

Evaluating the post-copulatory sexual selection hypothesis for genital evolution reveals evidence for pleiotropic harm exerted by the male genital spines of *Drosophila ananassae*

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 precopulatory adaptive function;
 sexual conflict.

Abstract

The contemporary explanation for the rapid evolutionary diversification of animal genitalia is that such traits evolve by post-copulatory sexual selection. Here, we test the hypothesis that the male genital spines of *Drosophila ananassae* play an adaptive role in post-copulatory sexual selection. Whereas previous work on two *Drosophila* species shows that these spines function in precopulatory sexual selection to initiate genital coupling and promote male competitive copulation success, further research is needed to evaluate the potential for *Drosophila* genital spines to have a post-copulatory function. Using a precision micron-scale laser surgery technique, we test the effect of spine length reduction on copulation duration, male competitive fertilization success, female fecundity and female remating behaviour. We find no evidence that male genital spines in this species have a post-copulatory adaptive function. Instead, females mated to males with surgically reduced/blunted genital spines exhibited comparatively greater short-term fecundity relative to those mated by control males, indicating that the natural (i.e. unaltered) form of the trait may be harmful to females. In the absence of an effect of genital spine reduction on measured components of post-copulatory fitness, the harm seems to be a pleiotropic side effect rather than adaptive. Results are discussed in the context of sexual conflict mediating the evolution of male genital spines in this species and likely other *Drosophila*.

Introduction

Male genital morphology exhibits a pattern of rapid evolutionary diversification among internally fertilizing animal species (Tuxen, 1970; Eberhard, 1985). Theoretical and empirical evidence supports an important role for sexual selection in this diversification (Stebbins, 1971; Waage, 1979; Shapiro & Porter, 1989; Ware & Opell, 1989; Porter & Shapiro, 1990; Arnqvist, 1998; Hosken & Stockley, 2004; Simmons *et al.*, 2009; Eberhard, 2010a,b), with the large majority of the effort focused on post-copulatory mechanisms of sexual selection (reviewed in Leonard & Córdoba-Aguilar, 2010). Although post-copulatory sexual selection is a wide-

spread and pervasive phenomenon likely responsible for much of the observed diversity in genital form and function, a growing body of evidence for the precopulatory adaptive function of animal genitalia is providing new insights into the mechanisms of genital trait evolution (Bertin & Fairbairn, 2005; Langerhans *et al.*, 2005; Moreno-García & Cordero, 2008; Kahn *et al.*, 2010; Miller, 2010; Polak & Rashed, 2010; Evans *et al.*, 2011; Grieshop & Polak, 2012; Mautz *et al.*, 2013). Still, the precise mechanism(s) by which sexual selection operates to drive the evolution of genital form and function remain unclear (Eberhard, 1985, 1993; Arnqvist, 1997, 1998; Eberhard, 2006, 2010a,b, 2011; Leonard & Córdoba-Aguilar, 2010; Rowe & Arnqvist, 2012; Evans *et al.*, 2013).

The three most commonly encountered hypotheses for genital evolution (Eberhard, 1993; Arnqvist, 1997; Hosken & Stockley, 2004; Leonard & Córdoba-Aguilar,

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2010) are (i) sperm competition: rivalry among the gametes of different males for access to female gametes, potentially involving other components of the male ejaculate and/or interactions with female reproductive anatomy and physiology (Parker, 1970; Waage, 1979; Birkhead & Møller, 1998; Simmons, 2001; Pitnick *et al.*, 2009; Pizzari & Parker, 2009; Manier *et al.*, 2010); (ii) cryptic female choice: differential sperm use by females in response to variation in male phenotype (Lloyd, 1979; Thornhill, 1983; Eberhard, 1985, 1996); and (iii) sexual conflict: fundamental differences in fitness acquisition between the sexes (Trivers, 1972; Clutton-Brock & Vincent, 1991) potentially generating a coevolutionary arms race of adaptations and counter adaptations (Parker, 1979; Arnqvist & Rowe, 1995, 2002a,b, 2005; Chapman *et al.*, 2003; Rönn *et al.*, 2007). Sperm competition and cryptic female choice are post-copulatory processes, whereas sexual conflict potentially operates before, during and/or after copulation.

Three additional post-copulatory sexual selection mechanisms that could drive genital evolution include (iv) the holdfast mechanism: male genital traits anchor the male securely to the female during copulation (Thornhill & Alcock, 1983; Simmons, 2001; Rönn & Hotzy, 2012); (v) traumatic insemination: male genital traits hypodermically transmit gametes through the female body wall where they migrate to the site of fertilization, thus bypassing the 'traditional' route of gamete transfer, and potentially avoiding the ability of female traits to influence the fate of male gametes (Michiels, 1998; Stutt & Siva-Jothy, 2001; Morrow & Arnqvist, 2003; Reinhardt *et al.*, 2003, 2007; Kamimura, 2007; Řezáč, 2009); and (vi) adaptive harm: male genital traits harm females, causally promoting male reproductive fitness (Michiels, 1998; Lessells, 1999; Johnstone & Keller, 2000; Morrow *et al.*, 2003; Lessells, 2005). That male traits can be harmful to their mates is a common suggestion gaining increasing support (Parker, 1979; Michiels, 1998; Lessells, 1999; Civetta & Clark, 2000; Crudgington & Siva-Jothy, 2000; Johnstone & Keller, 2000; Rice, 2000; Morrow *et al.*, 2003; Arnqvist & Rowe, 2005; Edvardsson & Tregenza, 2005; Lessells, 2005; Hotzy & Arnqvist, 2009; Hotzy *et al.*, 2012), but there is an important distinction between adaptive harm (above) and pleiotropic harm, in which male genital traits harm females as a side effect of their adaptive function, and the harm *per se* does not causally promote male fitness (Parker, 1979; Morrow *et al.*, 2003; Arnqvist & Rowe, 2005; Hotzy & Arnqvist, 2009). Pleiotropic harm is therefore not a mechanism of post-copulatory sexual selection, as the harm itself is not the target of selection. It is important to note that the list of hypotheses described above is not an exhaustive list of explanations for genital evolution and furthermore that none of these are necessarily

mutually exclusive from the others. In fact, there is considerable overlap among them, with most of them fitting into the broader context of sexual conflict (Arnqvist & Rowe, 2005).

The aim of the present study was to evaluate the hypothesis that the genital spines in *Drosophila ananassae* (Fig. 1) function in post-copulatory sexual selection. Females of this species are polyandrous; hence, the potential exists for post-copulatory sexual selection to influence the evolution of male genital spines (Eberhard, 1985; Simmons, 2001). The *Drosophila* genital spines exhibit a diversity of shapes and sizes and are widespread within the melanogaster species group (with over 40 species expressing them; Hsu, 1949; Bock & Wheeler, 1972; McEvey *et al.*, 1987; Schiffer & McEvey, 2006). The spines consist of 1–5 pairs (depending on species) of hard, sclerotized, claw-like structures that are external at rest, extending from the ventral cercal lobe (secondary claspers) (Hsu, 1949; Bock & Wheeler, 1972; McEvey *et al.*, 1987; Schiffer & McEvey, 2006), and move independently of the aedeagus and other genital structures. These spines have been demonstrated via manipulative experimentation to function in *pre*copulatory sexual selection in both *Drosophila bipectinata* and *D. ananassae* (Polak & Rashed, 2010; Grieshop & Polak, 2012). Males use their spines to grasp the female genitalia in order to initiate copulation (Polak & Rashed, 2010; Grieshop & Polak, 2012). Behavioural evidence has shown that efficient coupling (i.e. gaining the copulation in relatively few attempts) helps to prevent the usurpation of females by rival males, but that females (at least in *D. ananassae*) do not choose mates on the basis of genital spine morphology (Grieshop & Polak,

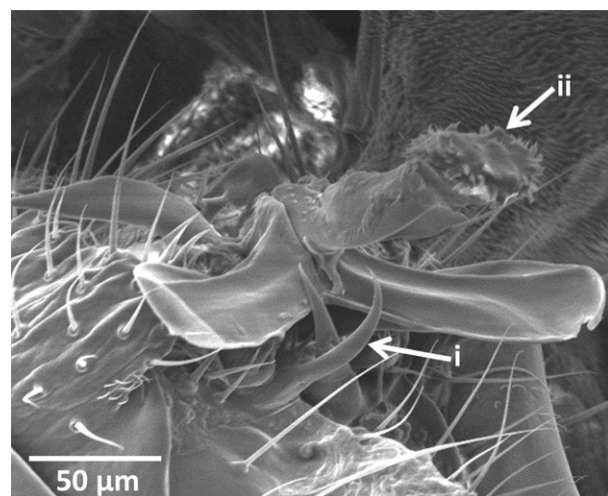


Fig. 1 Scanning electron micrograph of male *Drosophila ananassae* terminalia (500 \times); (i) genital spines (external at rest) in crossed orientation, (ii) everted aedeagus.

2012). Nevertheless, the spines are necessary to overcome females' general resistance to mating and thus seem to have evolved under the combined influence of intrasexual selection and sexual conflict (Polak & Rashed, 2010; Grieshop & Polak, 2012).

Although no evidence thus far indicates a post-copulatory function for the male genital spines of *Drosophila* (Polak & Rashed, 2010; Grieshop & Polak, 2012), such a function may have been missed (Eberhard, 2011) and requires further investigation. Although *Drosophila* genital spines have a similar pre-copulatory function to that of the genital claspers of many insects (e.g. Arnqvist & Rowe, 2002a; Bertin & Fairbairn, 2005; Moreno-García & Cordero, 2008), they also share morphological features with genital traits having post-copulatory functions (e.g. Kamimura, 2007; Hotzy *et al.*, 2012) in that they come to a sharp, potentially injurious point (Fig. 1), and insert into the female's external genitalia (but not the gonopore) during copulation (Grieshop & Polak, 2012). Furthermore, in *D. ananassae*, a substantial portion (approximately 35%) of the male genital spines must be ablated in order to elicit a statistically significant reduction in male copulation success (Grieshop & Polak, 2012; and the present study), indicating that spine length may have been selected beyond that which is necessary for copulation to perform some additional post-copulatory function.

Using a precision micron-scale laser surgery system as a means of phenotypic manipulation (Polak & Rashed, 2010; Grieshop & Polak, 2012), we first tested for the effect of surgical reduction of male genital spine length on female remating behaviour and egg deposition (hereafter: oviposition). Responses in either or both these female reproductive functions are predicted by the cryptic female choice hypothesis (Eberhard, 1985, 1996, 2011). Specifically, if the spines serve to stimulate the female to use the current male's sperm to fertilize eggs, it is predicted that experimental reduction in spine length would reduce post-mating oviposition and/or the interval of time to a second copulation with another male (Eberhard, 1985, 1996, 2011). We also tested for the role of the spines in promoting competitive male fertilization success, a critical prediction of both the cryptic female choice and sperm competition hypotheses (Eberhard, 1985, 1996, 2011; Birkhead & Møller, 1998; Simmons, 2001; Pitnick *et al.*, 2009; Pizzari & Parker, 2009). To this end, we assessed the effect of surgical reduction of male genital spine length on the proportion of a twice-mated female's clutch of eggs sired by her first (P_1) and second (P_2) mate. If the spines promote competitive fertilization success, surgical reduction of male genital spines should decrease male P_1 and/or P_2 (Eberhard, 1985, 1996, 2011; Birkhead & Møller, 1998; Simmons, 2001; Pitnick *et al.*, 2009; Pizzari & Parker, 2009).

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 h L (24 °C) : 12 h D (22 °C) photoperiod and temperature regime in 6–8 240-mL-glass milk bottles containing 6 g of Formula 4-24 Instant *Drosophila* Medium (Carolina Supply Co., Burlington, NC, USA), 20 mL water, 8 mL of banana/live yeast slurry (50 mL water: 25 g banana: 1.5 g live yeast). The base population was mass cultured in the laboratory for 3 years (≈ 90 generations) prior to 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 polystyrene shell vials lined with cornmeal-agar food medium (approximately 25 flies per vial) and allowed to age for 5 days prior to use in the experiments. Experimental flies were transferred to fresh food vials every other day until experimentation, and live yeast was added to vials containing females.

Laser manipulation

Virgin males were laser-treated within 22 h (± 2 h) of eclosion using the protocol described in Polak & Rashed (2010). Briefly, males were placed one at a time in a plexiglass surgical chamber while lightly anesthetized with humidified CO₂. Pulsed laser light ($\lambda = 532$ nm) was used to administer precision cuts to genital spines with little or no collateral damage to surrounding structures or bristles (see fig. 1c in Grieshop & Polak, 2012). The surgical treatment used for 'cut' males throughout this study entailed the removal of approximately $\frac{1}{3}$ of the length of both spines. A size reduction of this amount does not severely compromise males' ability to mate (Grieshop & Polak, 2012), thus assuring a sufficient number of matings for the present study. Surgical controls were generated by ablating two large, randomly chosen bristles on the seventh sternite of the ventral abdomen (Grieshop & Polak, 2012). Laser surgeries for all males, including the controls, took < 1 min per individual. Following surgery, males were held without females in food vials (approximately 20 males per vial) until experimentation. Surgically manipulated males were always 5 days old on the day they were used for mating. Throughout both experiments described below (involving > 350 individual surgeries), no males died in holding vials prior to experimentation.

Female remating and fecundity

Across three replicate blocks (B1–B3), a total of 80 virgin females from the base population (n 's: B1 = 39, B2 = 22, B3 = 19) were individually paired in agar vials with cut males, whereas 79 virgin females (n 's: B1 = 38, B2 = 22, B3 = 19) were individually paired with surgical control males; vials were continuously scanned for matings. Of the 80 females paired with cut males, 52 mated, whereas 57 of 79 mated with control males. Within 1 h of copulation, females were transferred to fresh oviposition vials to lay eggs and were then transferred to fresh oviposition vials every 24 h until they either remated or the experiment was terminated. The number of eggs deposited by each female during every 24-h period was counted. The term 'female fecundity' refers to the number of eggs deposited in the first 24-h period after the first mating.

Once-mated females were paired for remating with 5-day-old males at 3, 5 and 7 days after females' first matings, which resulted in at least 50% of the females remating in a particular block. Females that did not remate within 2 h on a given day were transferred to fresh oviposition vials until the next remating opportunity. 'Intercopulation interval' refers to the number of elapsed days between a female's first and second matings. 'Pre- P_2 eggs' refers to the number of eggs laid during the intercopulation interval. The latency to, and duration of, all copulations were recorded. This experiment was conducted blind with respect to male surgical treatment, which was identified after experimentation.

After use, males and females were preserved in 95% ethanol and later examined under an Olympus SZX12 stereomicroscope (Olympus Corp., Center Valley, PA, USA) to identify males' surgical treatment and verify the integrity of the surgical manipulation (without knowledge of copulatory status), as well as to measure male and female thorax lengths. Thorax length, an estimate of body size (Robertson & Reeve, 1952), was measured (in rehydrated specimens) from the tip of the scutellum to the anterior edge of the thorax using an ocular micrometre; independent repeated measurements of thorax lengths in a random sample of 10 males were highly repeatable among males (one-way ANOVA: $F_{9,10} = 516.7$, $P < 0.0001$).

Male competitive fertilization success

We investigated the effect of male genital spine reduction on defensive (P_1) and offensive (P_2) competitive fertilization success, the proportion of a twice-mated female's clutch of eggs sired by the first or second male, respectively (Simmons, 2001). The latency to, and duration of, all copulations for these experiments were recorded. The data for P_1 and P_2 determination were collected blind with respect to male surgical treatment, which was identified after experimentation when the

integrity of all surgical manipulations was verified and male thorax lengths were measured. The calculations of P_1 and P_2 (described below) assume that incidences of unhatched eggs owing to causes other than experimental treatments were randomly distributed across our treatment categories.

Defensive competitive fertilization success (P_1)

Across two replicate blocks (B1 and B2), a total of 50 virgin females from the base population (n 's: B1 = 25, B2 = 25) were individually paired in agar vials with cut males, and 50 virgin females (n 's: B1 = 25, B2 = 25) were paired with surgical control males; vials were continuously scanned for matings. Of these females, 26 of 50 mated with cut males and 34 of 50 mated with control males. Within 1 h of copulation, females were transferred to fresh oviposition vials to lay eggs and were then transferred to fresh oviposition vials every 24 h until they either remated or the experiment was terminated; the number of eggs deposited by each female during every 24-h period was counted. Mated females were paired for remating with 5-day-old irradiated virgin 'donor' males at 4, 5 and 6 days after their first mating, which resulted in at least 50% of the females remating. The donor males were irradiated with a 150 Gy dose from a ^{60}Co source (Polak & Simmons, 2009) when they were 2 days old; sperm of irradiated males fertilize eggs but zygotes die before hatching due to lethal mutations (Simmons, 2001). To check the efficacy of this irradiation dosage, we monitored 10 randomly selected females each mated once to an irradiated male and verified that no eggs from those matings hatched (mean number eggs \pm SD per female: 44.8 ± 6.56). Females that did not remate within 2 h were transferred to fresh oviposition vials until the next remating opportunity. The variables 'female fecundity', 'intercopulation interval', and 'pre- P_2 eggs' are defined as above.

Twice-mated females were transferred to fresh oviposition vials within 1 h of the second copulation and then transferred to fresh oviposition vials every morning until a minimum of 10 eggs were laid (mean \pm SD (n): 19.9 ± 7.9 (30)). P_1 was calculated as the number of eggs that hatched into larvae divided by the total number of eggs laid (Boorman & Parker, 1976; Polak & Simmons, 2009).

Offensive competitive fertilization success (P_2)

Across two replicate blocks, a total of 100 virgin females from the base population (B1 = 50, B2 = 50) were individually paired in agar vials with irradiated virgin donor males; 80 of these 100 females mated. Within 1 h of copulation, females were transferred to fresh oviposition vials to lay eggs and were then transferred to fresh oviposition vials every 24 h until they either remated or the experiment was terminated. The number of eggs deposited by each female during every 24-h period was

counted. Half of the mated females were then paired for remating with cut males and the other half with control males at 4, 5 and 6 days after their first mating, which resulted in at least 50% of the females remating in a particular block. Females that did not remate within 2 h on a given day were transferred to fresh oviposition vials until the next remating opportunity. 'Intercopulation interval' and 'pre-P₂ eggs' are defined as above.

Twice-mated females were transferred to fresh oviposition vials within 1 h of the second copulation and then transferred to fresh oviposition vials every morning until a minimum of 10 eggs were laid (mean \pm SD (n): 17.1 \pm 6 (44)). P₂ was calculated as the number of eggs that hatched into larvae divided by the total number of eggs laid (Boorman & Parker, 1976; Polak & Simmons, 2009).

Statistical analysis

Female remating and fecundity

Male mating success across treatments was analysed using binary logistic regression. Copulation latency exhibited a skewed frequency distribution, so was log-transformed, but copulation duration residuals were normally distributed so this variable was not transformed. Copulation latency and duration were analysed using ANCOVAs. The binary logistic regression and the ANCOVAs included block, surgical treatment, and male and female thorax length as independent terms. In total, nine matings were missing measurements for male and/or female thorax length so that analyses including those covariates will be lacking some observations.

Female fecundity data had an overabundance of zero values (treatments did not differ significantly in this respect, $\chi^2 = 1.12$, $P = 0.29$), causing models to have non-normally distributed residuals. The best fit model to these data was a standard least-squares ANCOVA on untransformed fecundity (compared to different data transformations and all suitable generalized linear models), which still yielded bimodally distributed residuals. Thus, 10 000 bootstrapped permutations of the model were performed using the residuals from the original model as the response variable – the preferred method of resampling multiple regressions and AN(C)OVA-type models (Ter Braak, 1992). The proportion of F statistics observed in 10 000 bootstrapped permutations that were \geq the F statistic produced by the original model represents the probability that the F statistic from the original model could occur from these data by random chance (i.e. the P -value for that F statistic). This analysis of fecundity included block, surgical treatment, and male and female thorax length as independent terms. We also present the results of this analysis as a two-way ANOVA (with only block and treatment as independent terms).

The effect of male treatment on the likelihood that a female would remate with a randomly selected virgin

male from the base population was analysed using binary logistic regression including block, surgical treatment, and male and female thorax length as independent terms. Of the females that did remate, the intercopulation interval was analysed using ordinal logistic regression with block, surgical treatment, male thorax length, and pre-P₂ eggs as independent terms.

Male competitive fertilization success

For the P₁ component, male mating success, copulation latency, copulation duration, female fecundity, female remating likelihood and female intercopulation interval across treatments were analysed as described above with minor exceptions: the covariate female thorax length was replaced by pre-P₂ eggs in the analysis of intercopulation interval and excluded from all remaining models, to enhance model fit. Treatments did not differ significantly in the proportion of matings producing zero female fecundity ($\chi^2 = 0.97$, $P = 0.326$). One measurement for male thorax length was missing so that analyses including male thorax length will be lacking one observation. Also, one female (mated to a control male) from this component of the experiment was removed from analyses involving egg deposition because she did not oviposit any eggs after her first or second mating.

For the P₂ component, the effect of surgical treatment on male mating success (with nonvirgin females that had been mated once to donor males) was analysed as described above (in the previous section). Copulation latency and duration (with nonvirgin females that had been mated once to donor males) were analysed as described above (in the previous section) except that the term female thorax length was removed to enhance model fit – females' copulation latencies and durations from their first matings, as well as pre-P₂ eggs, were considered as additional covariates in these models but were removed to enhance model fit. One female escaped from her holding vial after remating, so P₂ analyses will be lacking one observation.

P₁ and P₂ values (proportions) were analysed separately using generalized linear models with a binomial error structure and a logit link function (specifying 1 as the greatest possible value); these models included block, surgical treatment, male thorax length (of the treatment male) and pre-P₂ eggs as independent terms.

Combined analysis

The P₁ component of the competitive fertilization success experiment was identical to the female remating and fecundity experiment with the exceptions that the randomly selected virgin males presented to females for remating opportunities were irradiated in the competitive fertilization success experiment, and the schedule of females' remating opportunities differed between the experiments (although both experimental designs featured a 5-day median intercopulation interval). Thus,

these two data sets were pooled to offer a larger sample size. In these analyses, the factor 'experiment' distinguishes from which experiment the data originate.

Male mating success, copulation latency, copulation duration, female fecundity and female intercopulation interval were analysed as described above, except that the term 'block' was replaced by the following three terms: experiment, block nested within experiment, and experiment by treatment interaction. Treatments did not differ significantly in the proportion of matings producing zero female fecundity ($\chi^2 = 2.65$, $P = 0.104$).

Residuals for all reported models (where relevant) were normally distributed unless otherwise specified and homoscedastic across treatment categories. For all models, stepwise backward elimination of the most nonsignificant terms was applied to models containing all potentially relevant covariates to explore model robustness and parameter stability – all of the reported models provided the best fit to the data to which they were applied (determined by the Akaike information criterion). JMP (v. 10.0.0; SAS Institute Inc., 2012) statistical software was used in all reported models except the bootstrapped fecundity analyses, in which case SYSTAT (v. 11.00.01; SYSTAT Software, Inc. 2004), which employs the Mersenne-Twister random number generator, was used.

Results

Female remating and fecundity

Spine length reduction had a nonsignificant effect on male copulation success ($\chi^2_1 = 0.75$, $P = 0.39$; Table 1). Of those males that did copulate, cut individuals exhibited a nonsignificant increase in copulation latency ($F_{1,94} = 1.28$, $P = 0.261$) and duration ($F_{1,94} = 3.86$, $P = 0.053$; Table 1) relative to control males.

Females mated to cut males laid a significantly greater number of eggs over the first 24-h period following copulation than those mated to control males ($F_{1,94} = 4.05$, $P = 0.047$); an adjusted P -value for this F statistic based on a bootstrapped resampling (10 000 iterations) of the residuals confirms this result (Tables 1 and 2). A two-way ANOVA excluding male and female thorax length so as to include all observations (see Methods) yielded congruent results ($F_{1,105} = 4.34$, $P = 0.039$, bootstrap-adjusted $P = 0.038$; mean number of eggs laid ± 1 SE (n) for cut: 45.58 ± 2.64 (52), control: 37.65 ± 2.93 (57)).

Male treatment had a nonsignificant effect on the likelihood of females remating with a randomly selected virgin male from the base population ($\chi^2_1 = 0.33$, $P = 0.565$; Table 1). Of the females that did remate, there was no significant effect of male treatment on females' intercopulation interval ($\chi^2_1 = 1.79$, $P = 0.181$; Table 1) – pre- P_2 eggs was significantly

positively associated with intercopulation interval ($\chi^2_1 = 17.14$, $P < 0.0001$), but pre- P_2 eggs did not vary significantly across male treatments (ANOVA: $F_{1,56} = 0.02$, $P = 0.879$).

Male competitive fertilization success

The results for the P_1 component of this experiment largely matched those found in the female remating and fecundity experiment (Table 1). There was no significant effect of treatment on copulation success ($\chi^2_1 = 3.27$, $P = 0.071$), copulation latency ($F_{1,56} = 0.14$, $P = 0.705$), copulation duration ($F_{1,56} = 0.72$, $P = 0.401$), remating likelihood ($\chi^2_1 < 0.01$, $P = 0.969$) or intercopulation interval ($\chi^2_1 = 2.42$, $P = 0.119$; Table 1). Male thorax length (of the treatment/first male) and pre- P_2 eggs were significantly positively associated with intercopulation interval ($\chi^2_1 = 9.9$, $P = 0.002$, and $\chi^2_1 = 4.44$, $P = 0.035$, respectively), but neither male thorax length nor pre- P_2 eggs differed significantly between male treatments (ANOVAS: $F_{1,27} = 0.53$, $P = 0.471$, and $F_{1,27} = 0.39$, $P = 0.539$, respectively). In this experiment, the increase in fecundity of females mated to cut males relative to those mated to controls was marginally nonsignificant (Tables 1 and 2).

There was no significant difference between cut and control males in defensive sperm competitiveness (P_1) ($\chi^2_1 = 0.825$, $P = 0.364$; mean $P_1 \pm 1$ SE (n) for cut: 0.32 ± 0.09 (13), and control: 0.19 ± 0.06 (16)), but the deviance residuals of this model were not normally distributed. A potential outlier in which a cut male's P_1 score equalled 1 (indicating that the female's second mate may not have transferred an ejaculate) was removed and the model run again to reveal congruent results ($\chi^2_1 = 0.405$, $P = 0.524$; Table 1) – this model was a better fit to the data and provided normally distributed deviance residuals.

For the P_2 component of the experiment, spine length reduction had a significantly negative effect on male copulation success with nonvirgin females previously mated to an irradiated male ($\chi^2_1 = 4.17$, $P = 0.041$; Table 1). Of those males that did copulate, cut males exhibited a significantly longer latency to copulation ($F_{1,41} = 6.86$, $P = 0.012$; Table 1), but copulation duration did not differ between treatments ($F_{1,41} = 0.59$, $P = 0.443$; Table 1). There was no significant difference between cut and control males in offensive sperm competitiveness (P_2) ($\chi^2_1 = 0.035$, $P = 0.851$; Table 1).

Combined analysis for female remating and fecundity

Cut males exhibited a nonsignificant reduction in copulation success relative to control males ($\chi^2_1 = 3.36$, $P = 0.067$; Table 1). Cut and control males did not significantly differ in copulation latency ($F_{1,158} = 0.73$,

Table 1 Means and parameter estimates (cut-control) for measures exhibited/elicited by cut and control males.

Term	Trt.	Female remating and fecundity experiment					Male competitive fertilization success experiment					Combined analysis							
		\bar{x}	SE	<i>n</i>	<i>E</i>	UCI	LCI	\bar{x}	SE	<i>n</i>	<i>E</i>	UCI	LCI	\bar{x}	SE	<i>n</i>	<i>E</i>	UCI	LCI
Cop. success (frequency)	Cut	49	4.2	76	-0.16	0.2	-0.51	26	3.57	50	-0.38	0.03	-0.81	78	5.57	129	-0.25	0.02	-0.53
	Control	51	3.82	71			34	3.26	49				88	5.08	124				
Cop. latency (seconds)	Cut	1362	239	49	0.13	0.35	-0.1	1542	365	26	0.06	0.38	-0.26	1437	194	78	0.08	0.27	-0.11
	Control	1137	216	51			1287	250	34				1249	161	88				
Cop. duration (seconds)	Cut	271	6.22	49	9.26	18.6	-0.1	262	10.4	26	-6.39	8.73	-21.5	269	5.3	78	2.06	10.3	-6.19
	Control	253	6.93	51			272	11.1	34				260	6.06	88				
Fecundity (eggs)	Cut	45.4	2.76	49	4.17	8.28	0.06	30.2	3.29	26	4.11	8.44	-0.23	40.5	2.22	78	4.13	7.22	1.03
	Control	37.3	3.2	51			21.7	2.79	32				31.4	2.33	87				
Remating (frequency)	Cut	30	3.45	49	0.12	-0.29	0.54	13	2.6	26	-0.01	-0.53	0.51	43	4.42	78	-0.05	0.27	-0.38
	Control	28	3.59	51			17	2.96	34				45	4.72	88				
Interop. interval (days)	Cut	4	0.27	30	0.4	1	-0.18	4.31	0.17	13	0.78	1.96	-0.19	4.09	0.19	43	0.38	0.8	-0.03
	Control	4.6	0.33	28			4.63	0.18	16				4.64	0.22	44				
P ₁ (prop. sired)	Cut	-	-	-	-	-	0.19	0.06	16			1.39	-0.48	-	-	-	-	-	-
	Control	-	-	-	-	-	0.26	0.08	12	0.41	1.39	-0.48	-	-	-	-	-	-	-
Cop. success	Cut	-	-	-	-	-	18	3.15	39	-0.48	-0.02	-0.97	-	-	-	-	-	-	-
	Control	-	-	-	-	-	27	3	40	-	-	-	-	-	-	-	-	-	-
P ₂ (frequency)	Cut	-	-	-	-	-	3835	892	18	0.56	0.98	0.12	-	-	-	-	-	-	-
	Control	-	-	-	-	-	1264	306	27	-	-	-	-	-	-	-	-	-	-
Cop. latency	Cut	-	-	-	-	-	261	11.4	18	-5.65	9.09	-20.4	-	-	-	-	-	-	-
	Control	-	-	-	-	-	274	8.7	27	-	-	-	-	-	-	-	-	-	-
P ₂ (seconds)	Cut	-	-	-	-	-	0.63	0.09	18	-0.06	0.6	-0.73	-	-	-	-	-	-	-
	Control	-	-	-	-	-	0.65	0.06	26	-	-	-	-	-	-	-	-	-	-

\bar{x} , sample mean (number of successful events in the case of copulation success and remating likelihood); SE, 1 standard error of the mean (binomial standard error in the case of copulation success and remating likelihood); *n*, sample size; *E*, parameter estimate (cut-control); UCI and LCI, upper- and lower-95% confidence intervals, respectively; -, N/A. Significant differences in bold.

Table 2 Results of ANCOVAs on female fecundity (eggs laid in the first 24 h after mating), with adjusted *P*-values based on 10 000 bootstrap iterations performed on residual fecundity from the original ANCOVA models.

Term	Female remating and fecundity experiment				Male competitive fertilization success experiment				Combined analysis			
	d.f.	S.S.	<i>F</i> _{ratio}	<i>P</i>	d.f.	S.S.	<i>F</i> _{ratio}	<i>P</i>	d.f.	S.S.	<i>F</i> _{ratio}	<i>P</i>
Experiment	–	–	–	–	–	–	–	–	1	7930.58	21.68	< 0.0001
Block (experiment)	–	–	–	–	–	–	–	–	3	2636.97	2.4	0.102
Experiment × treatment	–	–	–	–	–	–	–	–	1	5.63	0.02	0.892
Block	2	1701.85	1.99	0.182	1	127.99	0.48	0.499	–	–	–	–
Treatment	1	1729.87	4.05	0.045	1	961.33	3.61	0.061	1	2539.67	6.94	0.006
Male thorax length	1	984.85	2.31	0.119	1	95.46	0.36	0.507	1	297.64	0.81	0.345
Female thorax length	1	887.86	2.08	0.091	–	–	–	–	–	–	–	–
Error	94	40146.7			54	14389.8			157	57426.6		

d.f., degrees of freedom; S.S., sum of squares; –, N/A. Significant differences in bold.

$P = 0.394$), or duration ($F_{1,158} = 0.24$, $P = 0.622$; Table 1).

Females mated to cut males exhibited significantly greater fecundity (a nearly 30% increase) relative to those mated to control males (Tables 1 and 2). There was no significant interaction between surgical treatment and experiment on female fecundity (Table 2).

Female remating likelihood did not vary significantly between treatments ($\chi^2_1 = 0.11$, $P = 0.742$), and of those females that did remate, the intercopulation interval did not vary significantly between male treatments ($\chi^2_1 = 3.23$, $P = 0.072$; Table 1). Although pre- P_2 eggs was significantly positively associated with intercopulation interval ($\chi^2_1 = 21.8$, $P < 0.0001$), it did not vary significantly across male treatments (ANOVA: $F_{1,85} = 0.14$, $P = 0.708$).

Discussion

This study investigated the relationship between male genital spine morphology in *D. ananassae* and four traits expected to mediate post-copulatory sexual selection: copulation duration, female oviposition and remating behaviour, and male competitive fertilization success. These spines are known to function in pre-copulatory sexual selection in this species, where males use their spines to grasp the female genitalia and initiate copulation in the face of rival competitors (Grieshop & Polak, 2012). The present study corroborates previous findings in that surgical reductions of male genital spines provided males with a slight, although nonsignificant, reduction in copulation success with virgin females, and a significant reduction in copulation success with nonvirgin females (Grieshop & Polak, 2012). Additionally, cut males exhibited a significantly longer latency to copulation with nonvirgin females, which was demonstrated by Grieshop & Polak (2012) to correspond to a greater number of (failed) copulation attempts. Thus, although the surgical manipulation employed

throughout the present study reduced genital coupling efficiency and mating success, it did not eliminate mating entirely, enabling the investigation of post-copulatory sexual selection.

We found no evidence for the male genital spines of *D. ananassae* functioning in post-copulatory sexual selection. Males with surgically reduced genital spines did not exhibit a decrease in copulation duration, or offensive or defensive competitive fertilization success (P_2 and P_1 , respectively) relative to control males, and females mated to cut vs. control males did not exhibit a significant decrease in short-term fecundity, pre- P_2 eggs, remating likelihood, or intercopulation interval. We emphasize that we cannot fully reject the post-copulatory sexual selection hypothesis for male genital spine function in *D. ananassae* (Eberhard, 2011). However, for the few, arguably vital, components of post-copulatory fitness that we did measure, we found no evidence of a post-copulatory adaptive value to male genital spines in this species.

The unexpected finding of our study was that females mated to males with surgically reduced genital spines deposited *more* eggs in the first 24 h after mating than females mated to control males. The direction of this finding was consistent between the two experiments (i.e. the female remating and fecundity experiment and the P_1 component of the male competitive fertilization success experiment). Although this effect was not statistically significant in the P_1 experiment alone, the combined analysis of the two data sets revealed a strongly significant positive effect of spine length reduction on female fecundity. This result suggests that the intact spines inflict harm to females. One of the critical predictions distinguishing adaptive harm from pleiotropic harm is that the former should consist of the harm itself eliciting a positive effect on at least some aspect of male fitness (Michiels, 1998; Lessells, 1999; Johnstone & Keller, 2000; Morrow *et al.*, 2003; Hotzy & Arnqvist, 2009). Genital spine ablation had no other effect on

any of the post-copulatory fitness components measured in this study; thus, we cannot conclude that the harm *per se* is adaptive. Instead, it would seem that the harm inflicted by male genital spines to female fecundity is likely a by-product of their adaptive function to promote competitive copulation success. Thus, without having performed an exhaustive investigation of post-copulatory fitness, the 'rescued' female fecundity in response to spine length reduction is most objectively interpreted as evidence for the pleiotropic harm hypothesis of genital function (Parker, 1979; Morrow *et al.*, 2003; Arnqvist & Rowe, 2005; Hotzy & Arnqvist, 2009).

Although we do not have any direct observations of spine-induced damage to females, the spines nevertheless do come to a sharp, potentially injurious point (Fig. 1) and are used to grasp females' external genitalia during copulation attempts that occur in rapid succession until copulation is achieved (Grieshop & Polak, 2012). Thus, spine-induced injuries are likely and may cause females to divert resources and/or time toward somatic tissue repair at the expense of egg provisioning and oviposition (Morrow *et al.*, 2003). Our surgical manipulations both shortened and blunted the spines, so during mating with treated males, females may have been protected from these damaging effects. Direct evidence of physical harm to females inflicted by male genital spines in *D. ananassae* would strengthen this interpretation.

The genital spines of male seed beetles in the genus *Callosobruchus* are a well-documented example of a harmful male genital trait (Crudgington & Siva-Jothy, 2000; Rönn *et al.*, 2007; Hotzy & Arnqvist, 2009; Hotzy *et al.*, 2012; Rönn & Hotzy, 2012) that offers insights/parallels to the genital spines of *D. ananassae*. Male *Callosobruchus maculatus* with relatively longer genital spines exhibit a sperm competition (P_2) advantage (Hotzy & Arnqvist, 2009; Hotzy *et al.*, 2012) and inflict greater damage to the female internal reproductive tract (Hotzy & Arnqvist, 2009) causing females mated to males with longer genital spines to have comparatively lower lifetime offspring production (Rönn & Hotzy, 2012). The sperm competition advantage bestowed upon male *C. maculatus* bearing longer genital spines may be mediated by nonsperm components of the ejaculate passing through the wounds (made by the spines) in the female reproductive tract and into the female haemocoel (Hotzy *et al.*, 2012). Because the adaptive mechanism behind the sperm competition advantage is mediated by components of the male ejaculate entering the female body cavity, and not by females' response(s) (physiological, behavioural, etc.) to the harm *per se* (Morrow *et al.*, 2003), the evolution of male genital spines in *C. maculatus* seems to have been influenced by sperm competition driving larger genital spines (Hotzy & Arnqvist, 2009; Hotzy *et al.*, 2012) and pleiotropic harm likely generating selection in females to counter

adapt (Parker, 1979; Morrow *et al.*, 2003; Arnqvist & Rowe, 2005; Hotzy & Arnqvist, 2009). Indeed, seed beetle species in which males have more 'spiny' genitalia consist of females that have thicker connective tissue in the area of their reproductive tract that is damaged by male genital spines (Rönn *et al.*, 2007). Thus, intra-sexual selection – sperm competition and mating competition – has been the driving force behind genital spine evolution in *Callosobruchus* (Hotzy & Arnqvist, 2009; Hotzy *et al.*, 2012) and *Drosophila* (Polak & Rashed, 2010; Grieshop & Polak, 2012), respectively. Likewise, the genital spines of both species evolved to become consequently harmful to females (Crudgington & Siva-Jothy, 2000; Rönn *et al.*, 2007; Hotzy & Arnqvist, 2009; Rönn & Hotzy, 2012; present study), likely setting an upper limit to their adaptive evolution. In seed beetles, this has generated a coevolutionary arms race between the sexes. In *Drosophila*, the spines have likely already been influenced by sexual conflict due to being necessary for overcoming female resistance to mating (Polak & Rashed, 2010; Grieshop & Polak, 2012), but the harm to females caused by male genital spines likely also has a role in sexual conflict and potentially generates further and/or different selection pressures favouring counter adaptations in female *Drosophila*. Investigations of the proximate mechanisms underlying the (competitive) mating advantage provided by *Drosophila* genital spines – as well as the harm caused by these spines – may clarify the pleiotropic constraints that limit genital spine evolution in *Drosophila* and help generate predictions for the type of female counter adaptations to be expected.

The field of genital evolution is becoming conceptually divided into pre- and post-copulatory traits that are, at least in insects, typically nonintromittent and intromittent structures, respectively (Simmons, 2014); but this can be misleading for some taxa (e.g. humans, primates, other mammals and fishes) exhibiting intromittent genitalia that also serve precopulatory adaptive functions (Langerhans *et al.*, 2005; Kahn *et al.*, 2010; Miller, 2010; Mautz *et al.*, 2013). Finding common evolutionary features between pre- and post-copulatory genital traits, such as the commonalities in genital spine evolution between *Drosophila* and *Callosobruchus* (see above), may help to advance and unify the interpretation of research into genital function and evolution.

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