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Individual and synergistic effects of male external genital traits in sexual selection

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Abstract

Male genital traits exhibit extraordinary interspecific phenotypic variation. This remarkable and general evolutionary trend is widely considered to be the result of sexual selection. However, we still do not have a good understanding of whether or how individual genital traits function in different competitive arenas (episodes of sexual selection), or how different genital traits may interact to influence competitive outcomes. Here, we use an experimental approach based on high-precision laser phenotypic engineering to address these outstanding questions, focusing on three distinct sets of micron-scale external (nonintromittent) genital spines in male *Drosophila kikkawai* Burla (Diptera: Drosophilidae). Elimination of the large pair of spines on the male secondary claspers sharply reduced male ability to copulate, yet elimination of the other sets of spines on the primary and secondary claspers had no significant effects on copulation probability. Intriguingly, both the large spines on the secondary claspers and the cluster of spines on the primary claspers were found to independently promote male competitive fertilization success. Moreover, when large and small secondary clasper spines were simultaneously shortened in individual males, these males suffered greater reductions in fertilization success relative to males whose traits were altered individually, providing evidence for synergistic effects of external genital traits on fertilization success. Overall, the results are significant in demonstrating that a given genital trait (the large spines on the secondary claspers) can function in different episodes of sexual selection, and distinct genital traits may interact in sexual selection. The results offer an important contribution to evolutionary biology by demonstrating an understudied selective mechanism, operating via subtle trait interactions in a post-insemination context, by which genital traits may be co-evolving.

KEYWORDS
animal genitalia, *Drosophila kikkawai*, laser phenotypic engineering, mating success, nonintromittent genital spines, post-insemination sexual selection, sexual selection and conflict

1 | INTRODUCTION

Male genitalia exhibit an extraordinarily rich morphological diversity, particularly among polygynous species with internal fertilization (Eberhard, 1985; Tuxen, 1970). This remarkable evolutionary trend has been of long-standing interest to biologists seeking to understand the underlying causal mechanisms that drive it (Dufour, 1844; Eberhard, 1985; Mayr, 1963) and to systematists, who rely

on features of male genitalia to distinguish species (McAlpine et al., 1981; Song & Bucheli, 2010; Tuxen, 1970). Whereas a variety of hypotheses addressing the evolutionary basis of divergent genital morphology has been proposed since the mid-1800s (Arnqvist, 1997; Eberhard, 1985; Masly, 2012), currently there is broad support for the role of sexual selection in this diversification, although the precise mechanisms by which this selection operates are debated (Eberhard, 2011; Hosken & Stockley, 2004; Leonard & Córdoba-Aguilar, 2010; Møller, 1998; Simmons, 2001, 2014).

Eberhard (1985), who was among the first to suggest that genitalia are under sexual selection, proposed that male intromittent genitalia (traits that are inserted into the female reproductive tract during mating) function as courtship devices that deliver sensory stimulation to the female during mating. According to this hypothesis, those males with particular intromittent features that provide the most appropriate stimulation to the female reproductive tract enjoy elevated paternity share and hence are favoured by "cryptic" female choice (Eberhard, 1985, 1996). Other post-insemination mechanisms affecting intromittent genitalia are recognized, namely sperm competition, as documented in some odonates and *Onthophagus taurus* dung beetles (Córdoba-Aguilar, 1999; House & Simmons, 2003), and sexual conflict (Hosken & Stockley, 2004), as shown in *Callosobruchus maculatus* seed beetles (Hotzy, Polak, Rönn, & Arnqvist, 2012). In vertebrate species, a role for post-insemination sexual selection in the evolution of intromittent genitalia is also recognized (e.g., Simmons & Firman, 2014). And in some cases, the phenotype of a male's intromittent genitalia may also influence male attractiveness (Mautz, Wong, Peters, & Jennions, 2013; but see Booksmythe, Head, Keogh, & Jennions, 2016) and thus in this way increase male mating success, for example, as shown in a beetle, *O. taurus* (Simmons, House, Hunt, & Garcia-Gonzalez, 2009).

The situation often differs, however, in the case of external (nonintromittent) genitalia (traits that remain external to the female gonopore during mating), such as genital claspers and spines, which, in contrast to intromittent traits, are generally regarded to operate in a pre-insemination context (Arnqvist, 1997; Darwin, 1874; Eberhard, 1985; Grieshop & Polak, 2014; Moreno-García & Cordero, 2008; Myers, Buckley, & Holwell, 2016). For example, mutually nonexclusive functions of nonintromittent genitalia include grasping and maintaining a hold of the female's body (Corbet, 1999), overcoming female resistance to mate (Bertin & Fairbairn, 2005; Sih, Lauer, & Krupa, 2002; Thornhill, 1983), opening protective structures that cover the female gonopore (Sirot, 2003; Wulff, Kamp, Santos Rolo, Baumbach, & Lehmann, 2017), positioning the male's body relative to that of the female for mating (Preziosi & Fairbairn, 2000) and grasping/securing the genitalia tightly together (Burke, Crean, & Bonduriansky, 2015; Polak & Rashed, 2010).

Notably, external genital traits are often observed to press and rub against the female during and/or after insemination, inviting the possibility that their function extends beyond the pre-insemination phase of the mating event (Eberhard, 1985, 1996). Moreover, females also often possess corresponding structures such as sensillae, grooves or depressions, at the sites of contact with male external genital structures (Sirot, 2003; Yassin & Orgogozo, 2013), further implying that such structures are stimulatory to the female, and subject to cryptic female choice. Yet, very few demonstrations of such an effect exist. In the fly *Dryomyza anilis*, males use their genital claspers to tap the female's external genitalia immediately after copulation; these tapping sequences have been shown to enhance male competitive fertilization success (Otronen, 1990). Experiments with the tsetse fly, *Glossina morsitans*, have shown that alteration of the genital structures that press against the ventral surface of the female's abdomen reduced uptake of the sperm of the current male and increased female receptivity to subsequent matings, suggesting that stimulation from the males' intact external genitalia elicits female neuromuscular responses that enhance male fertilization success via cryptic female choice (Briceño & Eberhard, 2009).

Species within the genus *Drosophila* exhibit a rich diversity of external genital traits and thus offer valuable opportunities for studying the function of this remarkable class of male genital ornamentation (Frazee & Masly, 2015; LeVasseur-Viens, Polak, & Moehring, 2015; Yassin & Orgogozo, 2013). Previous experimental work has examined the adaptive function of external genital spines (sharp, claw-like structures) in two species of *Drosophila* in the *ananassae* subgroup, *D. bipectinata* and *D. ananassae* (Grieshop & Polak, 2012, 2014; Polak & Rashed, 2010). The spines in these species occur as a single pair on the male secondary claspers (one spine on either clasper), and males with experimentally shortened spines by way of laser surgery failed to couple their genitalia with that of the female, whereas when surgically altered males did succeed to mate, their competitive fertilization efficiency was not significantly affected (Polak & Rashed, 2010). These results indicate that the secondary clasper spines in these *ananassae* subgroup species function in a pre-insemination context, serving as grasping and holdfast devices.

The approximately 100 described species within the *montium* subgroup of the *melanogaster* species group (Bächli, 2017) exhibit a particularly rich diversity of genital spines that differ dramatically in size, shape and number among species (Burla, 1954; Schiffer & McEvey, 2006; Tsacas, 1975, 1981; Tsaur & Lin, 1991). The evolutionary forces responsible for this remarkable morphological diversification remain unstudied. Here, we employed a high-precision surgical laser (Polak & Rashed, 2010) to experimentally manipulate and study the function in both a pre- and post-copulatory context of three distinct sets of conspicuous external genital spines in the *montium* subgroup species, *Drosophila kikkawai*. The spines that were studied include the pair of unequal spines on each secondary clasper, in addition to a group of *ca.* 8 irregularly spaced prominent spines on each primary clasper (Figure 1).

We first tested for a role of each of the three sets of spines on male copulation success, a function previously demonstrated for the secondary clasper spines in the two *ananassae* subgroup species, noted above (Grieshop & Polak, 2012, 2014; Polak & Rashed, 2010). Second, we evaluated the role of each set of spines in post-insemination sexual selection, by testing the prediction that eliminating or reducing the

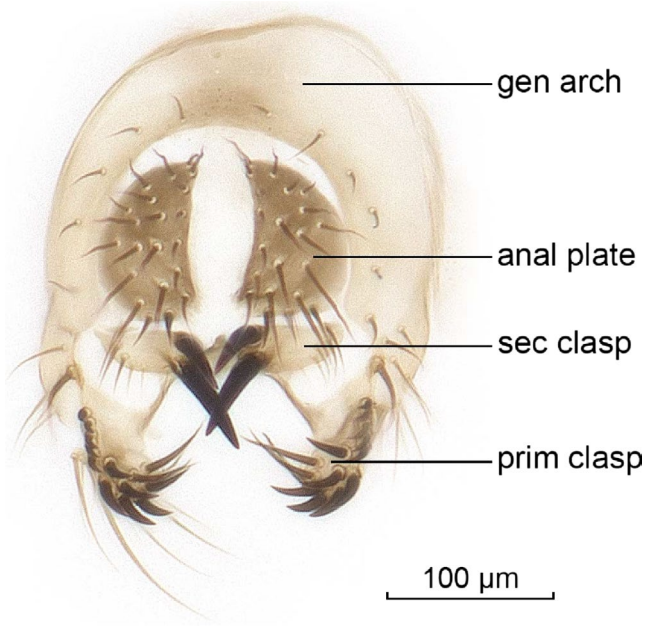

FIGURE 1 Nonintromittent (periphallic) traits of male *Drosophila kikkawai* Burla, caudal view. Abbreviations: gen arch, genital arch; sec clasp, secondary clasper; prim clasp, primary clasper (terminology following Bock & Wheeler, 1972). The spines on each secondary clasper and the irregularly spaced spines on each primary clasper were laser-manipulated in the present study. Specimen from Rarotonga, Cook Islands

size of the spines would impair male competitive fertilization success (Simmons, 2001). In a final experiment, we tested whether the two pairs of spines on the secondary clasper in *D. kikkawai* may interact in post-insemination sexual selection. Despite a growing number of manipulative experiments designed to identify the function of individual genital structures in sexual selection, few studies have tested for synergistic effects between genital traits (Simmons, 2014). Given the sheer complexity and multitude of such structures (Eberhard, 1985), synergistic effects should be expected. Our results indicate that external genital structures play, independently and synergistically, significant roles in different episodes of sexual selection and contribute to elucidating the causal bases of genital diversification.

2 | MATERIALS AND METHODS

2.1 | Experimental flies

All experimental flies were sourced from a base population of *D. kikkawai* Burla (Diptera: Drosophilidae) that had been established with 50 field-caught, mature female flies (plus an approximately equal number of males), collected in March 2013 in Taipei, Taiwan. Flies were mass cultured under laboratory conditions (12:12 L:D photoperiod and 24°C (L): 22°C (D) temperature regime) in 240-ml glass milk bottles containing cornmeal-agar food medium. Virgin individuals were collected from the base population within 6 hr of eclosion and maintained in sex-specific 35-ml disposable polystyrene shell vials containing cornmeal-agar food medium until use in the experiments. Live yeast was added to food vials containing females. Experimental flies were transferred to fresh food vials every other day until experimentation.

2.2 | Laser surgical manipulation

Virgin males were laser-treated within 24 hr of eclosion following Polak and Rashed (2010). Briefly, males were individually placed in a glass-bottomed acrylic chamber while anaesthetized with a light stream of humidified CO_2. Pulsed laser light (λ = 532 nm) emitted from a Vector 532-1000-20 Q-switched laser (Coherent), and focused through an UPlan Apo 20× objective lens of an IX71 inverted light microscope (Olympus), was used to carry out precise cuts to the genital spines. For each given trait, its right and left counterparts received a similar manipulation. After the surgical treatment, which required that males be anaesthetized for approximately 2 min each, males were aged for 5 days in treatment-specific groups of 3–5 flies per food vial before experimentation.

2.3 | Copulation success

Two time-blocks of an experiment to evaluate the effects of the spines on male ability to copulate with a receptive virgin female were conducted 7 weeks apart. In each block, laser cuts were administered to create three surgical treatments: "L-full cut" (Large-full cut), complete removal of the large spine on each secondary clasper; "S-cut" (Small-cut), complete removal of the smaller spine on each secondary clasper; and "Lobe-cuts," complete removal of eight of the irregularly clustered large spines on each primary clasper (Figure 2). Additionally, two control treatments were generated (Grieshop & Polak, 2012): "Surg-control" (surgical control), removal of 2–5 large nongenital bristles from the distal end of the abdomen; and "Sham-control", the laser pulse shot 2–5 times next to the specimen without contacting the fly.

On the evening prior to the copulation assay, 5-day-old test males were randomly assigned to numbered food vials lined up along a desktop, into which they were gently aspirated one at a time. When lights were turned on next morning at 08:00 hr, 6-day-old virgin females were individually aspirated into vials in sequential order. Each vial was scanned approximately once each minute from the time the female entered the vial, for a total of 1 hr (at 24.5–25.7°C), or until copulation occurred. Each time a vial was scanned, the observer recorded whether the male was engaged in courtship or attempted copulation (mounting the female but dismounting without securing the genitalia together). These behavioural data were collected to check whether any reductions in copulation success of treated males could merely be due to damaged courtship activity or eagerness to copulate.

2.4 | Competitive fertilization success

2.4.1 | Irradiation of males

We assessed the competitive fertilization success of treated males in double-mating trials using rival males that were irradiated the

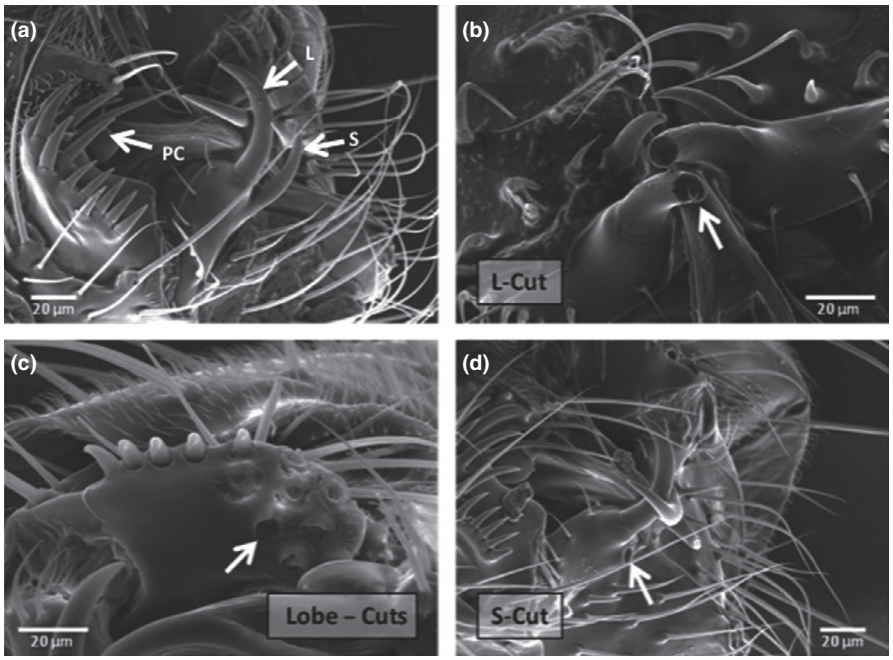

FIGURE 2 Scanning electron micrographs showing the results of the laser surgical manipulation on *Drosophila kikkawai* male genital spines. (a) Terminal segment of the male abdomen showing intact spines. S and L refer to the small and large secondary clasper spines, respectively, and PC refers to the group of primary clasper spines. (b) Shortened large spines (L-Cut). Arrow points to the cut end of one of the spines. (c) Ablated cluster of spines on the primary clasper (Lobe-cuts). Arrow points to the former insertion of one spine. (d) Ablated small spine (S-Cut). Arrow points to the former insertion point of a spine

day after collection with a 151 Gy dose from a ^{60}Co source (Polak & Simmons, 2009). The sperm of irradiated males fertilize eggs but zygotes die before hatching due to lethal mutations, which allows paternity to be assigned when a female mates with both an irradiated male and a normal male (Boorman & Parker, 1976; Simmons, 2001). To check the efficacy of the irradiation, we monitored the development of eggs laid by females after a single copulation with irradiated males. The hatching rate for these eggs was negligible in each experiment, but not zero as desired in such experiments (Polak & Simmons, 2009). After irradiation, male flies were transferred to cornmeal food vials and held for an additional 5 days.

2.4.2 | Experiment 1

We conducted two time-blocks (separated by 15 days) of an experiment testing for the effects of male surgical treatment on competitive fertilization success. Treatment categories of males were identical to that described in the "Copulation success" section above, except that the large spines were reduced in length by only 1/3 (referred to as the "L-Cut" treatment) (Figure 2b), because their complete removal sharply reduces a male's ability to copulate (see Results, and Polak & Rashed, 2010). In each block, irradiated, non-laser-treated, 5-day-old virgin males were individually aspirated into numbered food vials in the evening and left overnight. When lights were turned on next morning at 08:00 hr, 5-day-old virgin females were individually aspirated sequentially into the vials. Vials were scanned for 2 hr each or until copulation occurred. Time points at which each female was added to the vial and at which copulation began and terminated were recorded. After copulation, females were individually transferred to an oviposition vial (containing a grape juice-agar substrate) and held until the next morning when they were randomly assigned to a laser-treated male and allowed to copulate in a food vial. Vials were each scanned for 5 hr or until copulation occurred. As above, time points at which each female was added to the vial and at which copulation began and terminated were recorded. Females that mated with the laser-treated male were transferred to an oviposition vial for 24 hr and then to a second oviposition vial for an additional 24-hr period of egg laying; eggs deposited in these two vials were used for P_2 (competitive fertilization success of the second male to mate with the female) determination. Females that did not mate with a laser-treated male within 5 hr were transferred to an oviposition vial and paired again the next morning with the same male. Females that mated were sequentially transferred to two oviposition vials for P_2 determination, as above. The intermating interval for each female was the number of days (1 or 2 days) elapsed between her first and second matings. Females that failed to mate with the laser-treated male on their second attempt were discarded.

The total number of eggs deposited by a given female between her first and second matings is referred to as preP_2 eggs. For each doubly mated female, the number of eggs laid during her first and second 24-hr period of egg laying is referred to as eggs1 and eggs2, respectively. Total eggs refers to the sum of eggs1 and eggs2. For all copulations, we calculated copulation latency as the time elapsed between the introduction of the female to the vial and the onset of copulation, and copulation duration as the time elapsed between the start of copulation and when the pair disengaged. Thorax length, used as an estimate of body size, was measured using an ocular micrometer of a stereomicroscope for both males and females as the linear distance between the anterior edge of the thorax to the tip of the scutellum.

After all eggs deposited by doubly mated females were counted, vials were incubated for 24 hr at 25°C. All hatched and unhatched eggs were then counted within each vial. After this initial count, vials were incubated for another 24 hr and checked for any additional eggs that may have hatched. All counts of hatched and unhatched eggs and measurements of copulation dynamics were done "blind"

with respect to surgical treatment. At the terminus of each block, treatment males were each examined under a stereomicroscope to verify the integrity of the surgical cuts.

P_2, the competitive fertilization success of a laser-treated male, was initially calculated as the proportion of eggs that hatched (presumed to have been fertilized by the nonirradiated male) divided by the total number of eggs laid by the doubly mated female (Boorman & Parker, 1976). In addition to sperm competition processes, these values, however, could be influenced by hatching failures of eggs fertilized by the nonirradiated males (in our study the laser-treated males) (Garcia-Gonzalez, 2004; Morrow & Gage, 2001). To correct for this potential source of error, we estimated hatching rates of eggs laid by females mated to two nonirradiated, non-laser-treated, males (n = 15 females). Hatching success was very high (mean proportion ± *SE*: 0.987 ± 0.004, range: 0.96–1), and thus, the room for error due to infertility rates was very small. We nevertheless used correction factors to take into account background infertility rates as well as failures of the irradiation technique to render males sterile with 100% effectiveness (Boorman & Parker, 1976; Morrow & Gage, 2001). We first calculated the proportion of eggs fertilized by the irradiated male's sperm, P_R, as follows:

$$P_R = (1 - x/p) + (z/p) * \left(\frac{1-(x/p)}{1-(z/p)} \right),$$

where x is the observed proportion of developed eggs from a doubly mated female in the sperm competition experiment (i.e. observed hatching success for the doubly mated female, as a proportion), p is the proportion of eggs succeeding to develop when females mate with two nonirradiated males, which in our case was 0.99, and z is the proportion of developing embryos when females mate with two irradiated males, which in our case was conservatively approximated using the mean hatching success of eggs laid after the female's first mating (this value [0.0083] was very low, meaning that the irradiation technique was generally very effective). Once P_R for each double-mating trial was obtained, and given that the irradiated male mated first, P_2 was calculated as $P_2 = 1 - P_R$ (Boorman & Parker, 1976; Morrow & Gage, 2001). Due to the correction, some individual P_2 values may be slightly lower than 0 or higher than 1; in these cases, we rounded up to 0 or down to 1, respectively.

Corrected P_2 values were used to calculate the number of eggs sired by each male so that binomial models could be run on the data (see below). After obtaining the corrected P_2 value for each double-mating trial, we calculated the number of hatched eggs sired by the laser-treated male by multiplying corrected P_2 by total eggs. The resulting number was subtracted from total eggs to obtain the number of eggs sired by the irradiated male.

A total of 202 doubly mated females were initially available for the analysis (N = 87 and 115 in blocks 1 and 2, respectively). However, the final sample size was slightly lower for several reasons, including the death and loss of flies, and data exclusion owing to imperfections in the surgical manipulation detected after checking the individuals at the end of the assays (e.g., nondesired modification of surrounding structures by the laser shots). Final sample sizes across experimental groups are provided in the Results section.

2.4.3 | Experiment 2

We tested for an interactive effect between the small and large pairs of spines on competitive fertilization success. In addition to the large-cut (L-cut) and small-cut (S-cut) experimental groups used above, we created a group in which cuts were made to both large and small spines (LS-cut). This treatment consisted of the removal of 1/3 of the large spines and the complete removal of the smaller spines for each male. Only the surgical control (Surg-control) treatment was employed here. Thus, the treatment groups were L-cut, S-cut, LS-cut and Surg-control. A total of 278 doubly mated females were initially assayed in this experiment (N = 145 and 133 in blocks 1 and 2, respectively). This sample size was, however, reduced before the analyses, for the reasons noted above. The final sample sizes are provided in the Results section. We predicted that if the two pairs of spines interact to influence post-fertilization fitness, males with both pairs altered simultaneously should exhibit significantly reduced competitive fertilization success relative to either of the two individual manipulations.

2.5 | Statistical analysis

2.5.1 | Copulation success

We used logistic regression in JMP (JMP® version 12.1.0, SAS Institute Inc.) to evaluate the effects of surgical treatment, male thorax length and their interaction, on whether or not a male copulated.

2.5.2 | Competitive fertilization success (Experiments 1 and 2)

Any observation for a given variable away from the mean by more than two standard deviations was considered a potential outlier. Cook's distances were also calculated (Quinn & Keough, 2002), and observations over the threshold of 4/N, where N is the sample size, were identified (Cook & Weisberg, 1982). Observations that met both criteria were considered influential outliers and excluded prior to statistical analyses. The number of excluded outliers for laser-treated male copulation latency, laser-treated male copulation duration, P_2 and total eggs was 3, 3, 5 and 6, in Experiment 1, and 3, 2, 18 and 5, in Experiment 2, respectively.

All statistical modelling was carried out using R 3.4.1 (R Core Team, 2017). Copulation latency and copulation duration of laser-treated males were analysed using linear mixed models (LMMs), run using the function "*lmer*" implemented within the package "*lme4*" (Bates, Maechler, Bolker, & Walker, 2015). Prior to analysis, these variables were log-transformed to adequately meet the normality and homoscedasticity assumptions of the linear models.

Total eggs and P_2 were initially analysed using Poisson and binomial distribution of errors, respectively, in generalized linear mixed models (GLMMs) with the "*glmer*" function in "*lme4*" (Bates et al., 2015). For P_2, we modelled, using the command "*cbind*," the number of eggs sired by the laser-treated male over the number of eggs sired by the first male (irradiated), which effectively takes into account variation in sample size (number of offspring scored) associated with each paternity share value. To deal with issues relating to dispersion of the residuals in the *glmer* models, these were subsequently run using the function "*glmmPQL*" from the package "*MASS*" (Venables & Ripley, 2002). In these analyses, quasi-Poisson and quasi-binomial distributions were used for total eggs and P_2 analyses, respectively. Lastly, we compared the results from the above analyses to results from LMMs on total eggs and on P_2 as a proportion (after arcsin square-root transformation) (Zuur, Ieno, Walker, Saveliev, & Smith, 2009). In all cases, LMMs and GLMMs yielded similar results. For this reason, in addition to the fact that the validation of LMMs was slightly superior, we report the results pertaining to LMMs only.

P_2 data were analysed with and without zero values; zero values could be the result of infertility or failure to transfer ejaculate by the second male and could therefore obscure real patterns of sperm precedence (Garcia-Gonzalez, 2004). The results from these analyses were quantitatively and qualitatively similar, other than slight variations in the contribution of the covariates. For this reason, we report the results of analyses of the full data set. Models included the predictors laser treatment (fixed factor with 5 and 4 levels for the Experiments 1 and 2, respectively), time block (random factor with two levels) and the intermating interval (fixed factor with two levels), and relevant covariates of interest (see Tables 1 and 3); covariates were mean-centred (Schielzeth, 2010). Effects of time block and the intermating interval were invariably not significant and were thus removed from further consideration.

Model reduction and tests of significance of individual terms were carried out following two different methods depending on the type of model. For GLMMs and LMMs, we ran progressively simplified models by removing terms one at a time using the "*drop1*" function (argument test set to "*Chi*") from the package "*stats*" (R Core Team, 2017) and testing the effect of removal of each term on the change in model deviance using a likelihood ratio test (LRT) and maximum likelihood (Bolker et al., 2009). The reported LRT for a particular nonsignificant effect was calculated using the "*drop1*" function after adding the particular nonsignificant effect back into the final model (the reduced model containing only significant terms). For GLMM models involving a penalized quasi-likelihood (PQL) estimation, we ran progressively simplified models by removing effects when they were nonsignificant. In all cases, we started from the full model including all predictors but not interactions. There were no clear a priori predictions involving interactions, which were excluded to avoid inflation of the Type-I error rate. Nonetheless, we controlled for multiple tests using the Benjamini–Hochberg procedure (false discovery rate of 0.05) to correct for potential inflation of Type-I error (Benjamini & Hochberg, 1995). The validity of the

TABLE 1 Results of the competitive fertilization Experiment 1, showing the significance of effects assessed in linear mixed models (see text)

Response variable	Effect	LRT	*p*
Laser-treated male copulation latency	Laser treatment	5.05	.28
	Female body size	0.37	.54
	Laser-treated male body size	0.97	.33
	Irradiated male body size	2.06	.15
	Irradiated male copulation duration	0.96	.33
	PreP_2 eggs	4.56	.03
Laser-treated male copulation duration	Laser treatment	103.94	**<.0001**
	Female body size	5.92	**.01**
	Laser-treated male body size	0.46	.50
	Irradiated male body size	0.33	.57
	Irradiated male copulation duration	1.29	.26
	PreP_2 eggs	0.57	.45
	Laser-treated male copulation latency	0.23	.63
P_2	Laser treatment	49.06	**<.0001**
	Female body size	0.62	.43
	Laser-treated male body size	0.25	.62
	Irradiated male body size	0.05	.82
	Irradiated male copulation duration	0.21	.65
	Laser-treated male copulation duration	18.83	**<.0001**
	PreP_2 eggs	0.17	.68
Total eggs	Laser treatment	5.66	.23
	Female body size	0.63	.43
	Laser-treated male body size	0	.99
	Irradiated male body size	2.05	.15
	Irradiated male copulation duration	2.02	.15
	Laser-treated male copulation duration	0.07	.79
	PreP_2 eggs	0.16	.69

Note: "PreP_2 eggs" refers to the number of eggs laid by females in the interval between the two matings. "Total eggs" refers to the eggs laid by the doubly mated females. *p*-Values are highlighted in bold if they remained significant ($\alpha < .05$) after the Benjamini–Hochberg correction (see text).

models was checked by inspecting *qqplots* and plots of the distribution of the residuals against fitted values.

3 | RESULTS

3.1 | Copulation success

When males were each paired with a single virgin female, there was a significant effect of surgical treatment on male copulation probability (χ^2 = 23.30, *df* = 4, p < .0001) whereas the effects of thorax length (χ^2 = 0.0086, *df* = 1, p = .92) and the treatment-by-thorax length interaction (χ^2 = 1.702, *df* = 3, p = .79) were not significant.

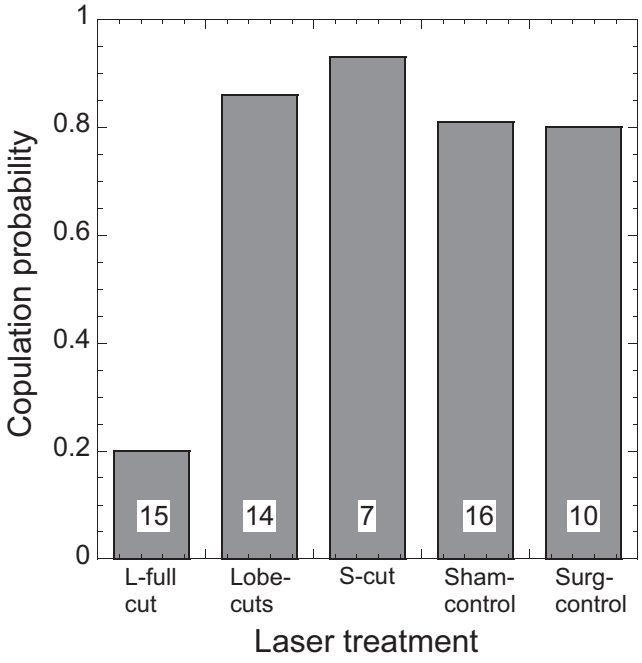

FIGURE 3 Effect of the laser surgical manipulation of males on the probability of copulation with virgin females. Numerals within each bar represent total sample sizes across two time-blocks

Males whose large spines were completely removed (L-full cut males) exhibited sharply reduced copulation success compared to the other experimental groups (Figure 3). When the data were re-analysed after excluding the L-full cut category, the significant treatment effect was lost (χ^2 = 1.06, *df* = 3, p = .79).

Of the 12 L-full cut males that failed to copulate, 11 (92%) made one or more copulation attempts (median attempts = 4.5, range 1–11), and 9 (75%) engaged in at least one bout of courtship that did not include a copulation attempt (median courtship bouts = 2.0, range 1–4). In contrast, of the five control males that failed to copulate, two males were seen to engage in 1 and 2 courtship bouts, and none were observed to make copulation attempts during the 1-hr observation period. Thus, despite exhibiting courtship and copulation *attempts*, males lacking large genital spines had significantly reduced copulation success.

3.2 | Competitive fertilization success: Experiment 1

3.2.1 | Copulation latency and duration

Variation in copulation latency was not significantly explained by any term in the model after controlling for multiple comparisons (Table 1). Copulation duration, in contrast, was significantly affected by laser treatment (p < .0001, Table 1), such that L-cut (large spine on each secondary clasper reduced in length by 1/3) and Lobe-cuts (eight spines removed from each primary clasper) males remained in copula significantly *longer* than other treatment groups (Figure 4a, Table 2). Female body size had a significant (p = .01, Table 1) and positive (Figure S1) effect on copulation duration. The remaining predictors did not significantly affect copulation duration (Table 1).

3.2.2 | Competitive fertilization success (P_2) and total eggs

Surgical treatment significantly affected P_2 (p < .0001, Table 1). P_2 of L-cut males was sharply reduced compared to all other groups (Figure 4b, Table 2). The Lobe-cuts group also exhibited

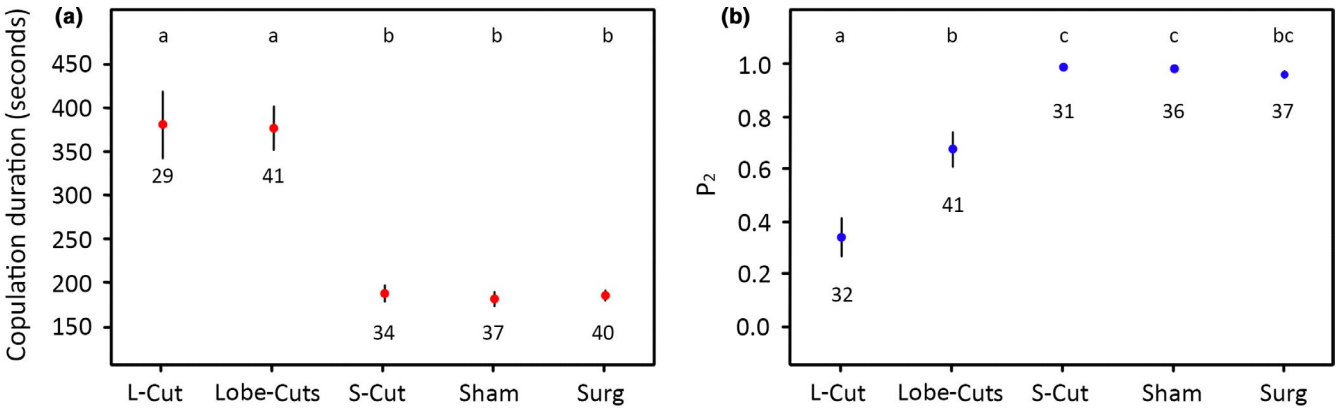

FIGURE 4 Effect of the laser surgical manipulation on (a) copulation duration and (b) the proportion of offspring sired by the second (laser-treated) male in competitive fertilization Experiment 1. Circles and whiskers represent mean ± 1*SE*, respectively. Different letters indicate significant differences (p < .05) among the treatment levels. Numerals below means indicate sample sizes

TABLE 2 Means ± 1*SE* and sample sizes (within brackets) for each behavioural and life-history trait analysed in Experiment 1

Response variable	L-Cut	Lobe-Cuts	S-Cut	Sham-control	Surg-control
Copulation latency (s) (181)	1,416.13 ± 314.94 (32)	1,861.42 ± 300.03 (41)	1,374.64 ± 287.30 (33)	2,240.32 ± 503.08 (37)	1,511.42 ± 359.06 (38)
Copulation duration (s) (181)	378.59 ± 38.27 (29)	375.71 ± 24.93 (41)	188.06 ± 10.26 (34)	182.35 ± 8.61 (37)	185.50 ± 6.26 (40)
P_2 (177)	0.33 ± 0.07 (32)	0.67 ± 0.07 (41)	0.98 ± 0.004 (31)	0.98 ± 0.007 (36)	0.95 ± 0.01 (37)
Total eggs (178)	72.43 ± 4.22 (30)	71.76 ± 3.66 (40)	79.55 ± 4.24 (33)	73.33 ± 4.06 (36)	67.53 ± 3.10 (39)

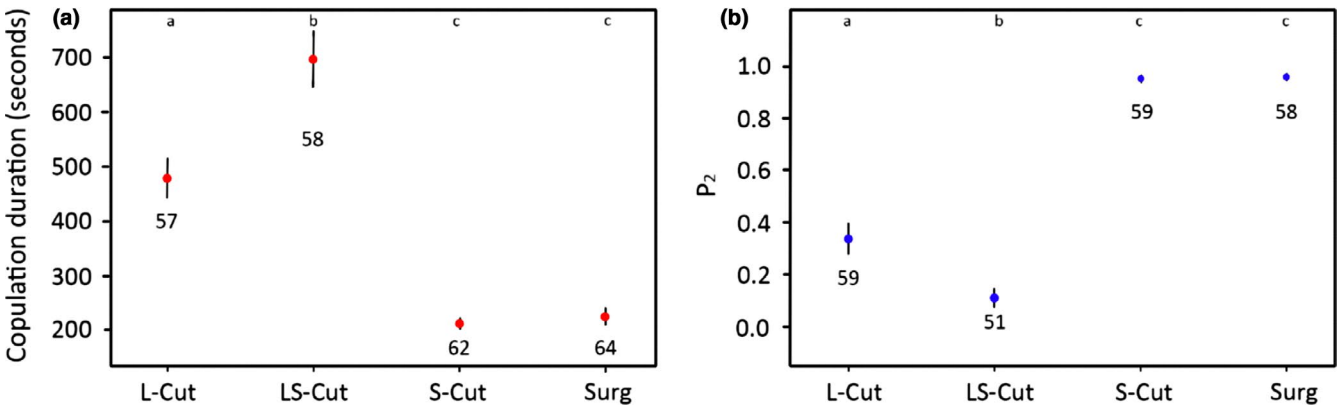

FIGURE 5 Effect of the laser surgical manipulation on (a) copulation duration and (b) the proportion of offspring sired by the second (laser-treated) male in competitive fertilization Experiment 2. Circles and error bars represent mean ± 1*SE*, respectively. Different letters indicate significant differences ($p < .05$) among the treatment levels. Numerals below means indicate sample sizes

significantly impaired fertilization success compared to controls, although the effect was not as pronounced as for the L-cut treatment; mean P_2 for the Lobe-cuts group was intermediate between L-cut and control males. S-cut (small spines removed) male P_2 did not differ from controls (Figure 4b, Table 2). Copulation duration of treatment males had a significant ($p < .0001$, Table 1) and positive effect on P_2. None of the remaining predictors significantly affected fertilization success (Table 1). For total eggs, none of the predictors included in the model had a significant effect (Table 1).

3.3 | Competitive fertilization success: Experiment 2

3.3.1 | Copulation latency and duration

Consistent with the results of Experiment 1, surgical treatment did not affect copulation latency, nor did any of the remaining predictors (Table 3). Copulation duration was significantly affected by surgical treatment ($p < .0001$, Table 3), also consistent with Experiment 1. Copulation duration of L-cut males was again significantly greater than for S-cut and surgical control groups (Figure 5a, Table 4). Likewise, copulation duration of LS-cut males was significantly greater than S-cut and control groups, and also greater than the L-cut group (Figure 5a, Table 4). None of the remaining predictors had significant effects (Table 3).

3.3.2 | Competitive fertilization success (P_2) and total eggs

There was a significant effect of laser treatment on P_2 ($p < .0001$, Table 3). P_2 for LS-cut males was significantly reduced relative to L-cut males, and P_2 values for both these groups, in turn, were significantly lower than for S-cut and control males (Figure 5b, Table 4). Copulation duration of treatment males also had significant effects on P_2 ($p = .0007$, Table 3): P_2 was negatively related to copulation duration of treatment males. None of the remaining variables exerted significant effects on P_2 (Table 3). For total eggs, neither laser treatment, nor any of the remaining predictors, had significant effects (Table 3).

4 | DISCUSSION

Male external genital traits, such as claspers and spines, are remarkably variable in size and shape across taxa (Eberhard, 1985), and there is now good evidence from a number of species that they function in pre-insemination sexual selection (Hosken & Stockley, 2004; Simmons, 2014). Also widely appreciated is that when such traits are harmful to female fitness, for example, when they involve grasping and/or coercing the female to mate (Burke et al., 2015; Polak & Rashed, 2010), they may have broader evolutionary consequences through selecting for counteradaptations in females and fuelling

TABLE 3 Results of the competitive fertilization success Experiment 2, showing the significance of effects assessed in linear mixed models (see text)

Response variable	Effect	LRT	*p*
Laser-treated male copulation latency	Laser treatment	3.16	.37
	Female body size	0.04	.84
	Laser-treated male body size	2.41	.12
	Irradiated male body size	3.01	.08
	Irradiated male copulation duration	1.25	.26
	PreP_2 eggs	1.65	.20
Laser-treated male copulation duration	Laser treatment	124.73	**<.0001**
	Female body size	2.66	.10
	Laser-treated male body size	0.02	.90
	Irradiated male body size	1.09	.30
	Irradiated male copulation duration	0.64	.43
	PreP_2 eggs	0.37	.54
P_2	Laser treatment	153.14	**<.0001**
	Female body size	0.31	.57
	Laser-treated male body size	0.76	.38
	Irradiated male body size	2.20	.14
	Irradiated male copulation duration	2.62	.11
	Laser-treated male copulation duration	10.98	**.001**
	PreP_2 eggs	0.01	.91
Total eggs	Laser treatment	2.87	.41
	Female body size	0.44	.51
	Laser-treated male body size	0	.99
	Irradiated male body size	0.14	.71
	Irradiated male copulation duration	2.77	.10
	Laser-treated male copulation duration	4.18	.04
	PreP_2 eggs	5.00	.03

Note: "PreP_2 eggs" refers to the number of eggs laid by females in the interval between the two matings. "Total eggs" refers to the eggs laid by the doubly mated females. *p*-Values are highlighted in bold if they remained significant (α < .05) after the Benjamini–Hochberg correction (see text).

co-evolutionary processes between the sexes (Arnqvist & Rowe, 2005; Sakaluk, Bangert, Eggert, Gack, & Swanson, 1995; Simmons & Fitzpatrick, 2019). The possibility that the adaptive function of external genital traits extends beyond pre-insemination sexual selection, however, is understudied, yet the complexity of such traits suggests that post-insemination mechanisms involving male–female interactions and cryptic female choice may often be at play. Here, we shed light on the role of different sets of external (nonintromittent) genital spines across episodes of sexual selection in a drosophilid fly. We provide novel insight into the evolutionary drivers of diversity in genital morphology by demonstrating, in addition to an influence of external genital traits on copulation success, independent and synergistic effects of these traits on competitive fertilization success.

We first showed that the full surgical excision of the larger of the two spines on the secondary claspers in *D. kikkawai* sharply reduced male mating success, by impeding ability of males to couple their genitalia with that of the female. This effect occurred despite males exhibiting courtship and mounting attempts, indicating that the effect was not the result of the laser surgery eliminating male courtship or motivation to mate (and see Polak & Rashed, 2010). Moreover, surgical- and sham-control males exhibited similar mating probabilities in this experiment, and mating latencies of treatment males did not differ from control groups in either of our two competitive fertilization success experiments, confirming that male sexual motivation was not impaired by contact with the laser light (Polak & Rashed, 2010). These comparisons solidify the conclusion that the excision of the large spines was itself the cause of the damaging effect on male mating success that we documented here, corroborating previous functional studies on the secondary clasper spines in two *ananassae* subgroup species, *D. bipectinata* and *D. ananassae* (Grieshop & Polak, 2014; Polak & Rashed, 2010). In these species, the spines on the secondary claspers are less pronounced than in *D. kikkawai*, and they occur as a single pair. Males of *D. bipectinata* and *D. ananassae* whose spines were surgically excised or reduced in size likewise failed to achieve copulation significantly more often than controls and, moreover, lost mating opportunities when placed in direct competition with rival males, demonstrating their role in pre-insemination intra-sexual selection.

Intriguingly, we found here that the large secondary clasper spines also mediate post-insemination sexual selection, indicating that this trait has diversified into an additional functional (adaptive) domain. Notably, the negative consequences of spine reduction on competitive fertilization success were detected in two independent experiments, indicating a robust effect. In contrast, in neither *D. bipectinata* nor *D. ananassae* was an effect of secondary clasper spine reduction on P_2 detected (Grieshop & Polak, 2014; Polak & Rashed, 2010), consistent with their relatively diminutive character in these species. In the present study, we additionally found that laser ablation of the cluster of stout spines on the primary claspers also impaired P_2, potentially through inadequate female stimulation or impaired ejaculate transfer owing to problems with genital coupling (discussed further below), identifying a second set of prominent external genital spines in *D. kikkawai* involved in post-insemination sexual selection. Thus, the results suggest that multiple genital traits in this species promote competitive fertilization success and that, moreover, a single trait (the large spines on the secondary claspers) functions in both episodes of sexual selection.

TABLE 4 Means ± 1*SE* and sample sizes (within brackets) for each behavioural and life-history trait analysed in Experiment 2

Response variable	L-Cut	LS-Cut	S-Cut	Surg-control
Copulation latency (s) (241)	2,281.85 ± 350.59 (58)	2,094.31 ± 298.16 (59)	1,833.25 ± 302.29 (60)	2,134.89 ± 304.21 (64)
Copulation duration (s) (241)	479.77 ± 36.98 (57)	700.32 ± 60.11 (58)	211.98 ± 8.67 (62)	223.67 ± 14.89 (64)
P_2 (227)	0.33 ± 0.06 (59)	0.12 ± 0.04 (51)	0.92 ± 0.02 (59)	0.94 ± 0.02 (58)
Total eggs (239)	59.97 ± 3.34 (58)	56.87 ± 3.53 (55)	61.72 ± 3.55 (62)	68.16 ± 3.36 (64)

Note: The values are provided for the total number of replicates and for the replicates in each level of the male laser treatments.

The causal basis(es) by which the genital spines in *D. kikkawai* promote competitive fertilization success is not yet understood, and at least two possible mechanisms exist. One is that the spines stimulate female external genitalia during mating and hence are subject to cryptic female choice. This possibility is consistent with the fact that surgical manipulation of either trait significantly prolonged copulation duration, indicating that the spines likely interact with the female sensory apparatus, which is a foundation stone of the cryptic female choice hypothesis for genital evolution (Eberhard, 1996). The idea is that the ablation resulted in impaired female stimulation, delaying both normal female response and the sensory feedback male would receive to trigger the dismount (and see Cocks & Eady, 2018; Eady & Brown, 2017). Copulation duration in *Drosophila* is arguably under male control (MacBean & Parsons, 1967; Jagadeeshan & Singh, 2006; Crickmore & Vosshall, 2013, but see Mazzi, Kesäniemi, Hoikkala, & Klappert, 2009; Edward, Poissant, Wilson, & Chapman, 2014), as a function of sensory cues males receive in the form of behavioural and/or neuromuscular activity produced by the female at an appropriate time post-insemination. Interestingly, the tight association we demonstrated between altered spine morphology and copulation duration suggests that copulation duration and spine configuration (as in size and number) have co-evolved, comparative evidence for which has been found in tettigoniid bushcrickets, where a male genital titillator structure exhibits correlated evolution with copulation duration across species (Vahed, Lehmann, Gilbert, & Lehmann, 2011).

The second possible mechanism for the negative effect of spine alternation on fertilization success is that the surgical manipulation interfered with ejaculate transfer, for example, by impeding male ability to open the female gonopore or to properly align and/or secure his genitalia with that of the female. Surgically altered males thus may have been placed at a disadvantage in sperm competition, due to reduced transfer of sperm or seminal plasma products (accessory gland proteins, Acps), which are known to mediate sperm storage and use, among other functions (Fiumera, Dumont, & Clark, 2005, 2007; Gillott, 2003; Wolfner, 2002, 2009). In *Callosobruchus* seed beetles, for example, male genital traits such as sclerotized spines and jaw-like clamps injure the female reproductive lining during copulation, which can facilitate the transfer of seminal fluid products from the male ejaculate to the female circulatory system, and enhance the copulating male's fertilization success in this way (Hotzy et al., 2012; Van Haren, Rönn, Schilthuzen, & Arnqvist, 2017). In a variety of species ranging from sea slugs (*Siphopteron*), bed bugs (*Cimex*), seed beetles (*Callosobruchus*) and fruit flies (*Drosophila*), there is convincing evidence that external and/or intromittent spinose structures are injurious to the female (Reinhardt, Anthes, & Rolanda, 2015; Siva-Jothy, 2009), and in some cases (of traumatic insemination), these injuries are inevitable outcomes of fertilization (Tatarnic, Cassis, & Siva-Jothy, 2014). However, there is no evidence that the *Drosophila* secondary clasper spines serve as a conduit for the ejaculate, and they do not insert into the female reproductive tract (rather, they embed into the female external genitalia). Thus, although potentially injurious to external female genitalia (Grieshop & Polak, 2014), the secondary clasper spines are unlikely to directly enable the transfer of ejaculate components such as Acps to the female haemolymph (cf. Kamimura, 2010).

A particularly noteworthy aspect of our work is that we demonstrated a synergistic effect of the small and large pairs of secondary clasper spines in promoting fertilization success; synergic effects among genital traits have rarely been observed in sexual selection (Arnqvist, 1997; Brennan & Prum, 2015; Simmons, 2014). Whereas large-cut (L-Cut) males suffered significant reductions in fertilization success, fertilization success of small-cut (S-Cut) males was unchanged relative to the appropriate control group, but when both pairs of spines were surgically manipulated simultaneously, these LS-Cut individuals suffered significantly stronger reductions in P_2 relative to their L-Cut counterparts. In other words, the combined effect of altering L and S spines was greater than the sum of their individual effects, indicating a synergistic effect. At least two possible mechanisms for such synergism exist. One is that the L and S spines interact during copulation to elevate paternity share, which is likely given their physical proximity (both occur on the secondary claspers; Figure 1). Alternatively, the results are also consistent with a threshold effect. According to this model, the alteration of S spines had no effect on its own, but altering both S and L spines simultaneously exceeded some sensory or mechanical threshold that resulted in a disproportionately damaging effect on P_2.

Notably, in this experiment copulation duration again displayed a highly congruent and opposite pattern of response to P_2, with the doubly cut males exhibiting significantly increased copulation duration relative to either of the two single-trait manipulations. Thus, these results further support the interpretation that the large and small pair of spines on the secondary claspers in *D. kikkawai* function together in some form of sensory and/or mechanical capacity to enhance paternity share and that these traits are synergistic and co-evolving. Our results applied more broadly suggest that synergistic effects among genital traits on cryptic female choice, sperm competition and/or sexual conflict may be more significant for genital

diversification via co-evolutionary processes than has been recognized. Such effects may have broader evolutionary consequences, such as on female genital anatomy. Complex multi-way interactive effects, between male traits and female anatomy, may have stronger effects on females than male traits singly interacting with the female and help explain why female genitalia, in some cases, show *greater* diversification than in males (e.g., Simmons & Fitzpatrick, 2019).

In conclusion, our study offers novel insight into the fitness consequences of multiple nonintromittent genital structures and into the relative contributions of pre- and post-insemination sexual selection on the evolution of this prominent and evolutionary labile class of phenotypic traits. Our major findings are that the functions of the potentially injurious male genital spines are complex and variable across the different traits and that they can exert profound effects on behaviour and physiology relevant to both pre- and post-insemination sexual selection. Indeed, we found that the most prominent set of spines on the secondary claspers enact a cascade of sequential events, influencing genital coupling, duration of copulation and processes that govern competitive fertilization success. In contrast, the small pair of spines on the secondary claspers functions in subtle yet also complex ways, acting synergistically with their larger counterparts to affect post-insemination fitness outcomes. Whereas our study was not designed to elucidate the causal basis(es) by which the spines affect fertilization success, clearly it is essential that future work uncovers the causal mechanisms underlying the post-mating outcomes of these ablations, as well as the effects of the different genital morphologies across species on mating, ejaculate transfer, storage and use within the female. The *montium* subgroup to which *D. kikkawai* belongs comprises many closely related species that differ in the number, size and shape of the claw-like genital spines (Hsu, 1949; Schiffer & McEvey, 2006; Tsacas, 1975, 1981), and offers valuable opportunities for functional and comparative studies.

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CONFLICTS OF INTEREST

The authors have no competing interests.

DATA AVAILABILITY STATEMENT

Data are made available at the Dryad Digital Repository https://doi.org/10.5061/dryad.g1jwstqmf.

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SUPPORTING INFORMATION

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