treatment	1.885	1	0.170	.
Pre-P_2 eggs	3.610	1	0.057	0.0200
Thorax length male 1	9.152	1	0.0025	0.00145
Copulation duration male 2	4.676	1	0.0306	0.00941

Range odds ratios are provided for significant and near-significant predictors.

range odds ratios were <1 (Table 1). Next, we analyzed continuous variation in P_2 (without zero values in the dataset), and found that, although there was a significant positive effect of pre-P_2 eggs on P_2 (slope ± SE, 0.0064 ± 0.0015, $F_{1,76} = 19.03$, $P < 0.0001$), there was no effect of surgical treatment on P_2 ($F_{1,76} = 0.0463$, $P = 0.83$; mean ± SE, lobe altered: 0.945 ± 0.0541; sham control: 0.929 ± 0.0495). We reran the analysis after expanding the surgical treatment categories to include slight and moderate alterations, and likewise found no significant effect of surgical treatment on P_2 ($F_{1,75} = 0.0775$, $P = 0.93$; Fig. S1). This outcome was confirmed in a generalized linear model with binomial errors (treatment effect, $\chi^2 = 0.0940$, df = 2, $P = 0.95$; pre-P_2 eggs effect, $\chi^2 = 26.24$, df = 1, $P < 0.0001$).

COPULATION DURATION BETWEEN MALE *D. mauritiana* AND FEMALE *D. simulans*

If the smaller lobe size of male *D. mauritiana* is responsible for the shortened copulation duration with female *D. simulans*, we predict that removal of the lobe would shorten copulations between these species even further. We found that full removal of both *D. mauritiana* posterior lobes did not further reduce copulation duration with *D. simulans* females (mean ± SE: 5.61 ± 1.84; $N = 9$) compared to what is observed when *D. mauritiana* males are unaltered (mean ± SE: 5.48 ± 0.95; $N = 12$; Mann–Whitney U: $z = -0.25$, $P = 0.803$; and see Cobb et al. 1988; Robertson 1988).

COPULATION DURATION IN *D. simulans*

To complement the interspecies results, above, we tested for the effect of altering the lobe size and shape in male *D. simulans* in intraspecific pairings. Altering *D. simulans* male lobes has the benefit of isolating the effect of lobe morphology on copulation duration, as these males should display all expected *D. simulans* mating characteristics with the exception of the shape and size of their genital lobes. The alteration of the lobes did not affect male

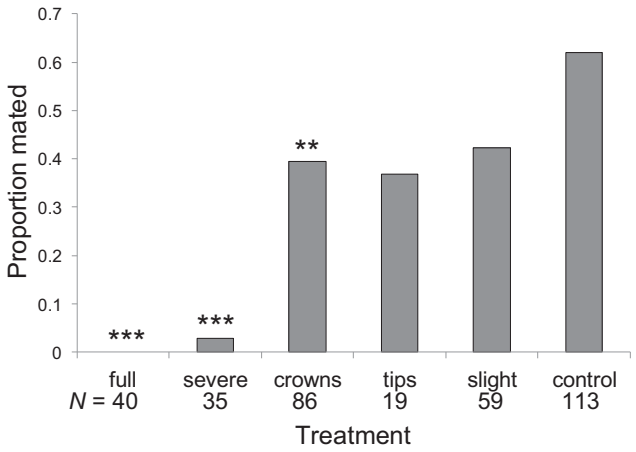

Figure 3. The proportion of *D. simulans* males who copulate with conspecific females when placed in a 1-h mating assay. Treatment groups were full lobe removal of both lobes, severe alterations to both lobes, crowns removed from both lobes, tips removed from both lobes, removal of only 5–10% of the lobe (slight), and sham surgery controls. ***P<0.0001, **P<0.005.

courtship occurrence (altered: $N = 154$, 64% courtship; surgical control: $N = 31$, 61% courtship; sham control: $N = 25$, 64% courtship), indicating that the surgery itself did not have a general effect on a male's ability or willingness to initiate courtship.

When *D. simulans* females were placed with *D. simulans* males that had the crowns or tips of the lobe removed (Fig. 1H, I, respectively), we found no effect on copulation duration (analysis of variance [ANOVA], $N = 101$, $F = 0.124$, df = 3, $P = 0.921$; Fig. S2). These results were replicated in a different laboratory (that of M. Polak) using a different laser apparatus, and similar results were obtained (altered: $N = 13$, duration mean ± SE = 25.59 ± 1.32; surgical control: $N = 8$, 26.36 ± 1.71; sham control: $N = 9$, 27.88 ± 1.38; $F = 0.709$, df = 2, $P = 0.50$).

COURTSHIP AND COPULATION ASSAYS

Our observations of courtship indicated that male and female genitals come into contact during copulation attempts, providing an opportunity for the female to assess the male's lobe shape and/or size prior to copulation. To assess copulation occurrence over shorter, and more biologically relevant, timescales, we altered both posterior lobes in *D. simulans* males to varying degrees, including slight alterations and more dramatic alterations (Fig. 1G–J; Polak and Rashed 2010). We then paired these males with conspecific females, and scored copulation occurrence in 1-h no-choice behavior assays. We found significantly reduced copulation occurrence (Fig. 3) for males with full lobe removal of both lobes (Fisher's exact test: $P < 0.0001$), severe alterations to both lobes (Fig. 1J; $P < 0.0001$), or crowns removed from both lobes (Fig. 1I; $P = 0.0025$). The less severe alterations of tips removed from both lobes (Fig. 1H; $P = 0.047$), and removal of

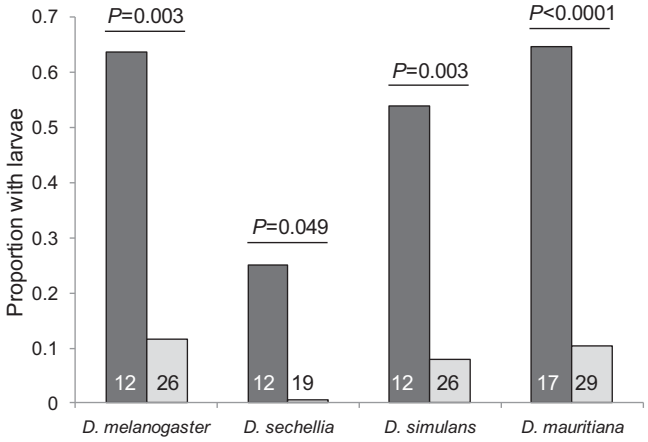

Figure 4. **The effect of posterior lobe alterations on reproductive success in conspecific pairings of *Drosophila* when males have slightly altered posterior lobes (dark gray) or full lobe removal (light gray; see Section "Methods"). Vials were scored for the proportion containing larvae after seven days. *N* is listed on each bar. Significant differences in reproductive success are listed above each comparison. The lack of larvae can be contributed to a lack of copulation, rather than shortened copulation duration, since shortened copulation duration has only been reported for the *Drosophila simulans–Drosophila mauritiana* interspecies pairing. Additionally, no mating was observed in 1-h mating assays for lobeless males who were courting females (*Drosophila melanogaster*: 0/26, *D. simulans*: 0/25, *D. mauritiana*: 0/29, *Drosophila sechellia*: 0/19), despite multiple copulation attempts, while many matings were observed for pairs where lobes were only slightly altered (8/14, 16/27, 6/20 and 7/15, respectively).**

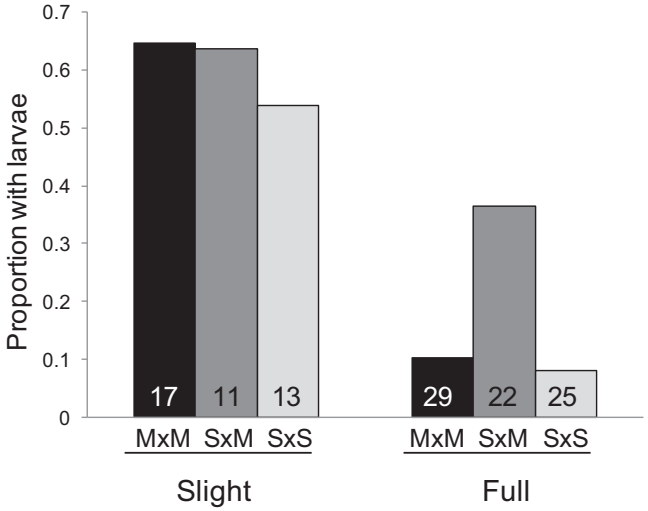

Figure 5. **The proportion of vials with larvae after seven to 10 days when the posterior lobes of males were slightly altered or fully removed, within one common experiment. Black, *Drosophila mauritiana* females with *D. mauritiana* males; dark gray, *Drosophila simulans* females with *D. mauritiana* males; light gray, *D. simulans* females with *D. simulans* males. Note that no copulations occur with the reciprocal interspecies pairing, and so we did not perform these assays. Fertilization success was significantly higher with only slight alterations compared to full lobe removal for pure-species *D. mauritiana* (black; $P = 0.0024$) and *D. simulans* (light gray; $P = 0.023$) pairings, but not for the interspecies pairing (dark gray; $P = 0.215$). When males have full lobe removal, *D. simulans* females mate significantly more with *D. mauritiana* than *D. simulans* males ("full," dark gray vs. light gray; $P = 0.049$). *N* for each group is listed over the corresponding bar.**

only 5–10% of the lobe ($P = 0.016$) did not significantly affect copulation occurrence after correction for multiple tests (Fig. 3). We then tested all of the members of the *melanogaster* complex for long-term copulation occurrence, and found that, in all cases, severe alterations to lobe shape significantly reduced reproductive success (Fig. 4).

We scored for the presence of female rejection behavior during copulation, and found that *D. simulans* females rarely displayed rejection behavior during copulation toward lobeless *D. simulans* males (3/44), while they displayed a high frequency of rejection behavior during copulation toward unaltered (10/11) and lobeless (17/17) *D. mauritiana* males (Fisher's exact test: $N = 72$, $P < 0.0001$), demonstrating that the lack of lobes does not itself induce female rejection behavior. The three conspecific pairs where females displayed rejection behavior toward lobeless males had copulation durations that were sufficient for sperm transfer to occur for fertilization (13.16, 15.38, and 25.01 min; Robertson 1988). It should be noted that females either displayed strong rejection behavior (as demonstrated by the female vigorously kicking or attempting to dislodge the male after a 1–2 min "settling in" period after copulation was initiated), or females displayed no rejection behavior, making this an easily scored, binomial trait.

LONG-TERM COPULATION OCCURRENCE ASSAYS

To test whether male copulation success over longer periods of time is affected by lobe shape, we first paired a single *D. mauritiana* or *D. simulans* male with slightly altered (removing only 5–10% of the lobe, which is within the outer limit of variation in lobe shape within the species; Coyne 1992; Liu et al. 1996) or fully removed lobes with a single conspecific or heterospecific female. We housed them together for seven to 10 days, which allows for repeated copulation attempts by the same male and scored the proportion of vials with larvae (as a proxy for successful reproduction). Reproductive success was significantly higher with slight alterations compared to full lobe removal for pure-species *D. mauritiana* (Mann–Whitney *U*: $z = -3.04$, $P = 0.0024$) and *D. simulans* ($z = -2.28$, $P = 0.023$) pairings, but there was no difference in reproductive success between these two treatments within the interspecies pairing ($z = -1.24$, $P = 0.215$; Fig. 5). The results also showed that *D. simulans* females had

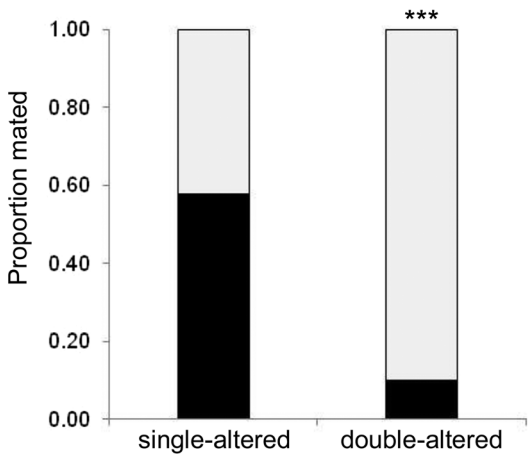

Figure 6. The proportion of single-altered (one lobe altered) or double-altered (both lobes altered) *Drosophila simulans* males who achieved copulation with conspecific females when placed in competition with unaltered *D. simulans* males. The single-altered male copulated as frequently as expected if females did not discriminate against altered males when in competition with unaltered males (56 vs. 50%; binomial test: $N = 19$, $P = 0.65$). In contrast, double-altered males copulated less frequently than expected (10 vs. 50%; binomial test: $N = 20$, $P < 0.0001$).

significantly higher reproductive success when paired with altered *D. mauritiana* males (36%; $N = 22$) than with altered conspecific males (8%; $N = 25$; Mann–Whitney U: $z = -1.65$, $P = 0.049$; Fig. 5).

We also individually paired approximately 30–40 altered *D. simulans* males (half were lobeless and half had varying degrees of alterations) with *D. mauritiana* females to qualitatively evaluate whether altering lobe shape or removing the lobes would eliminate the reproductive barrier between *D. mauritiana* females and *D. simulans* males; none of the vials had larvae, indicating that successful reproduction did not occur.

COMPETITIVE MATING ASSAYS IN *D. simulans*

It is possible that the "slightly" altered males are only able to gain copulations because the females are placed with the males in a no-choice experiment. We therefore also tested whether variation in *D. simulans* lobe morphology would eliminate a male's ability to gain copulations if he was placed in competition with another male (Grieshop and Polak 2012). Slight lobe alterations significantly reduced a male's success at achieving copulations when placed in competition, but only when both lobes were altered (binomial test: $N = 20$, $P < 0.0001$; Fig. 6), indicating that one intact lobe is sufficient to allow for copulation to occur at normal levels, even in a competitive environment, and that the laser surgery itself is not responsible for the reduction in mating observed in males that have both lobes altered.

Discussion

Drosophila mauritiana and *D. simulans* exhibit behavioral isolation in interspecies pairings whereby females of the former species refuse copulation attempts and females of the latter species reduce copulation duration when placed with heterospecific males (Robertson 1983; Cobb et al. 1988; Coyne 1989, 1992). This behavioral isolation is thought to be influenced by divergence in the male's external genitalia (Robertson 1988; Coyne 1993; Coyne and Orr 2004; Masly 2012): *D. mauritiana* has a stick-like shape, while *D. simulans* resembles a helmet (Fig. 1A–D). We used a microdissection laser to test the role of genital morphology in copulation and sexual selection in these species in four ways: (1) intraspecific effect on postcopulatory sexual selection, (2) interspecific effect on copulation, (3) intraspecific effect on copulation, and (4) intra- or interspecific effect on precopulatory sexual selection.

First, we tested whether the lobe is under postcopulatory sexual selection within *D. simulans*. *Drosophila simulans* females typically eject sperm out of the bursa within a few hours after copulation (Manier et al. 2013b), and the timing of this ejection can influence how many sperm make their way from the bursa into the spermathecae or SR sperm storage organs, affecting a male's direct fertilization success as well as displacement of competitor sperm (Lüpold et al. 2013). In the interspecies pairing of *D. simulans* females with *D. mauritiana* males, the females eject the sperm significantly more rapidly (Manier et al. 2013a), and significantly fewer sperm are transferred into the sperm storage organs, than when these females are paired with conspecific males (Price et al. 2001; Manier et al. 2013a,b). These females have also been shown to selectively fertilize eggs with conspecific sperm over heterospecific sperm (Manier et al. 2013a) as another mechanism of cryptic female choice. It is possible that variation in genital lobe morphology plays a role in the above mechanisms of cryptic female choice.

We measured the quantity of sperm present in the female sperm storage organs of *D. simulans* and found that alteration of genital morphology did not affect the location or total quantity of sperm stored. We then tested whether females exhibited preferential use of sperm based on divergence in male genital shape. We predicted that if the genital lobe is the target of postcopulatory sexual selection, then P_2 would be significantly lower for the altered males compared to controls. We found no significant effect of genital lobe treatment on paternity. Thus, the data weaken the hypothesis that lobe morphology is the target of cryptic female choice in *D. simulans* (for a description of additional possible mechanisms of cryptic female choice, see: Eberhard 2011). By extension, the data suggest that the lower rate at which *D. simulans* females store and use sperm from *D. mauritiana* males (Price et al. 2001; Manier et al. 2013a) is not due to the morphology of the posterior lobe.

We also tested whether lobe shape underlies the reduced copulation duration of the interspecies pairing between *D. mauritiana* males and *D. simulans* females, and found that further reduction of *D. mauritiana* male lobe size did not significantly affect copulation duration. However, it is possible that we did not observe a reduction in copulation duration in the above pairings because the lobes of *D. mauritiana* are already below a threshold size required for normal copulation with female *D. simulans*, so that greater reduction in lobe size would not be expected to reduce copulation duration further.

We then tested the intraspecific effect of lobe morphology on copulation duration by pairing *D. simulans* females with lobe-altered *D. simulans* males and measuring copulation duration. These pairings have the advantage over interspecies combinations, in that males should exhibit all species-specific mating signals, with the exception of stimuli delivered by intact lobes. Again, we found no effect of genital lobe manipulation on copulation duration. Thus, a *D. simulans* female does not shorten copulation duration in response to an aberrant male lobe, contrary to what would be expected if lobe shape or size is the primary cue for copulation duration in either the intraspecies or interspecies pairings. Interestingly, in the three conspecific pairs in which females displayed rejection behavior toward lobeless males, the copulation durations were still long enough for sufficient sperm transfer to occur for fertilization (Robertson 1988), indicating that the lobes themselves are not likely to be mechanically necessary for maintaining the genitals together once copulation has begun, even in the presence of strong female rejection behavior during copulation. Therefore, the posterior lobes either act as a secondary cue that is only assessed when other traits are aberrant, or do not act as cues for copulation duration at all. Our conclusions align with another study of intra- and interspecific mating behavior in the *D. melanogaster* species complex (Jagadeeshan and Singh 2006), which concluded, based on nonexperimental data, that copulation duration is likely largely dictated by factors other than the genital lobes.

We have thus demonstrated that divergent lobe morphology is unlikely to be the proximate factor responsible for the shortened copulation duration and overt rejection behavior exhibited by female *D. simulans* in interspecies pairing with male *D. mauritiana*. That genital morphology does not play a role in copulation duration runs counter to long-held beliefs for this species pair (Cobb et al. 1988; Robertson 1988; Coyne and Orr 2004; Masly 2012). Furthermore, removal of the *D. simulans* lobes does not eliminate reproductive isolation with *D. mauritiana* females, indicating that the increased size of the *D. simulans* lobe is not the primary barrier to this interspecies pairing.

We also measured whether the lobe is important for copulation to occur, and whether the lobe may be under precopulatory sexual selection. Although less commonly studied than genital shape and postcopulatory sexual selection, precopulatory sexual selection on genital shape has been previously demonstrated in both invertebrates (Michiels 1998; Bertin and Fairbain 2005; Polak and Rashed 2010; Grieshop and Polak 2012) and vertebrates (Langerhans et al. 2005; Kahn et al. 2010; Mautz et al. 2013). During our assays of copulation duration for lobe-altered males, detailed above, we often had to assay an excessive number of pairs to observe a copulation event, particularly for males with full lobe removal, indicating that the proper lobe morphology might be required for either female acceptance or mechanical pairing of the genitals. If the species-specific lobe morphology is necessary for copulation to occur, either through female discrimination or through mechanical incompatibility or insufficiency, we would expect males that have deviant lobe morphology to have a significantly reduced frequency of copulation. We would also expect that the frequency of copulation would be negatively correlated with the severity of alteration: males with severely deviant lobes would gain fewer copulations than males with only slight modifications to the lobes. Indeed, our results support both of these predictions (Figs. 3 and 5). However, males from all but the most severe two groups of alterations still achieved copulation approximately 40% of the time (Fig. 3). Although these males with "slight" alterations achieved copulation in a no-choice assay, their copulation success was significantly lowered when females were each placed with two males, one altered and one unaltered (Fig. 6). Thus, severe alterations almost eliminated copulation occurrences entirely, while slight to moderate variations in lobe morphology had a more subtle effect on a male's copulation success, with the strongest effect occurring when he was placed in competition.

Surprisingly, our results also show that *D. simulans* females mated significantly less often with altered conspecific *D. simulans* males than altered heterospecific *D. mauritiana* males (Fig. 5). What may explain these counterintuitive findings? A previous report indicated that *D. mauritiana* males attempt copulation sooner and more aggressively than other members of their subgroup (Robertson 1983). Thus, a possible explanation for these results is that the aggressive behavior of *D. mauritiana* males bypasses female *D. simulans* ability to resist mating attempts.

In summary, our results indicate that lobe morphology in this species pair (1) does not affect duration of interspecies mating, (2) does not affect duration of conspecific mating, (3) is not the target of postcopulatory sexual selection as measured by sperm storage and sperm use in fertilization, and (4) affects copulation occurrence and is thus under precopulatory sexual selection. In a separate laser ablation experiment in *Drosophila bipectinata*, the male's genital spines were also found to affect copulation occurrence, but not postcopulatory success (Polak and Rashed 2010), suggesting that external genitalia may be under precopulatory selection across multiple species of *Drosophila*. Since the lobes do not appear to play a role in well-defined aspects of copulatory

and postcopulatory sexual selection in the species pair, we examined, at least within a laboratory setting, further study is needed into the underlying cues involved in these processes. It will also be important to distinguish across this species group whether the lobes are used purely as a precopulatory device to achieve genital coupling, a cue by females in precopulatory mate assessment, or both. The genital lobes in the *D. melanogaster* species complex warrant more detailed exploration in this context.

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LITERATURE CITED

Arnqvist, G. 1997. The evolution of water strider mating systems: causes and consequences of sexual conflicts. Pp. 146–163 *in* J. C. Choe, and B. J. Crespi, eds. The evolution of mating systems in insects and arachnids. Cambridge Univ. Press, Cambridge.

Arnqvist, G., and L. Rowe. 2002. Sexual conflict. Princeton Univ. Press, Princeton, NJ.

Arnqvist, G., and R. Thornhill. 1998. Evolution of animal genitalia: patterns of phenotypic and genotypic variation and condition dependence of genital and non-genital morphology in water strider (Heteroptera: Gerridae: Insecta). Genet. Res. 71:193–212.

Bertin, A., and D. J. Fairbairn. 2005. One tool, many uses: precopulatory sexual selection on genital morphology in *Aquarius remigis*. J. Evol. Biol. 18:949–961.

Boorman, E., and G. A. Parker. 1976. Sperm (ejaculate) competition in *Drosophila melanogaster*, and the reproductive value of females to males in relation to female age and mating status. Ecol. Entomol. 1:145–155.

Burnham, K. P., and D. R. Anderson. 2002. Model selection and multimodel inference. 2nd ed. Springer, New York.

Cobb, M., B. Burnet, and K. Connolly. 1988 Sexual isolation and courtship behavior in *Drosophila simulans*, *D mauritiana*, and their interspecific hybrids. Behav. Genet. 18:211–225.

Coyne, J. A. 1989. Genetics of sexual isolation between two sibling species, *Drosophila simulans* and *Drosophila mauritiana*. Proc. Natl. Acad. Sci. USA 86:5464–5468.

———. 1992. Genetics of sexual isolation in females of the *Drosophila simulans* species complex. Genet. Res. 60:25–31.

———. 1993. The genetics of an isolating mechanism between two sibling species of *Drosophila*. Evolution 47:778–788.

———. 1996. Genetics of sexual isolation in male hybrids of *Drosophila simulans* and *D mauritiana*. Genet. Res. 68:211–220.

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer and Associates, Sunderland, U.K.

Coyne, J. A., J. Rux, and J. R. David. 1991. Genetics of morphological differences and hybrid sterility between *Drosophila sechellia* and its relatives. Genet. Res. 57:113–122.

De Wilde, J. 1964. Reproduction. pp 9–58 *in* M. Rockstein, ed. Physiology of insecta. Vol 1. Academic Press, New York.

Eberhard, W. G. 1985. Sexual selection and animal genitalia. Harvard Univ. Press, Cambridge, MA.

———. 1992. Species isolation, genital mechanics, and the evolution of species-specific genitalia in three species of *Macrodactylus* beetles (Coleoptera, Scarabeidae, Melolonthinae). Evolution 46:1774–1783.

———. 1996. Female control: sexual selection by cryptic female choice. Princeton Univ. Press, Princeton, NJ.

———. 2011. Experiments with genitalia: a commentary. Trends. Ecol. Evol. 26:17–21.

Grieshop, K., and Polak, M. 2012. The precopulatory function of male genital spines in *Drosophila ananassae* [Doleschall] (Diptera: Drosophilidae) revealed by laser surgery. Evolution 66:2637–2645.

House, C. M., Z. Lewis, D. J. Hodgson, N. Wedell, M. D. Sharma, J. Hunt, and D. J. Hosken. 2013. Sexual and natural selection both influence male genital evolution. PLoS One 8:e63807.

Hunter, F. M., and T. R. Birkhead. 2002. Sperm viability and sperm competition in insects. Curr. Biol. 12:121–123.

Jagadeeshan, S., and R. S. Singh. 2006. A time-sequence functional analysis of mating behaviour and genital coupling in *Drosophila*: role of cryptic female choice and male sex-drive in the evolution of male genitalia. J. Evol. Biol. 19:1058–1070.

Kahn, A. T., B. Mautz, and M. D. Jennions. 2010. Females prefer to associate with males with longer intromittent organs in mosquitofish. Biol. Lett. 6:55–58.

Kvarnemo, C., and L. W. Simmons. 2013. Polyandry as a mediator of sexual selection before and after mating. Philos. Trans. R. Soc. Lond. B 368:20120042.

Lachaise, D., M. L. Cariou., J. R. David, F. Lemeunier, L. Tsacas, and M. Ashburner. 1988. Historical biogeography of the *Drosophila melanogaster* species subgroup. Evol. Biol. 22:159–225.

Langerhans, R. B., C. A. Layman, and T. J. DeWitt. 2005. Male genital size reflects a tradeoff between attracting mates and avoiding predators in two live-bearing fish species. Proc. Natl. Acad. Sci. USA 102:7618–7623.

Liu, J., J. M. Mercer, L. F. Stam, G. C. Gibson, Z. B. Zeng, and C. C. Laurie. 1996. Genetic analysis of a morphological shape difference in the male genitalia of *Drosophila simulans* and *D mauritiana*. Genetics 142:1129–1145.

Lüpold, S., S. Pitnick, K. S. Berben, C. S. Blengini, J. M. Belote, and M. K. Manier. 2013. Female mediation of competitive fertilization success in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 110:10693–10698.

Macdonald, S. J., and D. B. Goldstein. 1999. A quantitative genetic analysis of male sexual traits distinguishing the sibling species *Drosophila simulans* and *D sechellia*. Genetics 153:1683–1699.

Manier, M. K., S. Lüpold, J. M. Belote, W. T. Starmer, K. S. Berben, O. Ala-Honkola, W. F. Collins, and S. Pitnick. 2013a. Postcopulatory sexual selection generates speciation phenotypes in *Drosophila*. Curr. Biol. 23:1853–1862.

Manier, M. K., J. M. Belote, K. S. Berben, S. Lüpold, O. Ala-Honkola, W. F. Collins, and S. Pitnick. 2013b. Rapid diversification of sperm precedence traits and processes among three sibling *Drosophila* species. Evolution 67:2348–2362.

Markow, T. A. 1996. Evolution of *Drosophila* mating systems. Evol. Biol. 29:73–106.

Masly, J. P. 2012. 170 Years of "lock-and-key": genital morphology and reproductive isolation. Int. J. Evol. Biol. 2012:247352.

Mautz, B. S., B. B. Wong, R. A. Peters, and M. D. Jennions. 2013. Penis size interacts with body shape and height to influence male attractiveness. Proc. Natl. Acad. Sci. USA 110:6925–6930.

McNeil, C. L., C. L. Bain, and S. J. Macdonald. 2011. Multiple quantitative trait loci influence the shape of a male-specific genital structure in *Drosophila melanogaster*. G3 (Bethesda) 1:343–351.

Michiels, N. 1998. Mating conflicts and sperm competition in simultaneous hermaphrodites. *In* T. Birkhead and A. P. Moller, eds. Sperm competition and sexual selection. Academic Press, New York, NY.

Parker, G. A. 1970. Sperm competition and its evolutionary consequences in insects. Biol. Rev. Camb. Phil. Soc. 45:525–567.

Polak, M., and A. Rashed. 2010. Microscale laser surgery reveals adaptive function of male intromittent genitalia. Proc. R. Soc. Lond. Biol. Sci. 277:1371–1376.

Polak, M., and L. W. Simmons. 2009. Secondary sexual trait size reveals competitive fertilization success in *Drosophila bipectinata* Duda. Behav. Ecol. 20:753–760.

Price, C. S. C., C. H. Kim, J. Posluszny, and J. A. Coyne. 2000. Mechanisms of conspecific sperm precedence in *Drosophila*. Evolution 54:2028–2037.

Price, C. S., C. H. Kim, C. J. Gronlund, and J. A. Coyne. 2001. Cryptic reproductive isolation in the *Drosophila simulans* species complex. Evolution 55:81–92.

Robertson, H. M. 1983. Mating behavior and the evolution of *Drosophila mauritiana*. Evolution 37:1283–1293.

———. 1988. Mating asymmetries and phylogeny in the *Drosophila melanogaster* species complex. Pac. Sci. 42:72–80.

Robertson, H. M., and H. E. H. Paterson. 1982. Mate recognition and mechanical isolation in *Enallagma* damselflies (Odonata: Coenagrionidae). Evolution 36:243–250.

Simmons, L. W. 2001. Sperm competition and its evolutionary consequences in the insects. Princeton Univ. Press, Princeton, NJ.

Tatarnic, N. J., G. Cassis, and M. T. Siva-Jothy. 2014. Traumatic insemination in terrestrial arthropods. Annu. Rev. Entomol. 59:245–261.

Thornhill, R. 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. Am. Nat. 122:765–788.

Zeng, Z. B., J. Liu, L. F. Stam, C. H. Kao, J. M. Mercer, and C. C. Laurie. 2000. Genetic architecture of a morphological shape difference between two *Drosophila* species. Genetics 154:299–310.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Least-squares mean competitive fertilization success as *P*2 across levels of surgical treatment in *D. simulans*.

Figure S2. Copulation durations for conspecific pairs of *D. simulans* after males were subjected to one of four laser treatments: both posterior lobes altered (double-altered), one posterior lobe altered (single-altered), surgical controls with hairs removed, and sham controls that have been in the laser microscope environment, but are unaltered.