doi:10.1111/j.1558-5646.2012.01638.x

THE PRECOPULATORY FUNCTION OF MALE GENITAL SPINES IN *DROSOPHILA ANANASSAE* [DOLESCHALL] (DIPTERA: DROSOPHILIDAE) REVEALED BY LASER SURGERY

Karl Grieshop[1,2] and Michal Polak[1]

[1]*Department of Biological Sciences, University of Cincinnati, Cincinnati, Ohio 45221-0006*

[2]*E-mail: karlgrieshop@gmail.com*

Received December 4, 2011
Accepted March 10, 2012
Data Archived: Dryad doi: 10.5061/dryad.g0v6h003

That male genital morphology evolves via postcopulatory sexual selection is a widely held view. In contrast, the precopulatory sexual selection hypothesis for genital evolution has received less attention. Here, we test the hypothesis that male genital spines of *Drosophila ananassae* promote competitive male copulation success. Using laser surgery to manipulate trait size, we demonstrate that incremental reductions of spine length progressively reduce male copulation success: males without spines failed entirely to copulate because of an inability to couple the genitalia together, whereas males with halfway ablated and blunted spines suffered reductions in copulation success of 87% and 13%, respectively. The decrease in copulation success resulting from spine length reduction was markedly stronger in sexually competitive environments than in noncompetitive environments, and females expressed resistance behaviors similarly toward competing male treatments, demonstrating directly the role of genital spines in promoting competitive copulation success. Because these spines are widespread within *Drosophila*, and because genital traits with precopulatory function are being discovered in a growing number of animal taxa, precopulatory sexual selection may have a more pervasive role in genital evolution than previously recognized.

KEY WORDS: **Adaptive function, animal genitalia, copulation success, functional morphology, laser ablation, sexual selection.**

The leading hypothesis to explain the remarkable diversification of male genital traits is that such complexity evolves in response to sexual selection (Eberhard 1985; Hosken and Stockley 2004; Leonard and Córdoba-Aguilar 2010). Specifically, postcopulatory mechanisms of sexual selection, including sperm competition (Parker 1970; Simmons 2001), cryptic female choice (Thornhill 1983; Eberhard 1985, 1996), and sexual conflict (Parker 1979; Andersson 1994; Arnqvist and Rowe 2005), have received the most attention (Hosken and Stockley 2004). Indeed, the last quarter century has seen considerable growth in the number of studies addressing the postcopulatory function of genitalia (Leonard and Córdoba-Aguilar 2010). In contrast, fewer studies have focused on the potential for precopulatory sexual selection to drive genital evolution (Eberhard 1993, 2010a,b; Arnqvist 1997; Simmons 2001; Hosken and Stockley 2004; Bertin and Fairbairn 2005). Although it is admittedly counterintuitive that genital traits would function prior to copulation, such traits do occur in a variety of animal taxa (e.g., flatworms: Michiels 1998; insects: Bertin and Fairbairn 2005; Polak and Rashed 2010; fish: Langerhans et al. 2005; Kahn et al. 2010; mammals: see Miller 2010), and thus they deserve greater empirical and theoretical consideration.

An effective way to study the adaptive function of a trait, and test ideas about the causal bases of its evolution, is to employ manipulative experimentation (Sinervo and Basolo 1996; Arnqvist 1997; Eberhard 2011). Although insects offer abundant examples of the rapid divergent evolution of animal genitalia (Eberhard 1985; Leonard and Córdoba-Aguilar 2010), the microscopic size of most insect genitalia impedes their phenotypic

EVOLUTION
INTERNATIONAL JOURNAL OF ORGANIC EVOLUTION

manipulation. The present study uses a precision laser surgery system, capable of ablating and altering the shape of micron-scale structures with little or no damage to surrounding structures (Polak and Rashed 2010), to study the adaptive function of male genital spines in the cosmopolitan fruit fly *Drosophila ananassae* (Tobari 1993). Specifically, we test the hypothesis that genital spines in *D. ananassae* function to promote competitive male copulation success. Male genital spines in *Drosophila* are a rapidly evolving and widespread trait within the melanogaster species group (with over 40 species expressing them), ranging from species that do not express them (e.g., *D. melanogaster*) to those exhibiting one to five pairs of spines (Hsu 1949; Bock and Wheeler 1972; McEvey et al. 1987; Schiffer and McEvey 2006). Polak and Rashed (2010) employed laser ablation in a study of the male genital spines of *D. bipectinata*, and found support for the hypothesis that genital spines promote competitive male copulation success. In the wild, members of both sexes of these species aggregate on fermenting fruit, where males chase, court, and attempt to copulate with females that come to the fruit to feed, mate, and oviposit. Typically, there are many males and females at these sites, where there is a premium on males to locate and mate with receptive females before they are usurped by rival males; the mating system of these flies is best described as scramble competition (Thornhill and Alcock 1983).

In *D. ananassae*, the genital spines are a single pair of hard, sclerotized, claw-like structures that are external at rest, extending from the ventral cercal lobe (or secondary claspers) (Fig. 1). These spines move independently of the aedeagus and other genital structures, and insert into the female's external genitalia (not the gonopore) during copulation. Although similar in appearance, the spines of *D. ananassae* are 21% longer (controlling for body size variation) than in *D. bipectinata* (Grieshop and Polak, unpubl. data). Thus, not only was it our intent to examine the function of the spines in *D. ananassae* in their own right, and hence to begin to assess the generality of the findings concerning *Drosophila* genital spine function reported in Polak and Rashed (2010), but also to compare their relative functional importance for copulation between these two species.

Experiment 1 of the present laboratory study investigates the effect of incremental reductions in spine length on male copulation success in a nonsexually competitive context. Experiment 2 investigates the effect of spine length reduction on male copulation success in two social contexts—noncompetitive and competitive—simultaneously. This experiment specifically tests the prediction that the negative consequence of spine reduction on male copulation success should be more pronounced in a competitive environment than in a noncompetitive environment. Experiment 3 repeats the test for the effect of spine length reduction on copulation success in these two social environments, but does so in smaller arenas to facilitate assessment of the potential role of female behavior in driving differential male copulation success between the surgical treatments.

Materials and Methods

EXPERIMENTAL FLIES

The base population of *D. ananassae* [Doleschall] (Diptera: Drosophilidae) was initiated with 100 inseminated females collected in February 2009 on the South Pacific island of Moorea (17°32′58.78″S, 149°52′59.29″W), Society Islands. Flies were mass cultured in the laboratory on a 12:12 h L:D photoperiod and a 24°C (L): 22°C (D) temperature regime in 240 mL glass milk bottles ($N = 6$) with 6 g of Formula 4–24 Instant Drosophila Medium (Carolina Supply Co., Burlington, NC), 20 mL water, 8 mL of banana/live yeast slurry (50 mL water : 25 g banana : 1.5 g live yeast), and autoclaved tissue paper. The base population was acclimated to these laboratory conditions for seven generations over 4 months before use in the experiments.

Virgin males and females were collected from the base population simultaneously within 4 h of eclosion, maintained separately as virgins in 35 mL disposable polystyrene shell vials lined with cornmeal-agar food medium ($N \sim 25$ per vial), and allowed to age for 6 days until use in a given experiment. Experimental flies were transferred to fresh food vials every other day until experimentation, and live yeast was added to vials containing females.

After each block of all experiments, males were preserved in 95% ethanol and later examined under an Olympus SZX12 stereomicroscope to verify treatment identity and the integrity of the surgical manipulation (without knowledge of copulatory status). Male thorax length, an estimate of body size (Robertson and Reeve 1952), was measured (in rehydrated specimens) from the tip of the scutellum to the anterior edge of the thorax using an ocular micrometer; independent repeated measurements of thorax lengths in a random sample of 10 males were highly repeatable among males (one-way ANOVA: $F_{9,10} = 516.7$, $P < 0.0001$). In Experiment 3, observation chambers were cleaned with water, and cover slips and filter paper were replaced between blocks.

LASER MANIPULATION

Virgin males were laser treated within 22 h (±2 h) of eclosion using the protocol described in Polak and Rashed (2010) (Fig. 1C). Experimental males had their spine lengths surgically reduced in a bilaterally symmetrical fashion, producing the surgical treatments as follows: full-cut, spines excised at the base; half-cut, half of spines excised; partial-cut, approximately one third of spines excised; and tips-cut, tips of spines excised (blunted). The control treatments were: surgical control, two bristles on the seventh sternite of the ventral abdomen excised at the base; and sham control, subject to the same conditions as all other

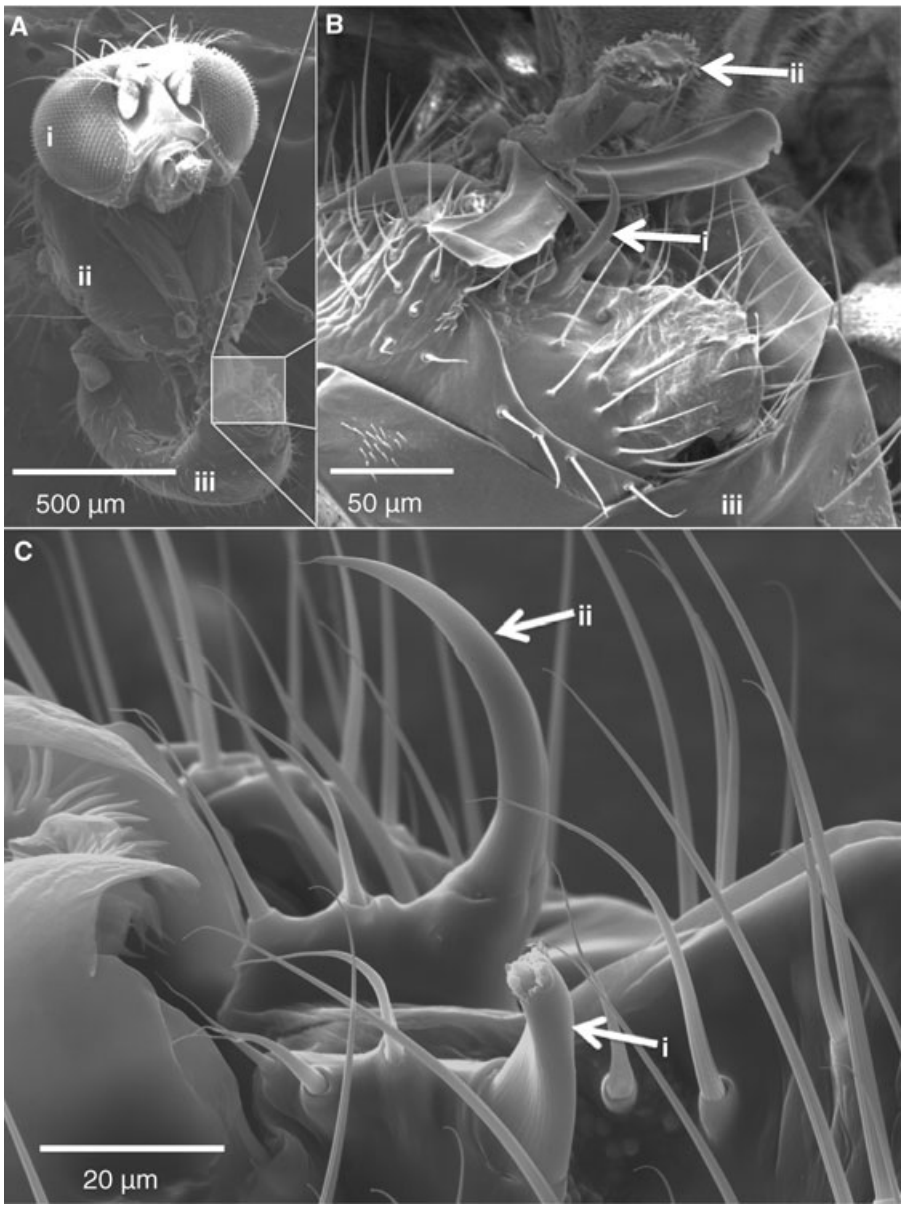

Figure 1. Scanning electron micrographs of male *D. ananassae* genitalia. (A) (50×) Male with legs removed, (i) head, (ii) thorax, (iii) abdomen. (B) (350×) Terminal segment of the male abdomen, (i) genital spines (external at rest) in crossed orientation, (ii) everted aedeagus, (iii) dorsal abdomen. (C) (1200×) Result of laser surgery, (i) partially ablated spine with no collateral damage to surrounding structures, (ii) opposing spine left intact for reference.

treatments but not actually contacted with the laser light (laser pulse shot just next to the specimen). Laser surgeries for all treatments took approximately the same amount of time to perform (<1 min per individual). Following surgery, experimental males were held separately (and without females) in food vials ($N \sim 20$ per vial) until experimentation. Across the three experiments described below (involving over 700 individual surgeries), a negligible number (<1%) of experimental males died prior to experimentation: two half-cut males (Experiment 1), two tips-cut males (Experiment 2), one partial-cut male and one surgical control male (Experiment 3).

EXPERIMENT 1

To investigate the effect of spine length reduction on copulation success in a noncompetitive context, observation vials lined with cornmeal–agar were placed in a row along a table. A female was aspirated into each of the vials at 2000 h, and the experiment commenced the following morning at 0800 h (23°C) when males were individually aspirated into the vials. Cut and control males were interspersed among the row of vials such that the following sequence repeated itself five times to constitute the 25 vials of each block: full-cut, half-cut, tips-cut, surgical control, and sham control. The time at which each male was introduced into a vial

with a female was recorded, and observers continually scanned the vials in successive order for 2 h, or until a copulation occurred. The start and stop times of all copulations were recorded. Copulation latency refers to the amount of time (s) elapsed between a male's introduction and the start time of copulation; and copulation duration refers to the amount of time (s) elapsed between the start and stop times of a copulation. Males that were not actively courting (<1%) were replaced. Three blocks of this experiment were conducted for a total $N = 75$ vials.

Copulation frequency data were pooled across blocks, as the heterogeneity χ^2 was nonsignificant ($P > 0.9$) (Zar 1999). The probability of copulation across treatments was analyzed using a subdivided χ^2 approach (Zar 1999): a χ^2-test including all treatments was first conducted, followed by χ^2-tests on subsets of the data to assess which treatments differed from each other. Log-transformed copulation latency and duration across treatments were analyzed separately using analysis of covariance (ANCOVA), with block and surgical treatment as factors and log-transformed male thorax length as the covariate. In this and the two following experiments, block had nonsignificant effects on copulation latency and duration (all Ps > 0.05), so it was removed from reported models.

EXPERIMENT 2

Here we investigated the effect of spine length reduction on copulation success in two social environments simultaneously. In the noncompetitive environment either a tips-cut or a surgical control male was placed individually in a vial with a female ($N = 15$ vials with a cut male and 15 vials with a control male, per block). In the competitive environment each vial contained a tips-cut male *and* a surgical control male with one female ($N = 15$ vials per block). The following sequence of vials repeated itself 15 times along the desktop to constitute the 45 vials of each block: noncompetitive (cut), noncompetitive (control), and competitive (cut plus control). Three blocks of this experiment were conducted for a total $N = 135$ vials.

Males were aspirated into vials at 2000 h, and the experiment commenced the following morning at 0800 h (23°C) when females were individually aspirated into the vials. The time at which each female was entered into a vial was recorded, and observers continually scanned the vials in successive order for 2 h, or until a copulation occurred. The start times of all copulations were recorded. Treatment identities of males were unknown to observers, so copulating pairs were aspirated out of vials as they formed; thus, although copulation latency was calculated as described in Experiment 1, copulation duration was not calculated here.

Fourteen vials were removed from relevant analyses for the following reasons: the copulating pair in a competitive vial separated before retrieval ($N = 2$); flies were accidentally killed or injured ($N = 2$ competitive vials); males of the tips-cut treatment were deemed (without knowledge of copulatory status) not to have enough spine length removed to constitute the treatment designation of "tips-cut" ($N = 7$ noncompetitive, and 3 competitive vials).

Copulation frequency data were pooled across blocks, as the heterogeneity χ^2 was nonsignificant ($P > 0.9$) (Zar 1999). χ^2 was used to analyze the effect of surgical treatment on copulation frequency separately for the two environment types. Log-transformed copulation latency across treatments was analyzed using ANCOVA, with block, treatment, environment, and treatment X environment interaction as factors, and log-transformed male thorax length as the covariate.

EXPERIMENT 3

This experiment was designed to investigate the potential influences of male and female behavior on any effect of surgical treatment and social environment on copulation success. Cut and control males were observed with females in noncompetitive and competitive environments simultaneously as in Experiment 2, with three major exceptions: (1) to facilitate behavioral observations, the experiment was conducted in small-cell mating chambers, which consisted of a plexiglass rectangle (75 mm × 25 mm × 6 mm) with a 12.5 mm diameter arena, a 2.5 mm diameter entrance tunnel in the side, a glass cover-slip ceiling fastened to the top with two-sided tape, a filter-paper floor fastened to the bottom with Scotch tape, and fine mesh plugging the entrance tunnel; (2) partial-cut males were used as the cut treatment in this experiment as opposed to the tips-cut males used in Experiment 2 (see Laser manipulation) to help ensure that treatment effect(s) would be detectable in these different mating arenas; and (3) males in both the noncompetitive and competitive chambers were distinguished by treatment with a small dot of colored paint on their dorsal thorax, which was randomly assigned to treatments. Three blocks of this experiment (45 chambers per block) were conducted for a total $N = 135$ chambers.

Males were aspirated into the mating chambers at 0700 h the morning of the experiment, which commenced at 0800 h (23°C) when females were individually aspirated into the chambers. The time at which each female was entered into a chamber was recorded, and observers continually scanned the chambers in successive order for 2 h, or until a copulation occurred, recording the start and stop times of all copulations, as well as the total number of times each chamber was scanned. Chambers were scanned on average 43 times (median: 54.5, range: 1–116). Male identities with respect to treatment were unknown to the observers.

During each scan of a given chamber, any occurrence of the following behaviors was recorded: male lunging, and female kicking, fleeing, decamping, and abdominal bending. Male courtship and female behavior in our laboratory population of *D. ananassae* is similar to that described by Spieth (1952). Males bend/curl their

Table 1. Least-squares mean ± 1 SE (*N*) copulation latency and duration (s) across surgical treatments from ANCOVAs for Experiments 1, 2, and 3. Data were $\log_e(y+1)$-transformed prior to analysis.

Response variable	Full-cut	Half-cut	Tips-cut	Part-cut	Surgical control	Sham control
Experiment 1						
Copulation latency	N/A	5.99±0.9 (2)	6.11±0.35 (13)	–	6.13±0.33 (15)	6.01±0.33 (15)
Copulation duration	N/A	5.55±0.18 (2)	5.44±0.07 (13)	–	5.49±0.07 (15)	5.52±0.07 (15)
Experiment 2						
(NC) Cop. latency[1]	–	–	6.39±0.19 (29)	–	5.87±0.18 (33)	–
(C) Cop. latency[1]	–	–	6.13±0.31 (11)	–	5.56±0.23 (21)	–
Experiment 3						
(NC) Cop. latency	–	–	–	6.37±0.38 (12)	5.55±0.24 (31)	–
(C) Cop. latency	–	–	–	7.11±0.96 (12)	6.31±0.25 (31)	–
(NC) Cop. duration	–	–	–	5.64±0.06 (2)	5.71±0.04 (29)	–
(C) Cop. duration	–	–	–	5.45±0.15 (2)	5.67±0.04 (28)	–

(NC) = noncompetitive social environment.

(C) = competitive social environment.

– = treatment not included in experiment. N/A = zero copulations for that treatment (see Fig. 2A).

[1]Significant differences revealed by ANCOVA (see Table 2).

abdomens underneath themselves, then lunge at a female, thrusting the tip of the abdomen forward in an attempt to bring the genitalia together. During lunges, the male probes the female's genitalia with his own, and after successfully coupling his genitalia to hers, completes the mounting process by climbing forward onto her abdomen between her wings. Failed copulation attempts involve males lunging only to achieve very brief and passing contact with the female's genitalia, sometimes lunging multiple times in rapid succession. Female resistance behaviors include bending/curling their abdomens underneath themselves away from the courting male, kicking with their hind limbs, fleeing while grounded, and decamping via flight.

Because female behaviors were exhibited infrequently, the frequencies of kicking, fleeing, decamping, and abdominal bending were summed for each female to yield a composite frequency of female "resistance behaviors." Behavioral frequencies, including male lunges and female resistance behaviors, were converted to behavioral rates by dividing them by the total number of scans per chamber. The resultant values closely estimated behaviors per unit time, as the amount of scans per chamber was highly correlated with the total amount of time each chamber was under observation ($r^2 = 0.96$, df = 127, $P < 0.0001$).

Seven chambers were removed from relevant analyses for the following reasons: flies were accidentally killed or injured ($N = 3$ noncompetitive chambers); the copulation began before any behaviors were recorded ($N = 2$ noncompetitive, and 1 competitive chamber); the stop time of the copulation was unknown ($N = 1$ competitive chamber).

Copulation frequency data were pooled across blocks, as the heterogeneity χ^2 was nonsignificant ($P > 0.4$) (Zar 1999). χ^2 was used to analyze the effect of surgical treatment on the frequency of copulation separately for the two environment types. Likewise, behavioral frequencies and rates were analyzed separately for the two environment types: a two-tailed Wilcoxon signed-rank test was used to analyze differences between treatments, and a Welch's ANOVA was used when variances between treatments were statistically unequal (Zar 1999). Log-transformed copulation latency and duration across treatments were analyzed using the ANCOVA model described in Experiment 2. JMP (version 8, SAS Institute Inc., 2009) statistical software was used throughout. Data archived in the Dryad repository: doi:10.5061/dryad.g0v6h003.

Results

EXPERIMENT 1

When males were placed individually with females, there was a highly significant effect of treatment on copulation frequency ($\chi^2_4 = 60.56, P < 0.0001$), attributable to a sharp reduction in copulation success of the full-cut and half-cut treatments. No male *D. ananassae* with their genital spines fully removed achieved copulation, and only 13% of half-cut males copulated (Fig. 2A). In contrast, 87% of tips-cut males, and 100% of both control treatments, copulated (Fig. 2A); copulation frequency did not differ among these three treatments ($\chi^2_2 = 4.19, P = 0.12$). ANCOVA revealed no significant differences in copulation latency ($F_{3,40} = 0.03$, $P = 0.99$) or duration ($F_{3,40} = 0.28$, $P = 0.84$) among treatments (Table 1).

EXPERIMENT 2

When placed individually with females in the noncompetitive social environment, tips-cut males exhibited a probability of copulation not significantly different from that of surgical control males

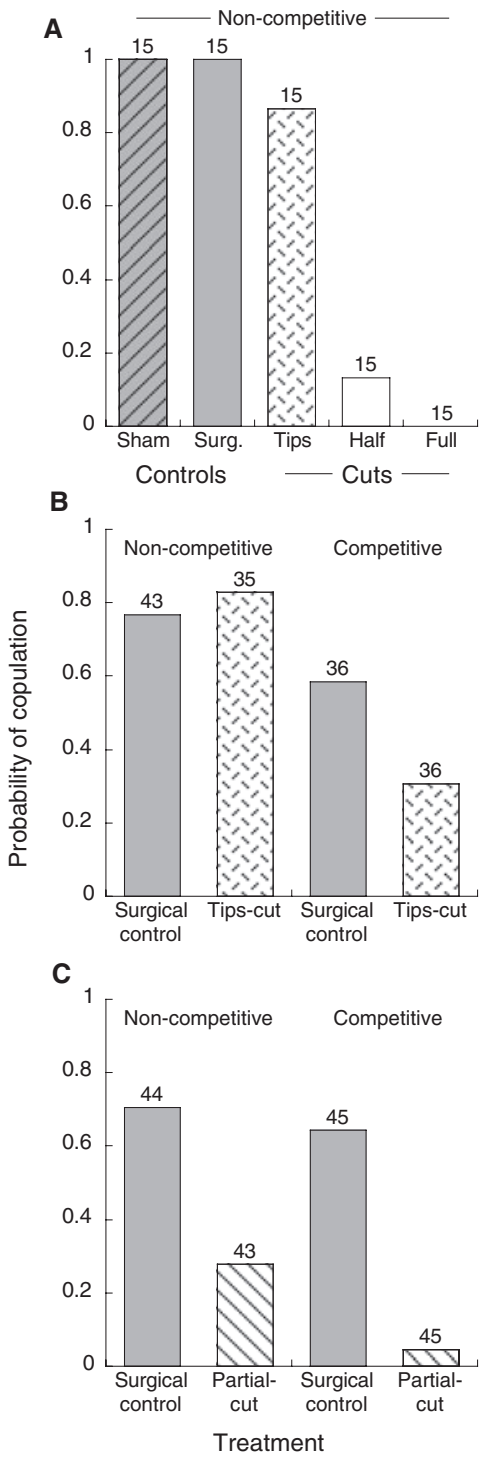

Figure 2. Probability of copulation by treatment and social environment. (A) Experiment 1, the effect of genital spine manipulation on copulation success in a nonsexually competitive social context. (B) Experiment 2, the effect of tip removal in both noncompetitive and competitive contexts. (C) Experiment 3, the effect of partial spine ablation in both social contexts performed in small-cell mating chambers. Numerals above bars represent sample sizes, pooled across three blocks for each experiment.

Table 2. Results of ANCOVA on copulation latency for Experiment 2.

Term	df	s.s.[2]	F	P
Male thorax length	1	2.6489	2.44	0.12
Treatment (Trt)[1]	1	5.9274	5.46	0.02
Environment (Env)	1	1.6249	1.49	0.22
Trt X Env	1	0.0111	0.01	0.92
Error	89	96.6232		

[1]Significant term.
[2]Sum of squares.

($\chi^2_1 = 0.44$, $P = 0.51$), consistent with the results of Experiment 1; however, tips-cut males did suffer a significantly reduced probability of copulation in the competitive environment when competing directly against surgical control males for access to individual females ($\chi^2_1 = 5.63$, $P = 0.02$) (Fig. 2B). Thus, whereas blunted genital spines did not reduce a male's probability of copulation in the noncompetitive context, this subtle manipulation did significantly reduce a male's probability of copulation when there was another male present to usurp the female, indicating social context interacts with spine manipulation to affect male copulation success.

ANCOVA revealed that tips-cut males exhibited a significantly greater latency to copulation than surgical control males (Table 2). That is, when control males gained copulations, they did so, on average, significantly sooner than tipless males (Table 1).

EXPERIMENT 3

When assayed in small-cell mating chambers, partial-cut males exhibited a significantly lower probability of copulation than surgical control males in both noncompetitive ($\chi^2_1 = 15.75$, $P < 0.0001$) and competitive environments ($\chi^2_1 = 35.87$, $P < 0.0001$) (Fig. 2C). In the absence of sexual rivals, partial-cut males were 61% less likely to copulate than controls, but when the two treatments competed directly for the same female partial-cut males were 93% less likely to copulate than controls. ANCOVA revealed no significant difference in copulation latency ($F_{1,69} = 1.89$, $P = 0.17$) or duration ($F_{1,68} = 2.47$, $P = 0.12$) between treatments; likewise, treatment X environment interaction had no significant effect on copulation latency ($F_{1,69} = 0.01$, $P = 0.91$) or duration ($F_{1,68} = 0.76$, $P = 0.39$) (Table 1). This lack of an effect on latency is contrary to that found in Experiment 2.

A two-tailed Wilcoxon signed-rank test revealed that partial-cut and surgical control males performed a similar number of copulation attempts per unit time (i.e., lunge rate) in noncompetitive ($Z_{43,42} = -0.66$, $P = 0.51$) and competitive chambers ($Z_{44,44} = 1.13$, $P = 0.26$). When lunges were analyzed without converting

them to a rate, Welch's ANOVA revealed that partial-cut males exhibited a significantly greater total number of lunges than surgical control males in both environment types (noncompetitive: $F_{1,48.4} = 9.38$, $P = 0.004$; and competitive: $F_{1,50.8} = 6.35$, $P = 0.015$). In noncompetitive chambers the mean number of lunges exhibited by partial-cut males ($N = 43$) was 2.88 (median: 5.5, range: 0–16), compared to 0.69 (median: 2.5, range: 0–6) for controls ($N = 42$); and in competitive chambers the mean number of lunges exhibited by partial-cut males ($N = 44$) was 2 (median: 5, range: 0–13), compared to 0.68 (median: 1.5, range: 0–3) for controls ($N = 44$).

Females in noncompetitive chambers that were paired with partial-cut males expressed resistance behaviors at a significantly greater rate (Wilcoxon: $Z_{42,43} = -2.85$, $P = 0.004$) and frequency (Welch's: $F_{1,46.8} = 4.3$, $P = 0.044$) than females paired with surgical control males. The mean number of resistance behaviors expressed toward partial-cut males ($N = 43$) was 2.12 (median: 5, range: 0–28), compared to 0.38 (median: 2, range: 0–7) for controls ($N = 42$).

In contrast, females in competitive chambers did not express a significantly different rate ($Z_{44,44} = 0.39$, $P = 0.69$) or frequency ($Z_{44,44} = 0.41$, $P = 0.69$) of resistance behaviors toward either treatment. The mean number of female resistance behaviors expressed toward partial-cut and control males ($N = 44$ each) was 0.61 (median: 2, range: 0–9) and 0.48 (median: 1.5, range: 0–8), respectively. This result did not change when the analysis was restricted to only those competitive chambers in which both males had been observed to lunge at least once (rate: $Z_{11,11} = -0.08$, $P = 0.94$; frequency: $Z_{11,11} = -0.12$, $P = 0.91$). Additionally, in all 74 copulations, females were never observed to exhibit resistance behaviors while in copula. Thus, females do not appear to discriminate between cut and control males.

Discussion

The data from the three experiments support the hypothesis that genital spines in *D. ananassae* function to promote competitive male copulation success. Experiment 1 revealed that incremental reductions in male genital spine length progressively reduced copulation success in a noncompetitive context, where one male was paired with one female. Whereas removing only the tips of the spines had a nonsignificant effect on male copulation success, removing half of both spines reduced male copulation success by 87% relative to controls, and full excision of the spines eliminated entirely the ability of males to copulate. Experiment 2, in turn, evaluated the effects of the "tips-cut" surgical manipulation on male copulation success simultaneously in competitive and noncompetitive contexts. The results indicate that only in the competitive social context was there a negative effect of this manipulation on male copulation success. Experiment 3 verified the existence of this apparent synergism between social environment and surgical treatment using much smaller observation chambers: partial-cut males suffered a significant reduction in copulation success compared to controls in both social contexts, but this effect was stronger in the competitive context. Furthermore, these reductions in the copulation success of partial-cut males occurred despite similar rates of male copulation attempts (lunges per scan) between the two treatment groups, indicating that the surgical reduction in spine length per se, and not any potential side effects of laser contact such as reduced male motivation to mate, caused the observed reduction(s) in copulation success. Thus, the results from Experiments 2 and 3 provide support for the prediction that the effect of spine reduction on male copulation success should be stronger in an environment where males compete for access to mates.

The behavioral observations conducted in Experiment 3 yield further insight into the function of male genital spines in *D. ananassae*. A first consideration is that in both social contexts of this experiment the partial-cut males exhibited a sharp increase in the frequency of failed copulation attempts (lunges) with virgin females. In other words, cut males exhibited a strong reduction in the efficiency with which they were able to couple their genitalia to that of the female's, which translated to longer copulation latency (although only significantly so in Experiment 2), and a significant loss of copulation success. These data indicate that the genital spines of male *D. ananassae* function in the mechanics of genital coupling, to which even subtle alterations have significant reproductive consequences in the face of direct sexual competition. Subtle variations in spine size and/or shape are therefore likely to have pronounced consequences for male reproductive fitness in natural populations of *D. ananassae* and other *Drosophila* species that exhibit a scramble competition mating system (Thornhill and Alcock 1983), where competing males search for receptive females on the surface of fruits and where efficient genital coupling is paramount for male copulation success.

A second consideration is that females exhibited statistically similar levels of resistance behaviors expressed toward partial-cut and control males in the competitive context, suggesting that female rejection of potential mates is not the cause of the impaired copulation success of cut males in sexually competitive contexts. We also checked for differences in resistance behaviors in the subset of cases in which both males of a competitive chamber were observed to lunge at the female, because such cases would have provided the female with a better opportunity to sense both males' genital spines. However, we likewise found no significant differences in the rate or frequency at which females expressed resistance behaviors toward cut and control males in this subset of the data.

Yet, the existence of female resistance behaviors suggests that sexual conflict (Parker 1979; Andersson 1994; Arnqvist and Rowe 2005) may help explain the evolution of male genital spine morphology within *Drosophila*, as discussed in Polak and Rashed (2010). If the present size and shape of male genital spines reflect their effectiveness at overcoming resistance behaviors exhibited by females during courtship and/or mating (Spieth 1952), then interspecific differences in spine morphology could at least in part represent differences in the intensity or form of female resistance across species (Arnqvist and Rowe 1995, 2002a,b). Indeed, the genital spines of male *D. ananassae* are 21% longer than that of male *D. bipectinata* (Grieshop and Polak, unpubl. data), and spine removal has a stronger detrimental effect on male copulation success in *D. ananassae* than it does in *D. bipectinata* (Polak and Rashed, 2010). Whereas relative spine size matches the trait's functional importance between these species, it remains unclear whether they also differ in the intensity of female mating resistance. Clearly, a broad range of species will need to be surveyed in terms of spine size, shape, and function, and of the intensity of female resistance, for a robust test of this idea.

For *D. ananassae*, further investigation of the adaptive function of male genital spines will require a thorough test of the postcopulatory sexual selection hypothesis (Eberhard 2011). Although such tests were not the focus of our study, we found no indication in the admittedly few variables we examined that spine reduction elicited any female behavioral responses during mating. Females almost invariably were motionless and exhibited no detectable resistance behaviors during copulation, and we consistently found no significant effect of spine manipulation on copulation duration. Similarly, Polak and Rashed (2010) found that although genital spines in *D. bipectinata* function to promote competitive male copulation success, spine length reduction had no detectable effect on sperm transfer, fertilization success, competitive fertilization success, fecundity, fertility, or copulation duration. Nevertheless, Polak and Rashed's (2010) study still was not an exhaustive test of the postcopulatory sexual selection hypothesis (see Eberhard 2011), so more work is needed to fully test this hypothesis with regard to *Drosophila* genital spines.

There are over 40 species of *Drosophila* with genital spines (Hsu 1949; Bock and Wheeler 1972; McEvey et al. 1987; Schiffer and McEvey 2006), and genital traits that function prior to copulation are taxonomically widespread outside of *Drosophila* as well (e.g., flatworms: Michiels 1998; insects: Bertin and Fairbairn 2005; Polak and Rashed 2010; fish: Langerhans et al. 2005; Kahn et al. 2010; mammals: see Miller 2010). Therefore, the precopulatory sexual selection hypothesis for genital trait evolution likely applies to very different animal taxa, and should weigh more heavily on future research into the remarkable diversification of male genitalia.

ACKNOWLEDGMENTS

The research was supported by a University Research Council (URC) Graduate Student Research Fellowship (to KG), the McMicken College of Arts and Sciences and the Department of Biological Sciences at the University of Cincinnati, and the National Science Foundation (DEB-1118599 to MP). We thank B. Hamilton, B. Cortright, and A. Warner for assistance with experiments, and Dr. N. Kaval of the University of Cincinnati's Department of Chemistry for the SEMs. We also thank Dr. A. Córdoba-Aguilar and reviewers for helpful comments on the manuscript.

LITERATURE CITED

Andersson, M. 1994. Sexual selection. Princeton Univ. Press, Princeton, NJ.

Arnqvist, G. 1997. The evolution of animal genitalia: distinguishing between hypotheses by single species studies. Biol. J. Linn. Soc. 60: 365–379.

Arnqvist, G., and L. Rowe. 1995. Sexual conflict and arms races between the sexes—a morphological adaptation for control of mating in a female insect. Proc. R. Soc. Lond. B. 261:123–127.

———. 2002a. Correlated evolution of male and female morphologies in water striders. Evolution 56:936–947.

———. 2002b. Antagonistic coevolution between the sexes in a group of insects. Nature 415:787–789.

———. 2005. Sexual conflict. Princeton Univ. Press, Princeton, NJ.

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.

Bock, I. R., and M. R. Wheeler. 1972. Pp. 1–102 The *Drosophila melanogaster* species group. Vol. 7213. University of Texas Publications. Austin, TX.

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

———. 1993. Evaluating models of sexual selection: genitalia as a test case. Am. Nat. 142:564–571.

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

———. 2010a. Evolution of genitalia: theories, evidence, and new directions. Genetica 138:5–18.

———. 2010b. Rapid divergent evolution of genitalia: theory and data updated. Pp. 40–78 *in* J. L. Leonard and A. Córdoba-Aguilar, eds. The evolution of primary sexual characters in animals. Oxford Univ. Press, New York, NY.

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

Hosken, D. J., and P. Stockley. 2004. Sexual selection and genital evolution. Trends Ecol. Evol. 19:87–93.

Hsu, T. C. 1949. Pp. 80–142. The external genital apparatus of male Drosophilidae in relation to systematics. Vol. 4920. University of Texas Publications. Austin, TX.

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.

Langerhans, R. B., C. A. Layman, and T. J. DeWitt. 2005. Mal 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.

Leonard, J. L., and A. Córdoba-Aguilar, eds. 2010. The evolution of primary sexual characters in animals. Oxford Univ. Press, New York, NY.

McEvey, S. F., J. R. David, and L. Tsacas. 1987. The *Drosophila ananassae* complex with description of a new species from French Polynesia (Diptera: Drosophilidae). Ann. Soc. Entomol. Fr. 23:377–385.

Michiels, N. 1998. Mating conflicts and sperm competition in simultaneous hermaphrodites. Pp. 219–254 *in* T. Birkhead and A. P. Moller, eds. Sperm competition and sexual selection. Academic Press, New York, NY.

Miller, E. H. 2010. Genitalic traits of mammals. Pp. 471–493 *in* J. L. Leonard and A. Córdoba-Aguilar, eds. The evolution of primary sexual characters in animals. Oxford Univ. Press, New York, NY.

Parker, G. A. 1970. Sperm competition and its evolutionary effect on copula duration in fly *Scatophaga stercoraria*. J. Insect Physiol. 16: 1301–1328.

———. 1979. Sexual selection and sexual conflict. Pp. 123–166 *in* M. Blum and N. Blum, eds. Sexual selection and reproductive competition in insects. Academic Press, New York, NY.

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

Robertson, F. W., and E. C. Reeve. 1952. Studies in quantitative inheritance. I. The effects of selection on wing and thorax length in *Drosophila melanogaster*. J. Genet. 50:416–448.

Schiffer, M., and S. F. McEvey. 2006. *Drosophila bunnanda*: a new species from northern Australia with notes on other Australian members of the *montium* subgroup (Diptera: Drosophilidae). Zootaxa 1333:1–23.

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

Sinervo, B., and A. L. Basolo. 1996. Testing adaptation using phenotypic manipulations. Pp. 149–186 *in* M. R. Rose and G. V. Lauder, eds. Adaptation. (Academic Press, San Diego, CA.

Spieth, H. T. 1952. Mating behavior within the genus *Drosophila* (Diptera). Bull. Am. Mus. Nat. Hist. 99:395–474.

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

Thornhill, R., and J. Alcock. 1983. The evolution of insect mating systems. 2001 reprint ed. iUniverse.com, Inc, Lincoln, NE.

Tobari, Y. N., ed. 1993. *Drosophila ananassae*: genetical and biological aspects. Japan Scientific Societies Press/Karger, Tokyo/Basel.

Zar, J. H. 1999. Biostatistical analysis. Prentice Hall, Upper Saddle River, NJ.

Associate Editor: A. Cordoba-Aguilar