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Microscale laser surgery reveals adaptive function of male intromittent genitalia

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The leading hypothesis for the evolution of male genital complexity proposes that genital traits evolve in response to post-insemination sexual selection; that is, via cryptic female choice or sperm competition. Here, we describe a laser ablation technique for high-precision manipulation of microscale body parts of insects, and employ it to discern the adaptive function of a rapidly evolving and taxonomically important genital trait: the intromittent claw-like genital spines of male *Drosophila bipectinata* Duda. We demonstrate experimentally and unambiguously that the genital spines of this species function to mechanically couple the genitalia together. The excision of the spines by laser ablation sharply reduced the ability of males both to copulate and to compete against rival males for mates. When spineless males did succeed to copulate, their insemination success and fertilization rate were not statistically different from controls, at odds with the post-insemination sexual selection hypothesis of genital function and evolution. The results provide direct experimental support for the hypothesis that genital traits evolve in response to sexual selection occurring prior to insemination.

Keywords: genitalia; function; sexual selection; laser ablation; *Drosophila*

1. INTRODUCTION

The mechanisms responsible for the remarkable diversification of genital morphology in animal species with internal fertilization remain unresolved, despite over a century of debate (Darwin 1871; Mayr 1963; Eberhard 1985; Arnqvist 1997; Hosken & Stockley 2004). A modern perspective that has emerged over recent decades holds that genital diversification results from sexual selection (Hosken & Stockley 2004).

The evolution of intromittent genital traits, structures that are inserted into the female genitalia or reproductive tract during sperm transfer and that exhibit an unusually high degree of morphological complexity, is ascribed to the action of post-insemination sexual selection (Eberhard 1985; Hosken & Stockley 2004; but see Kahn *et al*. in press), which includes sperm competition (where ejaculates of different males compete for fertilization; Parker 1970) and cryptic female choice (where females bias fertilization of ova in favour of particular phenotypes; Eberhard 1985). The operation of either of these post-insemination processes is predicted to result in significant associations within species between variation in male genital morphology and fertilization success (Arnqvist 1997), for which correlational evidence is accumulating (e.g. House & Simmons 2003; Hotzy & Arnqvist 2009). Moreover, two experimental studies have reduced the size of intromittent genital traits with instruments such as fine scissors and reported negative effects of their respective manipulations on sperm storage efficiency (Takami 2003; Rodríguez *et al*. 2004).

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However, many genital traits of insects and other arthropods, such as a remarkable variety of spines, 'teeth' and elaborate genital devices associated with copulation and/or sperm transfer, are far too minute and inaccessible with mechanical surgical instruments, a practical impediment that continues to frustrate efforts to elucidate genital trait function. For example, in the bruchid beetle, *Callosobruchus maculatus*, a model system for studies of sperm competition and sexual conflict (Hotzy & Arnqvist 2009), the male intromittent organ possesses microscale sclerotized spines that inflict injury and scarring to the female reproductive tract during mating (Crudgington & Siva-Jothy 2000; Hotzy & Arnqvist 2009). Whereas male spine length variation correlates positively with male competitive fertilization success, as predicted by the post-insemination sexual selection hypothesis, experimental studies involving the manipulation of the spines in *C. maculatus* are lacking, precluding definitive conclusions about their proximate function (Hotzy & Arnqvist 2009).

Here, we employ a laser surgical technique to manipulate and study the function of the intromittent genital spines in *Drosophila bipectinata* Duda (Diptera: Drosophilidae), the claw-like projections from the male ventral cercal lobes (figure 1*a*). We refer to these genital spines as intromittent because they insert into female external genitalia during copulation, and not because they insert into the reproductive tract. The spines exhibit a pattern of pronounced diversification among closely related species, and they are of notable taxonomic importance (Bock & Wheeler 1972).

It has been suggested that these spines, by breaching the female body wall during copulation, inflict wounds to the female (Kamimura 2007), as in the *C. maculatus* beetles noted above. Additionally, it has been claimed that sperm are injected into the female reproductive tract across the female body wall via the wound sites, thereby

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bypassing the female's genital orifice (Kamimura 2007), the usual route of sperm transfer in *Drosophila*. Thus, the current view being promulgated is that the genital spines in *D. bipectinata* and related *Drosophila* species function in an unusual and extreme mode of sperm delivery: traumatic insemination (Kamimura 2007; Bonduriansky *et al.* 2008; Řezáč 2009). In the present study, we demonstrate that the spines in *D. bipectinata* promote copulatory success, and not insemination or fertilization, contrary to the post-insemination sexual selection hypothesis for genital trait function and evolution.

2. MATERIAL AND METHODS

(a) *Flies*

All experimental flies were sourced from a general laboratory stock established from 200 flies of each sex collected at Cape Tribulation, Australia, in January 2003, and mass-cultured under conditions previously described (Polak & Simmons 2009). In all experiments, flies were aged, observed and allowed to lay eggs in eight fluid dram polystyrene shell vials containing banana–yeast–agar substrate.

(b) *Laser ablation*

The following protocol generated the treatment groups used in all experiments. Males were collected as virgins from culture and, at 24 h of age, anaesthetized under a light, humidified stream of CO_2 in a Plexiglas chamber with a thin glass bottom. The male was positioned ventral side down and leaning slightly to one side, so the genital spines were visible from below. The chamber was mounted on a Prior (Rockland, MA, USA) H117 motorized stage fitted to an Olympus (Center Valley, PA, USA) IX71 inverted light microscope. Pulsed laser light ($\lambda = 532$ nm) from a Vector 532-1000-20 Q-switched laser (Coherent, Santa Clara, CA, USA) was focused through an Olympus Uplan-Apo 20× objective, and used to administer precision cuts to the genital spines with little or no damage to adjacent bristles. Males from general culture were randomly assigned to treatment groups, as follows. Full cut: each spine completely excised by optically cutting it off at its base (figure 1*b*). Partial cut: each spine shortened and blunted by cutting off one-third its length (figure 1*c*). Surgical control: two randomly selected large bristles on the terminalia, each sliced off at the base. Sham control: males treated identically as those in other groups, except the laser beam was focused 10–12 μm away from the tip of the abdomen.

(c) *Non-competitive mating success and courtship behaviour*

A total of 50, 60 and 55 pairs of flies were monitored in time blocks 1, 2 and 3, respectively. In each block, 3-day-old males from each of the four treatment categories were individually aspirated into vials at 19.00, and vials were interdigitated at random in a row along a desktop. When lights were turned on at 07.00 the next morning, 4-day-old virgin females were individually introduced into each vial. Vials were scanned continuously in successive order by two observers at room temperature (24.5–25.4°C) for 2 h, or until a copulation occurred. Copulations last approx. 10 min; therefore, none were missed. The frequency at which males did not copulate was contrasted between experimental categories using χ^2 testing. Data across the three blocks were pooled as the heterogeneity χ^2 (Zar 1998) was non-significant ($p > 0.5$). In block 1 only, the occurrence of specific behaviours

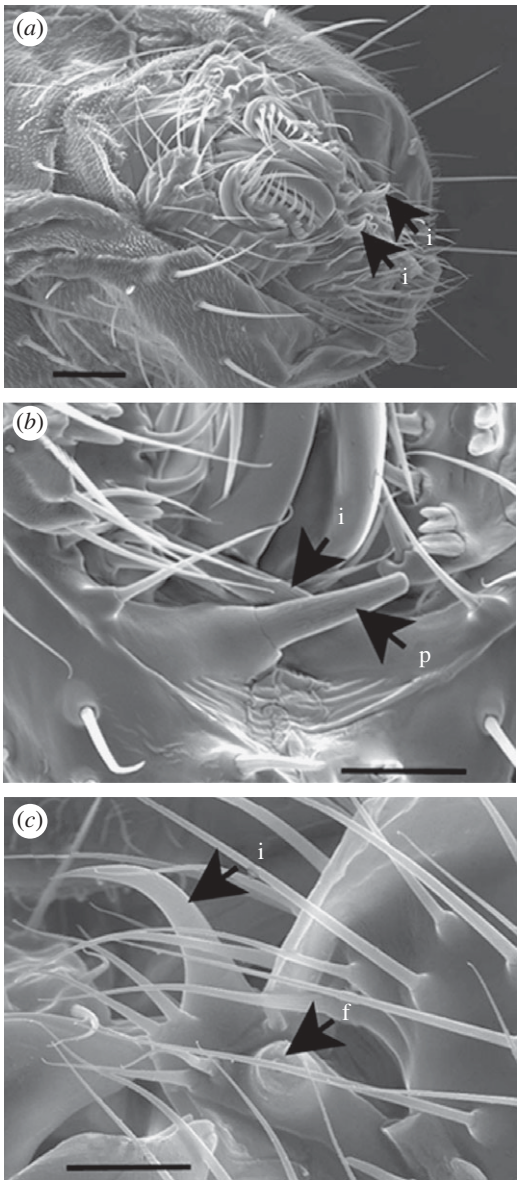

Figure 1. Scanning electron micrographs of male genitalia in *D. bipectinata*, illustrating intact and optically engineered spines precisely ablated without damage to adjacent structures. (*a*) Both spines intact (650×); (*b*) partially ablated spine, with the intact spine lying in crossed orientation behind it (1500×); (*c*) previous location of a fully excised spine (1500×). Scale bars: (*a*) 50 μm; (*b*,*c*) 20 μm. Abbreviations: i, intact; p, partially ablated; f, fully excised.

was recorded. One observer conducted 14 scans, recording (i) male courtship (chasing the female, tapping her abdomen and 'singing'; Cooperman *et al.* 2007), (ii) copulation failure (mounting the female and dismounting without copulating) and (iii) copulation (mounting leading to successful genital coupling).

(d) *Competitive mating success*

The above experiment does not directly test the involvement of the spines in sexual selection because males were paired singly with females. Thus, the present experiment consisted of competitive dyadic mating trials, as follows. One full cut male and one surgical control were paired in individual vials ($n = 33$) at 19.00 on the evening prior to the mating trial. When lights were turned on at 07.00 the next morning, a single 4-day-old virgin female was aspirated into each vial,

and all vials were monitored at room temperature by scanning them one at a time in successive order by one observer. Each vial was monitored for 2 h or until a copulation occurred. All copulating pairs were removed from vials within 1 min of the onset of copulation, and both males were identified by treatment category. The null hypothesis of equal mating frequency between experimental categories was evaluated with a likelihood ratio test, adjusted by the continuity correction (Sokal & Rohlf 1995). The adjusted value of G (G_{adj}) was compared with a critical value of χ^2 for 1 d.f.

(e) *Success in sperm transfer*

In each of three time blocks, 4-day-old males were individually aspirated into numbered vials and lined up along a desktop the evening prior to mating. In each block, males from all treatment groups were represented and interdigitated at random among the vials. Next morning, 5-day-old virgin females were aspirated individually into vials, and pairs were continually monitored for 2 h. All copulations and their durations were recorded ($n = 25$, 14 and 9 in blocks 1, 2 and 3, respectively). Immediately after a copulation, the female was anaesthetized with ether, and the ejaculate released from her uterus into a drop of physiological saline on a gelatin/chrome alum-coated glass slide (DeVries 1964). During each dissection, it was verified that no sperm occurred elsewhere in the reproductive tract where sperm are stored, the ventral receptacle and spermathecae. The sperm mass was gently teased apart, fixed and stained with a 5×10^{-7} Hoechst (Sigma, Lot 044K4097) solution (Polak *et al.* 2001). Sperm were visualized and counted using epifluorescence under an Olympus BX60 light microscope. Counts were conducted blind with respect to treatment. Based on two independent counts of eight preparations, repeatability (Lessels & Boag 1987) was high ($r = 0.77$, $F_{7,8} = 25.9$, $p < 0.0001$). Thorax lengths of males and females were taken as measures of body size. Sperm count data were analysed using analysis of covariance (ANCOVA), with block and treatment as fixed factors, and copulation duration and thorax lengths of both sexes as covariates. Female thorax length and the block × treatment interaction were non-significant ($p = 0.82$ and 0.70, respectively), and were excluded from the final model. Residuals were normally distributed ($W = 0.98$, $p = 0.58$) and homoscedastic across levels of treatment (Levene's test: $F_{3,44} = 0.99$, $p = 0.40$). Interaction terms between the covariates and factors were non-significant ($p > 0.05$), confirming the homogeneity of slopes assumption of the analysis (Sokal & Rohlf 1995).

(f) *Non-competitive fertilization success*

In each of the two time blocks, 4-day-old males were individually aspirated into numbered vials as above (in each block, all treatment groups were represented). Next morning, 8-day-old virgin females were aspirated individually into vials, and pairs continually monitored for 2 h. All copulations and their durations were recorded. Females ($n = 32$ and 47 in blocks 1 and 2, respectively) were transferred to a fresh oviposition vial within 1 h after copulation, and then daily for a total of 5 consecutive days. For each day, the number of eggs laid (fecundity) and the number of larvae produced (fertility) were counted. All counts were conducted blind with respect to treatment. Fecundity and fertility were separately analysed with a repeated-measures analysis of variance (ANOVA), with time (i.e. days 1–5) as the within-subject factor, and block and treatment as fixed between-subject factors. The time × treatment interaction was of particular interest to assess whether responses in fecundity and fertility over time differed among treatments. For fecundity, residuals were normally distributed ($W = 0.98$, $p = 0.14$) and homoscedastic across levels of treatment (Levene's test: $F_{3,75} = 1.24$, $p = 0.31$). Interactions between covariates and factors were non-significant. For fertility, residuals were likewise normally distributed ($W = 0.97$, $p = 0.13$) and homoscedastic across levels of treatment (Levene's test: $F_{3,75} = 1.33$, $p = 0.27$). Interactions between covariates and factors were non-significant. In a separate analysis, similar in structure to the above but without the time factor, we evaluated the effect of treatment on fecundity and fertility specifically for the first 24 h post-mating.

(g) *Competitive fertilization success*

Three time blocks were conducted in which a total of 50 (block 1), 60 (block 2) and 65 (block 3) 5-day-old virgin females from general culture were individually mated to 4-day-old irradiated general-culture males in vials. Males were irradiated with a 150 Gy dose from a ^{60}Co source (Polak & Simmons 2009), thus the zygote dies before hatching because of lethal mutations (Simmons 2001). Within 1 h after copulation, females were transferred to vials with banana–agar substrate for oviposition, and were transferred to fresh vials each morning for 7 consecutive days. The variable 'pre-P2 eggs' refers to the total number of eggs laid by females over this 7-day period (Polak & Simmons 2009). On the morning of the eighth day, females were randomly paired with males from treatment groups in vials. The duration of all copulations and thorax lengths of each female's first and second mate were measured. Immediately following her second copulation, the female was transferred to an oviposition vial and allowed to lay a minimum of 20 eggs (mean ± s.d.: 38.4 ± 8.0, range: 23–56; $n = 54$ females). P_2, the proportion of offspring sired by a female's second mate, was calculated as the number of eggs that hatched divided by the total number of eggs (Boorman & Parker 1976). Counts of eggs and larvae were conducted blind with respect to treatment. The P_2 data were arcsine-square root-transformed and analysed using ANCOVA with the thorax length and treatment of the second male as independent terms. The original full model contained seven independent terms that could explain the variation in P_2 (block, treatment, thorax length of male 1, thorax length of male 2, copulation duration of male 1, copulation duration of male 2, pre-P_2 eggs). Least significant terms were sequentially eliminated from the model; none of the removed terms had alpha values <0.2. Interaction between thorax length and treatment was non-significant. For the final model that we report, residuals were reasonably close to normally distributed ($W = 0.90$, $p = 0.002$), and they were homoscedastic across treatment categories (Levene's test: $F_{3,53} = 0.38$, $p = 0.77$). Statistical analyses throughout were conducted in SAS (2001).

3. RESULTS

(a) *Non-competitive mating success and courtship behaviour*

In the experiment where males were paired singly with virgin females, there was a strongly significant effect of surgical treatment on copulation probability ($\chi^2 = 92.73$, d.f. = 3, $p < 0.0001$). Males whose genital spines

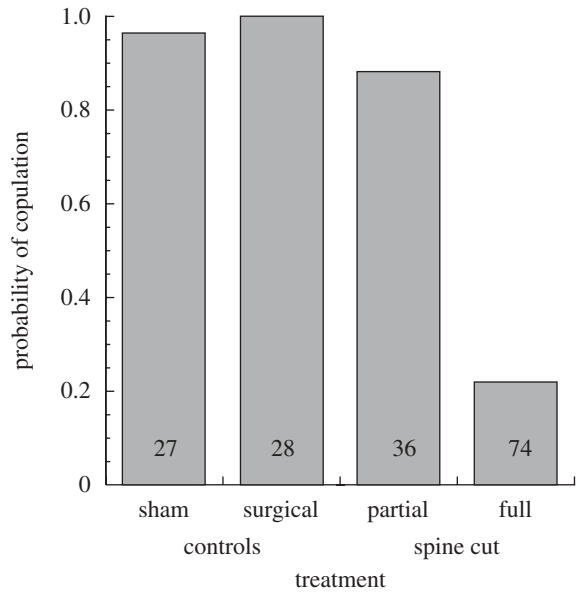

Figure 2. Treatment effects on the probability of copulation. Numbers are sample sizes, pooled across three time blocks.

were fully ablated exhibited a precipitous drop in the probability of copulation compared with all other categories (figure 2). When the data were analysed without the fully ablated category (Zar 1998), the significant treatment effect was lost ($\chi^2 = 3.98$, d.f. $= 2$, $p = 0.14$).

We also examined the data from the first block separately because this was the block in which sexual behaviours were monitored. These results mirrored that of the full dataset: 20 per cent of males within the fully ablated category copulated, whereas for controls this value was 100 per cent. Of the 24 males whose genital spines were ablated, 100 per cent courted the female at least once (median number of courtship bouts: 4; range: 1–11), and 96 per cent (23/24) attempted copulation but failed (median number of copulation failures: 2.5; range: 1–9). Males exhibiting copulation failure grasped the female's abdomen with their front legs aided by their sex combs, curled their abdomen downward and forward, probed the female's terminalia repeatedly with their own genitalia and, after repeated probings (which we interpret as copulation attempts), dismounted without copulating (contrast movies S1 and S2 in the electronic supplementary material). Thus, direct observations of male behaviour revealed that spineless males, despite exhibiting vigorous courtship and attempts to copulate, failed to do so because of the inability to achieve genital coupling.

(b) *Competitive mating success*

This experiment consisted of competitive dyadic mating trials, where a spineless male and a surgical control competed for access to one virgin female. In 32 (97%) of 33 trials, it was the surgical control that copulated ($G_{adj} = 33.54$, $p < 0.0001$). We conclude that the genital spines in *D. bipectinata* confer an advantage in pre-insemination sexual selection.

When intact males were lightly anaesthetized with CO_2, their spines were seen to fold and unfold in a rhythmic fashion, suggesting muscular control of their movements. Figure 1*b* illustrates how the spines are able to fold over each other. Indeed, in pairs ($n = 5$) that had been flash frozen in copula and partially separated under a stereomicroscope, the spines were similarly observed to be folded over each other while entrenched in the female external genitalia.

(c) *Success in sperm transfer*

We did not detect a significant effect of surgical treatment (table 1) or of the treatment × block interaction (table S1 in the electronic supplementary material) on the response variable. We note that sperm numbers transferred by males in the partial and full ablation categories were marginally *elevated* relative to controls (table 1). There was a significant effect of male body size on the number of sperm transferred to females (table S1 in the electronic supplementary material); these variables were positively related (slope: $\hat{b} = 43.81$; s.e.: 20.18, $p = 0.036$).

(d) *Non-competitive fertilization success*

ANCOVA revealed non-significant effects of laser treatment on female fecundity and fertility (table 1). The effects of time and, importantly, its interaction with treatment, were also non-significant for both traits (tables S2 and S3 in the electronic supplementary material). These non-significant interactions indicate that there was a statistically homogeneous lack of response in the pattern of expression of these reproductive traits across 5 consecutive days post-mating. Copula duration significantly positively affected both fecundity and fertility (tables S2 and S3 in the electronic supplementary material, respectively). Treatment effects on fecundity and fertility over females' first 24 h post-mating were likewise not significant ($F_{3,69} = 0.40$, $p = 0.75$ and $F_{3,69} = 0.55$, $p = 0.65$, respectively).

(e) *Competitive fertilization success*

Results of ANCOVA on offensive sperm competitive ability, measured as P_2 (the proportion eggs fertilized by the second male), revealed no significant effect of surgical treatment (table 1). In contrast, there were significant effects of the body size of the second male to mate (table S4 in the electronic supplementary material), with larger males fertilizing a greater proportion of eggs (slope: $\hat{b} = 0.038$; s.e.: 0.016; $p = 0.024$).

4. DISCUSSION

The power of the laser ablation technique resides in its ability to excise or alter the shape of traits of interest by ablating the smallest of structures, down to less than 1 μm in size, with little or no collateral damage to adjacent structures. Also, it has advantages over genetic engineering, which cannot easily exclude confounding effects arising from pleiotropic transgenes or from the genetic background of mutant stocks (Chapman *et al.* 1995; Ng & Kopp 2008). With this laser technique, we have opened up for experimental study a rich diversity of microscale morphological traits previously inaccessible with mechanical tools.

Arguably the most popular hypothesis for the evolution of male genital complexity is cryptic female choice, according to which genitalia function as internal courtship devices, with those males best able to stimulate

Table 1. A comparison of reproductive trait means ± s.e. (*n*) among treatments. d.f., degrees of freedom. Full results of statistical analyses provided in tables S1–S4 in the electronic supplementary material.

	controls		genital spine manipulations				
trait	sham	surgical	partial cut	full cut	*F*	d.f.	*p*
non-competitive							
sperm transferred ($\times 10^2$)	9.81 ± 0.78 (12)	9.38 ± 0.79 (12)	10.81 ± 0.78 (13)	11.31 ± 0.75 (11)	1.46	3, 40	0.24
fecundity	91.53 ± 4.05 (24)	89.57 ± 4.09 (23)	83.44 ± 4.95 (19)	82.49 ± 5.44 (13)	1.15	3, 69	0.33
fertility	77.21 ± 4.79 (24)	72.36 ± 4.83 (23)	67 ± 5.85 (19)	71.79 ± 6.43 (13)	0.62	3, 69	0.61
competitive							
P_2	0.99 ± 0.087 (14)	0.82 ± 0.088 (14)	0.81 ± 0.079 (17)	0.84 ± 0.095 (12)	0.93	3, 52	0.43

the female siring a disproportionate fraction of offspring (Eberhard 1985, 1996; Hosken & Stockley 2004). In contrast, we demonstrate experimentally that the primary role of the intromittent genital spines in *D. bipectinata* is for securing copulations, and that they confer an advantage in sexual competition occurring prior to insemination. Our results support the hypothesis that male genital morphology evolves in response to direct competition among males for securing mates (Thornhill & Alcock 1983; Eberhard 1985; Simmons 2001).

Our key finding is that laser excision of the genital spines sharply reduced the ability of males to secure a mate. In trials where males were paired singly with females, the copulation rate of spineless males was 22 per cent, compared with 98 per cent for controls (surgical and sham controls combined). Direct observations of sexual behaviours revealed that spineless males were unable to lock into the female genitalia with their own genitalia, thus they failed to achieve genital union. Observations of the range of movements of the spines in live and intact males under CO_2 anaesthesia, and of the orientation of the spines during copulation, lead us to the model that the folding action of the spines over each other (figure 1*b*) draws the genitalia together.

In mating trials where two males were allowed to compete for a single virgin female, the copulation rate of the spineless male was 3 per cent, indicating that in these competitive trials the spineless male almost invariably lost the receptive female to the intact control. This result demonstrates directly, albeit under laboratory conditions, that the genital spines in *D. bipectinata* function in pre-insemination sexual selection.

In the relatively rare event that spineless males did succeed to copulate, there was no evidence that they had decreased fertilization or paternity success. We first showed that males with either fully or partially ablated spines did not transfer significantly different quantities of sperms to the reproductive tract of females compared with controls; in fact, both spine-ablated groups transferred marginally, but not significantly, more sperms than controls. This result indicates that the spines do not serve to promote insemination, contradicting the suggestion that the spines function to inject semen into the female reproductive tract across her body wall via traumatic insemination (Kamimura 2007).

We likewise found no effect of the surgical spine manipulations on female fecundity or either measure of fertilization success. Whereas the lack of these 'downstream' effects could be predicted from the sperm data, they were nevertheless important to quantify, as any wound inflicted by the sharp tips of these spines conceivably could facilitate the delivery of bioactive compounds into the female circulatory system and enhance paternity (Eberhard 1998; Hotzy & Arnqvist 2009). The seminal fluid in *Drosophila* contains a cocktail of accessory gland proteins (Acps) known to influence a variety of female physiological and reproductive traits, some of which were specifically selected for examination here. For example, the male seminal peptides ovulin and sex peptide, both of which enter the female circulation from the reproductive tract after mating, stimulate oogenesis and egg deposition, with effects of ovulin occurring quickly, in the first 24 h post-mating (Hendorn & Wolfner 1995). In addition, some Acps participate in sperm storage, and possibly sperm competition as well (Ram & Wolfner 2007). However, our results failed to show effects of the genital manipulation on female short-term (up to 24 h post-mating) or longer-term (up to 5 days post-mating) fecundity or fertility, or on competitive fertilization success. Thus, this set of tests strongly suggests that the genital spines in *D. bipectinata* do not serve to increase fertilization gains once copulation has been achieved.

Sexual conflict occurring *prior* to insemination (Arnqvist & Rowe 2005) may help explain the interspecific diversification of the spines. *Drosophila bipectinata*, like other species within the genus, has a promiscuous mating system, generating the potential for conflict between the sexes over reproduction, such as over the timing of copulation (Rice 2000). The occurrence of such conflict is suggested by the common phenomenon in *Drosophila* (Spieth 1952) and other species (Crudgington & Siva-Jothy 2000) of the female kicking against the male during courtship, among other behaviours. Thus, if the spines act to counter such forms of female sexual resistance, coercing females to mate, differences in spine morphology in *Drosophila* may represent divergent adaptations in males matched to different forms and intensities of female resistance; antagonistic coevolution between the sexes (Arnqvist & Rowe 2005) may be contributing to the rapid diversification of the *Drosophila* spines. Additionally, if the spines act to secure the female against takeover attempts, thus acting as holdfast devices (Darwin 1871; Simmons 2001),

variation among species in the intensity and form of direct intrasexual rivalry, in turn influenced by variability in ecological factors and social organization, may be contributing to the diversification of this remarkable genital armament.

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