



Sexual selection on the genital lobes of male *Drosophila simulans*

Running title: Sexual selection on fly genital lobes

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Competing interests

We have no competing interests.

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Abstract

Sexual selection is thought to be responsible for the rapid divergent evolution of male genitalia with several studies detecting multivariate sexual selection on genital form.

However, in most cases, selection is only estimated during a single episode of selection, which provides an incomplete view of net selection on genital traits. Here we estimate the strength and form of multivariate selection on the genitalia arch of *Drosophila simulans*

when mating occurs in the absence of a competitor and during sperm competition, in both sperm defence and offense roles (i.e. when mating first and last). We found that the strength of sexual selection on the genital arch was strongest during non-competitive mating and weakest during sperm offense. However, the direction of selection was similar across selection episodes with no evidence for antagonistic selection. Overall, selection was not particularly strong despite genitals clearly evolving rapidly in this species.

Introduction

The male genitalia of animals with internal fertilization have been found to be strikingly different, even among closely related species (Eberhard 1985; Hosken and Stockley 2004; Simmons 2014). This rapid divergent evolution is increasingly thought to be driven by sexual selection (Eberhard 1985; Arnqvist 1997; Hosken and Stockley 2004; Mendez and Cordoba-Aguilar 2004; Simmons 2014; Hosken et al. 2019). In some cases, selection on male genitalia can occur if genital form affects mating success (Hosken et al. 2019). However, post-copulatory sexual selection (i.e. competitive sperm success) seems to be the most universal driver of genital evolution (e.g. Eberhard 1985, 2004; Hosken & Stockley 2004; Simmons 2014; Hosken et al. 2019). Despite this prevailing wisdom, much of the evidence for sexual selection on genital form is indirect and there have been relatively few formal (Lande and Arnold 1983; Arnold and Wade 1984) estimates of multivariate selection on male genitals.

The most comprehensive estimates of the strength and form (i.e. linear and nonlinear) of sexual selection come from studies of insect genitalia (Simmons 2014). Selection on male genitalia during sperm competition is most frequently linear (damselflies: Cordoba-Aguilar 1999, 2002; 2009; Waage 1979; water striders: Arnqvist and Danielsson 1999; Danielsson and Askenmo 1999; Praying mantis: Holwell et al. 2010; oriental beetle: Wenninger and Averill 2006; earwig: van Lieshout 2011; van Lieshout and Elgar 2011), whereas during sexual coupling and insemination, sexual selection is largely non-linear in form (seed bug: Tadler 1999; Dougherty and Shuker 2016; dung beetle: Simmons et al. 2009; millipede: Wojcieszek and Simmons 2011a, b; broad horned beetle: House et al. 2016). Evidence for sexual selection acting on genitalia is rarer in vertebrates, but studies have been undertaken in reptiles (King et al. 2009), fish (Devigili et al. 2015; Head et al. 2015), birds (Brennan et al. 2007) and mammals (Mautz et al. 2013; Stockley et al. 2013).

Finally, direct evidence for sexual selection on genitals also comes from experimental evolution studies where the strength of sexual selection is manipulated experimentally and microevolutionary responses to this manipulation are assessed (insects: Cayetano et al. 2011; Hotzy and Arnqvist 2009; House et al. 2013; Hopwood et al. 2016; fish; Langerhans et al. 2005; mammals; Simmons and Firman 2014). While these contrasting empirical approaches provide compelling evidence that sexual selection acts on genitalia, surprisingly few studies have quantified the strength and form of sexual selection across several episodes of selection (but see Devigli et al. 2015; Dougherty and Shuker 2016; House et al. 2016). This is despite the fact that to fully understand genital evolution, identifying which episodes of selection contribute most to the evolved phenotype is paramount (Hunt et al. 2009).

This importance is highlighted by the fact that different episodes of sexual selection (i.e. pre- and postcopulatory) could act antagonistically (Hunt et al. 2009). Furthermore, theory suggests that during sperm competition there may also be antagonistic selection on males (Parker 1984). Selection is predicted to favour 'defensive' traits that protect sperm from being usurped and 'offensive' traits that overcome these defences and it may be difficult to maximise both functions (Parker 1984). Evidence from insects supports this conjecture. In water striders, beetles, and earwigs different genital components improve fertilization success during paternity defence (i.e. P1: the siring success of the first of two males to mate with a female) (Wenninger and Averill 2006; Lieshout and Elgar 2011) and offense (i.e. P2: the siring success of the second of two males to mate with a female) (Arnqvist and Danielsson 1999; House and Simmons 2003). These studies suggest that males may specialize in paternity defence or offense, consistent with the notion that selection on

genital components can be antagonistic. However, the generality of this pattern is unclear as too few studies have been undertaken.

Reflecting the general pattern (Eberhard 1985), the posterior lobes (i.e. secondary genital grasping devices) of male *Drosophila* differ between closely related, sister species (e.g. Coyne 1993; Jagadeeshan & Singh 2006). This is suggestive of a history of divergent directional selection, an inference supported by QTL analysis of *D. mauritiana* and *D. simulans* (Coyne 1993) and experimental evolution in *D. simulans* found that the male posterior and ventral lobes evolved via sexual and natural selection (House et al. 2013). During mating, the posterior lobes insert into the female's abdominal segments (VII and VIII) and although they are not directly involved in sperm transfer, variation in the posterior lobes is thought to be functionally significant (Price et al. 2001; Jagadeeshan & Singh 2006; Polak and Rashed 2010; House et al. 2013; Grieshop and Polak 2010, 2014; Frazee and Masly 2015; LeVasseur-Viens et al. 2015; Rice et al. 2019). A systematic analysis of *D. melanogaster* and *D. simulans* posterior lobes during copulation confirmed this suggestion by showing that male lobes were important in securing mounting and genital coupling (Jagadeeshan and Singh 2006). Additionally, the experimental micro-ablation of genital structures reduced male mating success in both *D. bipectinata* and *D. ananassae* (Polak and Rashed 2010; Grieshop and Polak 2010, 2014), and similar results were found in *D. pachea*, (Rhebergen et al. 2016). Posterior lobe alteration (i.e. surgical and genetic modification) was used in *D. simulans* and *D. melanogaster* to test whether the lobe morphology influenced mating and fertilization success (Frazee & Masly 2015; LeVasseur-Viens et al. 2015). In *D. simulans*, the findings support that of Jagadeeshan & Singh (2006), altered lobes reduced mating success but did not influence competitive fertilization success during sperm defence.

However, the influence of lobe alteration during sperm defence was not assessed. In a similar study, the genetically modified lobe of male *D. melanogaster* significantly reduced mating success and competitive fertilization success during sperm defence but had no influence when males mate second (sperm offence) (Frazee & Masly 2015). Again we note that *D. simulans* lobe form evolved due to experimental manipulation of sexual selection strength (House et al. 2013), but currently it is not clear which elements of sexual selection cause this evolution. However, post-copulatory sexual selection is a likely mechanism to have caused the microevolutionary shape changes of the posterior lobe (House et al. 2013).

These studies suggest that sexual selection targets the lobes during mounting and coupling (Polak and Rashed 2010; Grieshop and Polak 2010, 2014; LeVasseur-Viens et al. 2015; Jagadeeshan and Singh 2006; Frazee & Masly 2015) and may be specialized to function in competitive fertilization. However, to date, the strength and form of selection has not been directly estimated across multiple episodes of sexual selection for any one species (i.e. across multiple reproductive events that potentially select on genital form) and therefore previous studies are unable to test whether sperm competition antagonistically selects on the lobe. It is also possible that cryptic female processes select on the genital arch, but the form of selection that this may impose is unknown. Here we addressed this knowledge gap by estimating the direction and strength of selection acting on the posterior and ventral lobes of *D. simulans* across several episodes of selection, to determine the relative importance of each in driving male genital evolution. We also test the theoretical prediction (Parker 1984) that sperm offence (P2) and defence (P1) may act antagonistically on genital form. Initially, we estimated multivariate linear and nonlinear sexual selection in matings with virgin females in the absence of male-male competition. We then estimate

selection on male genitals during competitive fertilizations, both in sperm defence and offense.

Methods

Fly stocks

Our wild-type population of *D. simulans* was originally collected from Tuncurry, Eastern Australia and from this population, 20 isolines were maintained at the Centre for Environmental Stress and Adaptation Research, La Trobe University, Australia. In our laboratory, these isolines were mixed and maintained for at least 9 years prior to the start of this study. These lines have been found to be phenotypically and genetically variable (e.g. Hosken et al. 2008; Wright et al. 2008; Okada et al. 2011; Sharma et al. 2011). Furthermore, multivariate sexual selection and abundant genetic variation has been found in the sex-combs of the wild-type flies that were measured at the same time as the posterior and ventral lobes (Maraqa et al. 2017). The ebony flies – ebony are homozygous for a recessive body colour marker – were derived from Tucson stock centre and maintained for over 50 generations. The body-colour marker permits quick identification of paternity when ebony females are mated to an ebony and wild-type male. All wild-type and *ebony* population cages had an excess of 600 flies, overlapping generations and free mate choice. Cages and experimental animals were maintained at 25°C under a 12:12-h light-dark cycle and maintained on an excess of *Drosophila* culture medium (Jazz Mix *Drosophila* Food, Fisher Scientific; and *Drosophila* Quick Mix Medium, Blades Biological).

Multivariate sexual selection

Experimental flies

Excess experimental flies were collected by placing egg laying vials in wild-type cages ($n = 4$) and *ebony* cages ($n = 4$) daily for 24 hrs over a 6-week period. These vials were incubated for 8 days until eclosions peaked and virgin flies were collected from these vials and separated by sex. Virgin males ($n = 40$) were housed in large vials (> 30 vials that were randomly used for the mating experiments) until 3 – 4 days of age to ensure they were sexually receptive. Virgin females were aspirated directly into individual vials (> 700 tubes for each virgin mating trial) containing *Drosophila* culture medium until 3 days of age when they were used in mating trials.

All mating trials commenced when the flies are most sexually active at the beginning of photophase (Manning 1967). Virgin males were aspirated into vials containing a single female and observed for 2 hrs. No-choice mating assays were used for these trials, a standard approach in sexual selection studies (e.g. Chenoweth and Blows 2005; Shakeleton et al. 2005; Narraway et al. 2010) and identical outcomes are reported in choice and non-choice assessments of *Drosophila* female mate-preference (e.g. Avent et al. 2008; Taylor et al. 2008). It should also be noted that in our stock populations females are reluctant to remate (Taylor et a. 2008) so initial matings are likely to be a source of considerable selection. Males have a repertoire of courtship behaviours (i.e. wing flicking, wing vibrations, leg rubbing and licking) and females indicate their mate choice by mating or rejecting males based on these signals (Spieth, 1974; Hosken et al. 2019). We observed male and female behaviour and recorded successful mating.

Genital morphology and selection during non-competitive matings

We initially tested whether variation in the posterior and ventral lobes of the genital arch was associated with variation in offspring number when a male courted and/or mated a virgin female during a non-competitive mating. This is because previous work has suggested genital form affects mating and genital coupling (Jagadeeshan and Singh 2006). To do this, we recorded whether wild-type male *D. simulans* courted but was rejected ($n = 154$) or courted and mated ($n = 340$) with a virgin, wild-type female. At the conclusion of the 2hr observation period these males were separated from the female and frozen and stored at -20°C for morphometric measurement ($n = 494$). Pairs that neither courted or mated were excluded from the study.

Genital morphology and post-copulatory sexual selection

Here we tested whether variation in the posterior and ventral lobes of the genital arch were associated with competitive fertilization success when the focal male mated first (i.e. defensive role, P1) or second (i.e. offensive role, P2). Mating trials were conducted as described above except that ebony females mated to two virgin males; the focal male was always the wild-type and his competitor was always ebony and they were used in one trial only. During day 1 of mating trials, males that courted and mated were separated from the female and stored for morphometric measurement. Once mated females are reluctant to re-mate immediately (Taylor et al. 2008) and were transferred to a fresh food vial daily to oviposit. On the 5th day, mated females and virgin males were aspirated into fresh food vials and pairs were observed for mating as described previously. Females that did not mate twice (together with their first mate) were excluded from the study ($n \sim 600$). Twice mated females were transferred to fresh vials daily to oviposit (as before) until the 8th day when

they were aspirated into an Eppendorf vial and frozen at -20°C . Each female's vials (i.e. 8 vials) were incubated at 25°C and checked daily for eclosion. Seven days after the first emergence, the vials were inverted and stored in the freezer for processing. Subsequently the number of offspring sired by the focal male (i.e. wild-type offspring) during defensive (P1, $n = 313$) or offensive (P2, $n = 378$) mating were counted.

Dissection and Morphometric Measurement

Body size measurement

Wing length was measured as a proxy of body size (Taylor et al. 2008; House et al. 2013).

Digital images of the left and right wing were captured using a Leica M125 microscope with a mounted camera that was linked to PC. The left and right wing vein (L3) was measured using Image J and the average length of both was used in our analysis.

Genital morphometric measurement

The external genitalia were detached from the abdomen and soaked in 50:50 lactic acid and glycerol for 60 min to soften the tissues prior to dissection. The genital arch is a paired structure that is delicate and prone to tear and therefore the intact, left or right lobe was mounted in Hoyers solution and a digital image was captured.

Geometric morphometric analysis was used to quantify the size and shape of the posterior and ventral lobe of the genital arch. We identified 4 landmarks along the outline that could be found consistently across all specimens and another 30 semi-landmarks were placed around the outline (House et al. 2013). The repeatability of placing landmarks along the genital lobe is high (House et al. 2013). Morphometric analysis was conducted on the complete data set (i.e. non-competitive mating, paternity defence and offense combined),

so that centroid size and the relative warps (RW) were in the same geometric space to allow comparison of the direction and form of selection in three contexts. The landmarks and semi-landmarks were applied using TPSUTIL (version 1.46) and TPSDig (version 2.14) programs (Rolf 2009). tpsRELW 1.46 (Rolf 2008) was used to extract cartesian coordinates of the landmarks and normalized them for position, orientation and scale (generalized least squares superimposition; Adams et al. 2009). tpsRELW 1.46 (Rolf 2008) was also used to estimate centroid size (which is the square root of the sum of squared distances of the landmarks from the centroid; Cardini 2012) and for calculation of relative warps (Adams et al. 2009) and visualize the shape of the genital arch as shape deformations of thin plate splines. Although our shape analysis returned a total of 64 RW scores, we only used the first four as they accounted for more than 70% of the shape variation and subsequent RW scores explained progressively smaller amounts of variation (from 5.15% to 0%) and the interpretation of very subtle shape variation is difficult.

Statistical Analysis

Multivariate Selection Analysis

We used standard multivariate selection analysis to estimate linear and nonlinear sexual selection on male body size (i.e. wing length, WL), genital size (CS) and shape (RW1, RW2, RW3 & RW4) during a non-competitive mating with a virgin female or competitive fertilization with a twice mated female during sperm defence (i.e. P1) or offense (i.e. P2). Male fitness was assigned a continuous fitness score that was the total number of offspring sired as we reasoned that offspring number was a common outcome across our three episodes of selection and captures more of the variation in male fitness – from those males that courted and mated or courted but failed to mate (non-competitive matings; i.e. 0 to

122 offspring), to those that mated but sired few or many offspring (competitive fertilizations; i.e. 0 to 250 offspring). For each bout of selection, we transformed the response variables to relative fitness by dividing individual scores by the mean fitness of the population and standardized the male phenotypic traits to zero means and unit variances (Lande and Arnold 1983). We then fitted separate, linear and polynomial regression models for each of the three bouts of selection to estimate linear and nonlinear (i.e. quadratic and correlational) selection gradients for male size and genital size and shape during non-competitive (β_V and γ_V) and competitive (β_{P1} , β_{P2} , γ_{P1} and γ_{P2}) mating (Lande and Arnold 1983, see Hunt et al. 2009 for details). All quadratic selection gradients were doubled as stabilizing or disruptive selection is underestimated by a factor of 0.5 (Stinchcombe et al. 2008).

As our continuous fitness measures were not normally distributed, we assessed the significance of our linear and nonlinear selection gradients using a re-sampling procedure where the original measures were randomly shuffled to de-couple the individual fitness score from the original male phenotype to obtain a null distribution for each gradient where there is no relationship between trait and fitness (Mitchell-Olds and Shaw 1987). We then tested the probability that the linear gradients of the pseudo-estimates (out of 9,999 permutations) was equal or less than the original estimated gradients for each episode of selection. The same procedure was repeated for the full quadratic model (i.e. models containing linear, quadratic and correlational terms).

As interpretation of individual γ -coefficients is difficult and may underestimate the strength of nonlinear selection (Phillips and Arnold 1989; Blows and Brooks 2003), we conducted canonical analyses to locate the major axes of selection using the Reynolds et al. (2010) approach. The analysis generates a new matrix of vectors of linear selection described by theta (θ) and nonlinear selection that are described by eigenvalues (λ) and

their corresponding eigenvectors (m_i). The significance of the eigenvalues was tested using the permutation procedure outlined in Reynolds et al. (2010) using the `car` function (Fox and Weisberg 2011). To visualize the major axes of selection that were extracted from the canonical rotations of θ_V , θ_{P1} , θ_{P2} , λ_V and λ_{P1} , we used thin-plate splines (Green and Silverman 1994) using the `Tps` function in the `fields` package (Nychka et al. 2017) of R (R Core Team 2018). The spline surfaces were fitted using the value of the smoothing parameter (λ) that minimized the general cross-validation (GCV) score. In R, we plotted the perspective and contour map of the surfaces. Finally, the difference in the linear, quadratic and correlational selection gradients in non-competitive and competitive fertilization (i.e. P1 and P2) were tested using a sequential model building approach (partial F-test) (Draper and John 1988; see Chenoweth and Blows 2005 for a detailed description of this procedure).

Results

Variation in genital shape

Our first four measures of genital shape (i.e. RW1, RW2, RW3 and RW4) explained more than 72.5% of the variation and these were used in subsequent analyses. RW1 explained 32.70% of the total variance in genital shape with positive values corresponding with a narrow space between the posterior process and ventral lobe and negative values with a large space between the posterior process and ventral lobe that is down-ward facing (Figure 1). RW2 explained 18.83% of the variation in genital shape with positive values corresponding with an elongated, long posterior process and negative values corresponding with a thicker, wider posterior process (Figure 1). RW3 explained 12.68% of the variation in genital shape with positive values corresponding with a posterior process with a laterally elongated tip and negative values corresponding with a posterior process with a shortened

tip (Figure 1). Finally, RW4 explained 8.29% of the variation in genital shape with positive values corresponding with a shallow, 'hook-like' posterior process and negative values corresponding with a deep, 'hook-like' posterior process (Figure 1).

Sexual selection during non-competitive mating

Standardized linear, quadratic and correlational selection gradients are presented in Table 1A. During non-competitive matings with a virgin female, linear selection on the genital posterior and ventral lobe was weak and non-significant except for RW4. Nonlinear selection was weak and non-significant for all traits except for disruptive selection on wing length (WL) and positive correlational selection on RW1 and RW2 (Table 1A). Canonical analysis of the λ matrix of quadratic selection gradients revealed significant positive, directional selection along vectors \mathbf{m}_1 and \mathbf{m}_6 , stabilizing selection along \mathbf{m}_4 and \mathbf{m}_5 and non-significant selection along vectors \mathbf{m}_2 and \mathbf{m}_3 (Table 2A). Visualization of the fitness surface against the significant axes of linear selection (\mathbf{m}_1 & \mathbf{m}_6) show a region of highest fitness at positive values of m_1 and intermediate values of m_6 (Figure 2A & B). \mathbf{m}_1 is most heavily influenced by negative values of RW1 (down-ward facing ventral lobe) and RW2 (thicker, wider posterior process) and \mathbf{m}_6 is most heavily influenced by positive wing length (i.e. larger body size) (Figure 2A & B). Stabilizing selection on \mathbf{m}_4 and \mathbf{m}_5 was most heavily influenced by genital size (CS), RW1, RW2 and RW4 that favoured intermediate genital size and shape (Figure 2C & D). In sum, this combination of linear and stabilizing selection would seem to favour a thicker, wider posterior process with a down-ward facing ventral lobe that converges on the consensus genital size and shape.

Sexual selection during competitive fertilization

During competitive fertilization, when a male mated in a defensive role (P1), linear selection on the genital posterior and ventral lobe was significant and negative for genital size (CS) and positive for RW4. Nonlinear selection was non-significant for all other traits (Table 1B). Canonical analysis of the λ matrix of quadratic selection gradients revealed significant positive, directional selection along vector \mathbf{m}_1 , disruptive selection along \mathbf{m}_2 and non-significant selection along vectors \mathbf{m}_3 , \mathbf{m}_4 , \mathbf{m}_5 and \mathbf{m}_6 (Table 2A). Visualization of the fitness surface along the significant axes of linear selection (\mathbf{m}_1) and disruptive selection (\mathbf{m}_2) showed a region of highest fitness at intermediate, positive values of \mathbf{m}_1 and extreme, negative values of \mathbf{m}_2 (Figure 3A & B). \mathbf{m}_1 is most heavily influenced by negative values of CS (small genital size) and RW1 (ventral lobe that is down-ward facing) and \mathbf{m}_2 is most heavily influenced by positive values of WL (i.e. larger body size) and RW2 (elongated, long posterior lobe) (Figure 3A & B).

When males mated in an offensive role (P2), linear selection on the genitalia was significant and negative for RW1 and positive for RW3. Nonlinear selection was non-significant for all other traits (Table 1C). Overall, canonical analysis of the λ matrix of quadratic selection gradients revealed that selection was very weak during paternity offense. Selection along all vectors was non-significant for all vectors, except for \mathbf{m}_4 (Table 2C) and visualization of the fitness surface against a non-significant, vector \mathbf{m}_1 , shows a region of highest fitness at negative values of \mathbf{m}_4 (Figure 3C & D). This vector is most heavily influenced by positive values of RW1 (narrow space between the posterior and ventral lobe) and negative values of RW3 (posterior lobe with a shortened tip) (Table 2C).

The strength of selection across episodes

Gradients of linear sexual selection differed significantly during sperm defence and offense due to a marginal difference in selection on RW4, with positive selection during defence (P1) and almost no selection during offense (P2) (Table 3). Quadratic selection differed significantly in non-competitive mating and competitive fertilization during sperm defence, with disruptive selection on body size (WL) when males mated virgin females and virtually no selection during sperm defence (P1) (Table 3). Finally, correlational selection differed significantly in non-competitive and competitive fertilization during paternity defence with positive correlational selection between RW1 and RW2 in non-competitive mating and negative correlational selection (albeit non-significant) between these same traits during defence (P1). More striking than the changes in selection on individual traits - across different episodes of selection, selection was weak across vectors during sperm defence and offence (P1 and P2).

Discussion

Sexual selection often acts on traits across multiple episodes of selection to determine sexual fitness (Hunt et al. 2009). Unfortunately, most genital studies (but see House et al. 2016; Devigili et al. 2015; Dougherty and Shuker 2016) have estimated linear and nonlinear selection during just a single episode of selection and this may not provide a complete view of net selection (Hunt et al. 2009). Under the standardized conditions in our laboratory, we showed how successive episodes of sexual selection act on the size and shape of the male posterior and ventral lobe and body size, although, if genital morphology is correlated to another trait that influences mating and/or fertilization success, selection may be indirect (Grafen 1988; Wade & Kalisze 1990; Krakauer et al. 2011). Furthermore, our estimates of

selection are limited to our rather simple paradigm and it is possible that variation in the environment and social sexual environment could change our estimates of selection strength and form. For example, it has been found that aspects of the posterior and ventral lobes changed in response to 47 generations of decreased or increased temperature (i.e. 25°C or 27°C) and a lifetime of monogamy or polyandry (House et al. 2013). Nonetheless, our findings are a useful complement to the experimental studies of *D. melanogaster* (Frazee & Masly 2015) and *D. simulans* (LeVasseur-Viens et al. 2015) that manipulated the posterior lobe to test the importance of this trait for mating and fertilization success. In these studies, it could be argued that the significant influence of lobe morphology was a by-product of the extreme reductions in the size/shape of the lobe compared to the 'natural' lobe. However, in our naturally occurring lobe phenotypes, our findings are remarkably consistent with Frazee & Masly (2015) and LeVasseur-Viens (2015) and demonstrate how selection analyses and experimental manipulation can be effectively used to isolate the effects of single traits and verify that they are the target of selection (Grafen 1988; Wade & Kalisz 1990; Krakauer et al. 2011). Furthermore, our findings are consistent with past experimental evolution work which documented micro-evolution of the lobes in response to variation in sexual (and natural) selection (House et al. 2013). The lobe shape that evolved through sexual selection in that work is remarkably similar to the prominent posterior and downward facing ventral lobe favoured by pre-copulatory and postcopulatory sexual selection during paternity defence that we document in the current study.

Selection was strongest during non-competitive mating and suggests that mounting and genital coupling is important for the evolution of the posterior and ventral lobes.

Perhaps this is not surprising as this component of the male genitalia does not enter the female and previous work found these genital structures were important in securing

mounting and in genital coupling (Jagadeeshan and Singh 2006). A combination of linear and stabilizing selection favoured a thicker, wider posterior process with a down-ward facing ventral lobe that converges on the consensus genital size and shape. This finding is consistent with work on *D. melanogaster* which reported variance in mating success that is the strongest source of selection acting on males (Pichedda and Rice 2012). During post-copulatory, competitive fertilization, when males mated in a defensive role (P1), linear and disruptive sexual selection favoured a smaller genital arch with a similarly shaped ventral lobe as during non-competitive mating but with a more elongated posterior lobe. Whereas, when males mated in an offensive role (P2), selection favoured a lobe with a shortened tip and a narrow space between the posterior and ventral lobe. However, despite these subtle differences, we find that there is little difference in the strength and direction of linear and non-linear selection on individual components of the genital lobes across the three episodes of selection. Therefore, there is little evidence of antagonistic selection on the genital arch when males compete, primarily because selection was weak during sperm defence and offense. For example, the average linear selection during non-competitive mating is $\beta = |0.0246|$ compared to $\beta = |0.0187|$ during sperm defence and $\beta = |0.007|$ during offense.

Selection on the genital lobe during non-competitive mating is consistent with previous studies that show that components of the external genitalia of *Drosophila* are important to establish genital coupling. For example, the asymmetrical epandrial lobe bristles (*D. pachea*; Rhebergan et al. 2016) and genital spines (*D. bipectonata*; Polak & Rashed 2010) are specialized to grasp the female during mating and stabilize coupling. Correlational studies such as ours do not elucidate the functional mechanism(s) that are driving selection (we only looked at outcome not process), but Jagadeeshan et al. (2006) argued that variation in the morphology of the genital lobe is not driven by male-female

conflict as the female apparently lacks genital modifications that reduce the likelihood of coupling, which contrasts to systems where morphological adaptations and counter-adaptations have probably evolved via sexual conflict (e.g. Arnqvist and Rowe 2002; Crudginton and Siva-Jothy 2000; Cayetano et al. 2011; Hotzy et al. 2012). Instead, it has been argued that relatively large and broad posterior lobes of the *Drosophila* genitalia act as hold-fasts that grasp the female oviscape so that copulation and sperm transfer is successful (Jagadeeshan et al. 2006; House et al. 2013; LeVasseur-Viens et al. 2015). Alternatively, it is equally possible that cryptic female choice (Eberhard 2009) imposes selection on the posterior lobe when the lobes contact the oviscape and potentially stimulate females and influence oviposition (Frazee & Masly 2015). As has been pointed out elsewhere, it is extremely difficult, and perhaps even fruitless, to try and separate these processes (Pitnick & Hosken 2010), but the main conclusion of our investigation, genital lobe shape impacts male sexual fitness-components, remains unchanged regardless of the relative contribution of these potential mechanistic explanations for the effects we document.

It has also been suggested that sperm competition generates antagonistic selection on males to both protect paternity and overcome paternity assurance adaptations (Parker 1984). Here, we find no evidence of antagonistic selection on the posterior lobe during non-competitive mating and sperm competition, which is consistent with work on *D. melanogaster* (Frazee & Masly 2015). Instead, we found that selection during sperm defence favoured a similar posterior lobe shape to that favoured during non-competitive mating (i.e. thicker, wider lobe), albeit selection was weaker during defence. Interestingly, in *D. melanogaster*, a prominent hook-like lobe morphology was also associated with greater reproductive success during sperm defence, suggesting that sexual selection tends to favour more exaggerated lobe phenotypes (Frazee & Masly 2015). In our study, selection during

sperm offense was weakest and overall we find limited evidence that lobe morphology influences this element of sperm competition. This is consistent with previous studies that also found little evidence that the lobes influence sperm offense in *D. simulans* (LeVasseur-Viens et al. 2015) or *D. melanogaster* (Frazee & Masly 2015). Nonetheless, our study shows considerable variation in fertilization success (i.e. the least and most fit males have a difference of 200 offspring), consistent with the fact that male traits other than genital form, like behaviour (i.e. Hosken et al. 2008), morphology (i.e. body size; Taylor et al. 2008a) and physiology (i.e. Hosken et al. 2008; Taylor et al. 2008b) also affect male sexual fitness.

In other arthropod systems, evidence for antagonistic sexual selection on genital traits is weak even when post-copulatory selection on genitals has been documented. For instance, in the oriental beetle, *Anomala orientalis* (Wenninger and Averill 2006) and earwig, *Euborellia brunneri* (van Lieshout 2011; van Lieshout & Elgar 2011), the aedeagus is under selection during sperm defence, but selection on genitals during sperm offense was not detected (Wenninger and Averill 2006; van Lieshout 2011; van Lieshout & Elgar 2011). In the water strider, *Gerris lateralis* and dung beetle, *Onthophagus taurus* the aedeagus have pairs of sclerites that function in either sperm defense or offense, and superficially, it appears that selection on the sclerites may be antagonistic. However, in *O. taurus*, this hypothesis is not supported as the genetic correlations between sclerites suggest that the size of the sclerites is optimized so that males may be successful in both roles (House et al. 2005). Conclusive evidence for antagonistic selection on genital structures ideally requires empirical studies that combine estimates of selection on genitalia across more than one episode (Parker 1984; Hunt et al. 2009). Therefore, whilst there is no evidence of antagonistic selection on the posterior lobe in some *Drosophila* species, general conclusions cannot be made until more evidence is gathered.

In a separate study in *D. simulans*, we found that the morphology of the genital lobes evolved in response to experimentally manipulated sexual and natural selection (House et al. 2013). In this study, there were similarities in the lobe shape favoured by sexual selection (i.e. non-competitive mating and sperm defence) and the shape that evolved under elevated sexual (i.e. polyandry) and natural selection (House et al. 2013). In both studies, a thicker wider posterior lobe with a down-ward facing ventral lobe were favoured. Thus, selection that we documented here is similar with aspects of the evolutionary divergence that has been documented previously (House et al. 2013).

Finally, although we detect linear selection on some aspects of the genital arch, overall, selection was not especially strong (median, $\beta = |0.009|$) compared with estimates of selection on non-genital, morphological traits (median, $\beta = |0.16|$) across species (Kingsolver et al. 2001). Weak linear selection on the posterior and ventral lobe is likely to limit the response to selection. This was unexpected because an experimental evolution study documented significant and rapid microevolution of the lobe (House et al. 2013) as expected if selection acted on it and there was (appropriate) genetic variation in the posterior lobe. More broadly a finding of weak selection on the lobe seems somewhat paradoxical as *Drosophila* species are morphologically similar but have strikingly different genitalia across species (e.g. Coyne 1993; Eberhard 1985; Arnqvist 1998; Simmons et al. 2009) - which seems to imply relatively strong selection on genitals – although we cannot know whether this divergence is due to strong selection in the past, and this appears to be a general pattern for genitals (Hosken et al. 2019). Perhaps the genital arch is relatively free from constraining genetic correlations, for which there is evidence as the lobe is less sensitive than other traits to genetic regulators of size (Dreyer and Shingleton 2011; Dreyer and Shingleton 2019; Shingleton et al. 2008). Additionally, perhaps selection and the G

matrix of the posterior lobe are aligned such that evolution is facilitated (Blows et al. 2004) despite of weak selection. This would be consistent with findings that sexually selected traits in animals tend to evolve faster than life history traits and morphological traits even though selection on them does not appear to be stronger (Pitchers et al. 2014). In short, we believe that examination of how the G matrix is aligned with the major axes of selection on the posterior lobe is required to reliably conclude that the lobe will or will not evolve in our population and this will be the subject of a future study.

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FIGURE LEGENDS

Figure 1. Frequency distribution of the four relative warp (RW) scores characterizing the variation in male genital shape (A, B, C and D). For each RW, we provide thin-plate spline visualizations (inset) that characterize a positive and negative score.

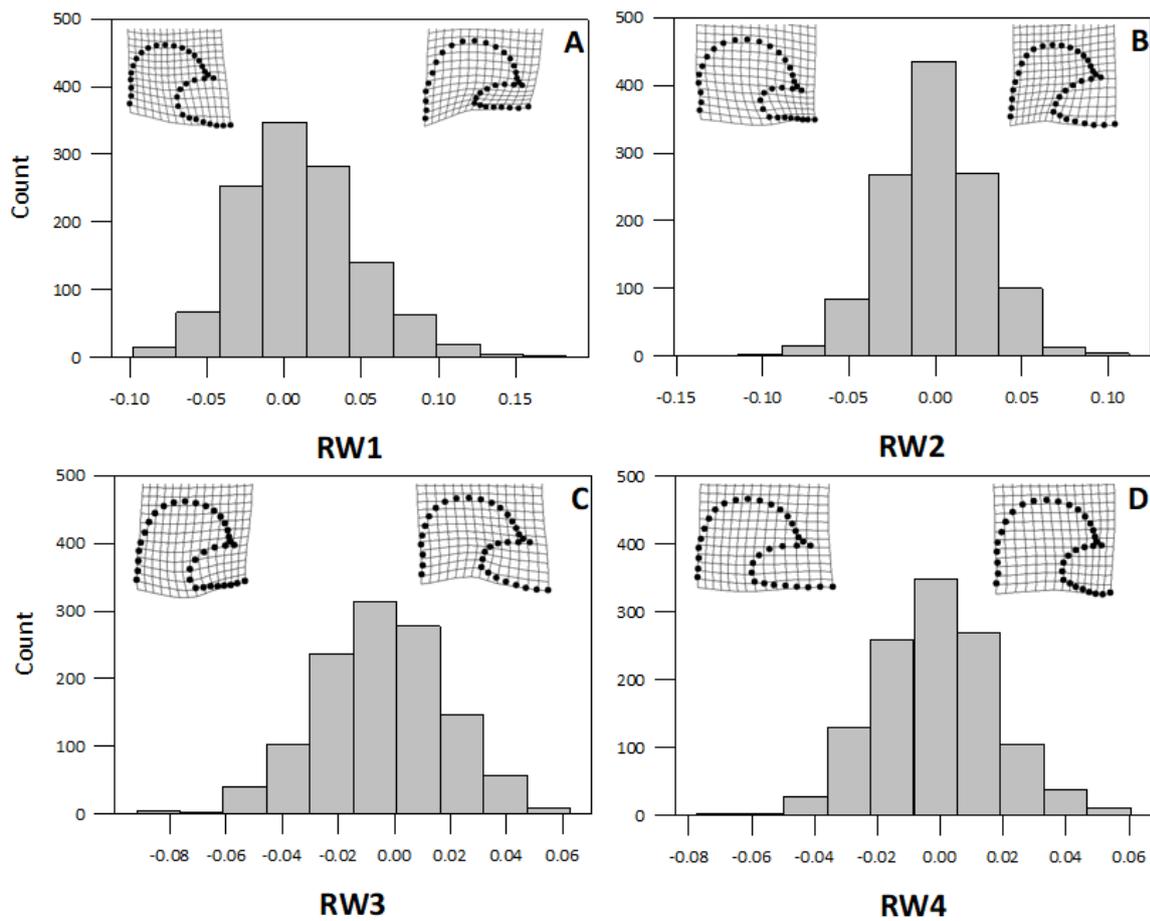


Figure 2. Thin-plate spline visualizations of the two major axes of linear (m_1 and m_6) and nonlinear (m_4 and m_5) selection on the fitness surface for males during non-competitive mating. The three-dimensional surfaces on the left (A & C) show a perspective-view while the contour plots on the right (B and D) show the same surface from above. In each contour plot, white colouration represents regions of highest fitness, whereas red colouration represents regions of lowest fitness. Individual data points are provided as black circles on the surface.

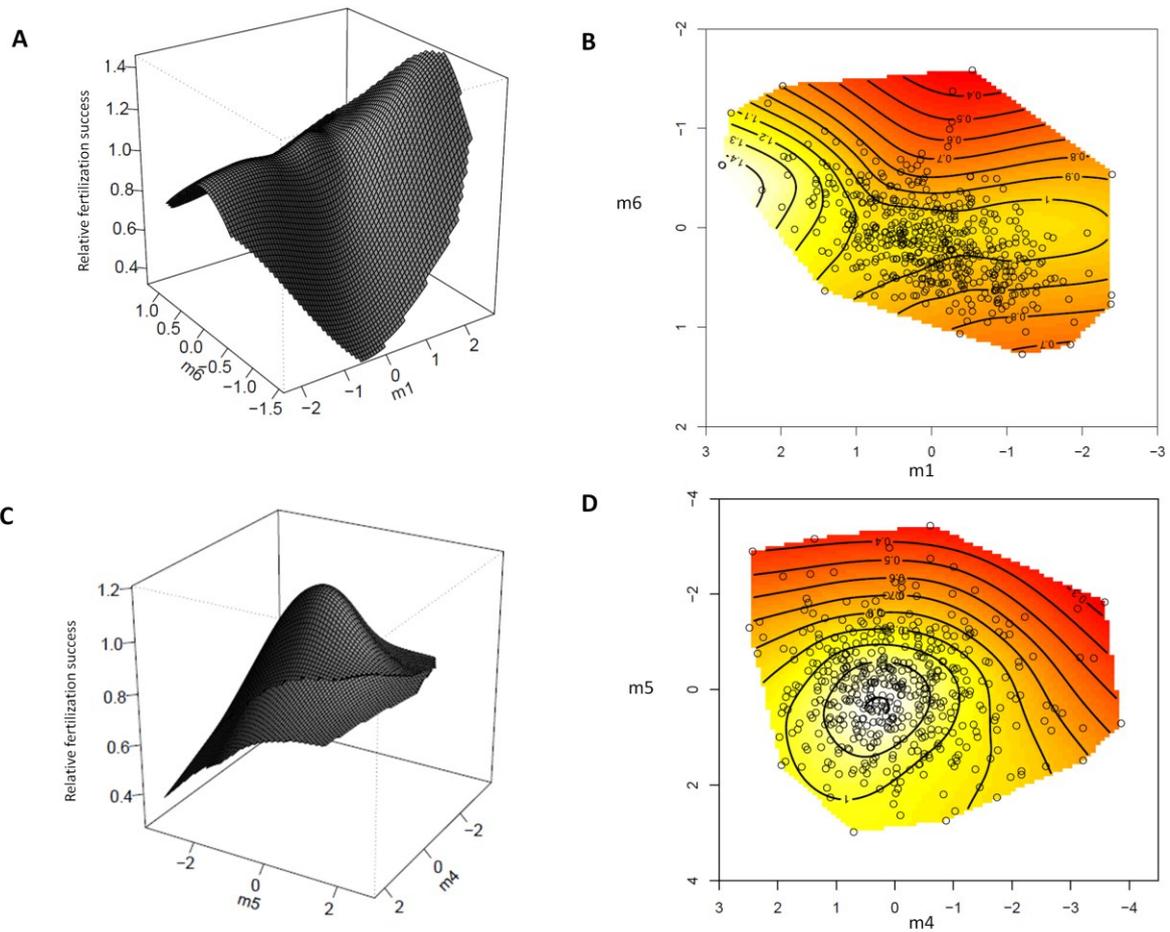


Figure 3. Thin-plate spline visualizations of the two major axes of linear and disruptive selection during paternity defence (m_1 and m_2) and two axes of linear selection (m_1 and m_4) during paternity offense – only selection along m_4 is significant. The three-dimensional surfaces on the left (A & C) show a perspective-view while the contour plots on the right (B and D) show the same surface from above. In each contour plot, white colouration represents regions of highest fitness, whereas red colouration represents regions of lowest fitness. Individual data points are provided as black circles on the surface.

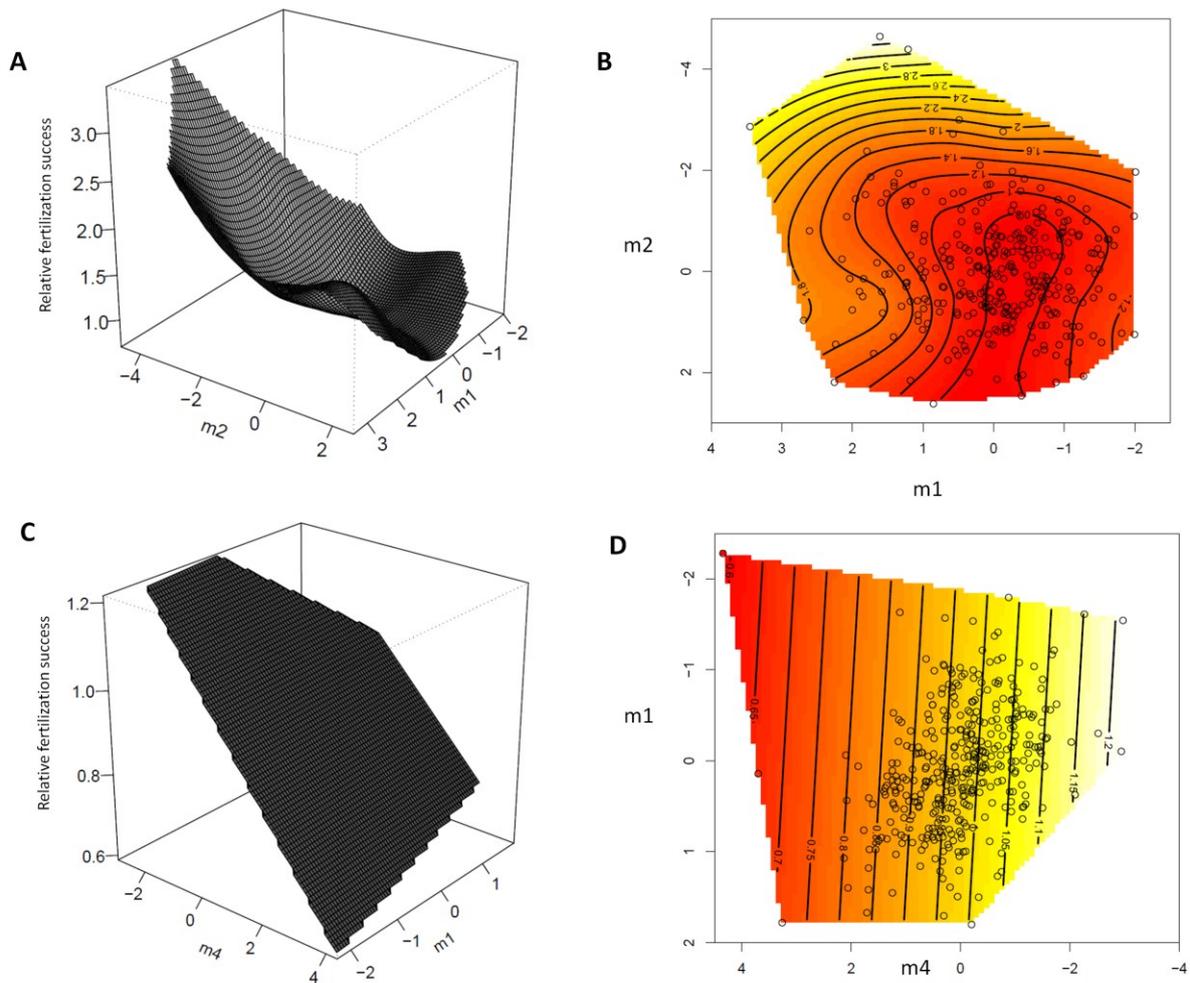


Table 1. The vector of standardized linear selection gradients (β) and the matrix of standardized quadratic and correlational gradients (γ) for body size (WL) and genital size (CS) and shape (RW1, RW2, RW3 & RW4) in male *D. simulans* during non-competitive mating when a male courted and/or mated a (A) virgin female or during post-copulatory sexual selection in a competitive fertilization role when the focal male mated (B) first (i.e. defensive role, P1) or (C) mated second (i.e. offensive role, P2). Randomization test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

| | β | Γ | | | | | |
|---|----------------|----------------|--------|---------------|--------|--------|--------|
| | | WL | CS | RW1 | RW2 | RW3 | RW4 |
| A. Standardized selection gradients when a male courted and/or mated a virgin female | | | | | | | |
| WL | 0.095 | 1.028** | | | | | |
| CS | -0.009 | 0.082 | -0.022 | | | | |
| RW1 | -0.055 | -0.026 | 0.101 | -0.036 | | | |
| RW2 | -0.068 | -0.247 | 0.118 | 0.180* | 0.048 | | |
| RW3 | -0.086 | -0.144 | 0.033 | -0.034 | -0.048 | 0.016 | |
| RW4 | 0.115* | -0.093 | -0.061 | 0.008 | 0.061 | -0.076 | -0.120 |
| B. Standardized selection gradients when a male mated in a defensive role (P1) | | | | | | | |
| WL | -0.097 | 0.096 | | | | | |
| CS | -0.123* | 0.016 | 0.212 | | | | |
| RW1 | 0.036 | 0.064 | 0.109 | -0.150 | | | |
| RW2 | -0.050 | 0.042 | -0.008 | -0.067 | 0.106 | | |
| RW3 | 0.036 | -0.075 | -0.036 | 0.031 | -0.049 | 0.032 | |
| RW4 | 0.108* | 0.033 | -0.066 | -0.075 | 0.001 | -0.010 | 0.014 |
| C. Standardized selection gradients when a male mated in an offensive role (P2) | | | | | | | |
| WL | 0.008 | 0.166 | | | | | |
| CS | -0.032 | -0.021 | 0.074 | | | | |
| RW1 | -0.077* | -0.026 | 0.038 | -0.002 | | | |
| RW2 | 0.005 | 0.003 | -0.007 | -0.011 | 0.016 | | |
| RW3 | 0.067* | -0.001 | 0.020 | -0.027 | -0.037 | 0.006 | |
| RW4 | -0.008 | 0.116 | -0.061 | -0.027 | -0.051 | -0.018 | 0.004 |

Virgin mating: fitness measure = number of offspring produced

P1: fitness measure = number of offspring produced by wild type male

P2: fitness measure = number of offspring produced by wild type male

Table 2: Linear (θ_i) and nonlinear (λ_i) selection gradients and the M matrix of eigenvectors from the canonical analysis of Y for (A) non-competitive, virgin mating success (B) paternity defence (i.e. P1) and (C) paternity offense (i.e. P2) in male *D. simulans*. The sign of λ_i describes the form of quadratic selection acting along each eigenvector, with a positive λ_i indicating disruptive selection and a negative λ_i indicating stabilizing selection.

Randomization tests: *P < 0.05, **P < 0.01, ***P < 0.001

| | θ_i | λ_i | M | | | | | |
|---|-----------------|-----------------|--------------|---------------|---------------|--------------|---------------|--------|
| | | | WL | CS | RW1 | RW2 | RW3 | RW4 |
| A. Canonical analysis of non-competitive, virgin mating success | | | | | | | | |
| \mathbf{m}_1 | 0.071* | 0.296 | 0.118 | -0.377 | -0.534 | -0.731 | 0.114 | -0.109 |
| \mathbf{m}_2 | -0.120 | 0.083 | -0.019 | 0.441 | 0.029 | -0.065 | 0.781 | -0.435 |
| \mathbf{m}_3 | -0.040 | -0.030 | -0.251 | -0.559 | -0.120 | 0.364 | 0.565 | 0.398 |
| \mathbf{m}_4 | 0.059 | -0.166** | -0.024 | 0.496 | -0.770 | 0.247 | -0.059 | 0.309 |
| \mathbf{m}_5 | 0.084 | -0.205* | 0.047 | 0.310 | 0.327 | -0.472 | 0.184 | 0.734 |
| \mathbf{m}_6 | 0.075*** | -1.120 | 0.959 | -0.094 | -0.0001 | 0.213 | 0.139 | 0.080 |
| B. Canonical analysis of P1 | | | | | | | | |
| \mathbf{m}_1 | 0.143* | 0.279 | -0.156 | -0.884 | -0.304 | 0.094 | 0.106 | 0.284 |
| \mathbf{m}_2 | -0.089 | 0.198* | 0.603 | -0.010 | -0.089 | 0.605 | -0.480 | 0.179 |
| \mathbf{m}_3 | 0.029 | 0.084 | 0.621 | -0.179 | 0.202 | -0.694 | -0.037 | 0.242 |
| \mathbf{m}_4 | 0.057 | 0.003 | -0.371 | 0.290 | -0.267 | -0.229 | -0.463 | 0.662 |
| \mathbf{m}_5 | 0.028 | -0.021 | 0.164 | 0.261 | 0.017 | 0.245 | 0.719 | 0.572 |
| \mathbf{m}_6 | -0.091 | -0.231 | 0.249 | 0.180 | -0.887 | -0.178 | 0.157 | -0.249 |
| C. Canonical analysis of P2 | | | | | | | | |
| \mathbf{m}_1 | 0.023 | 0.250 | 0.795 | -0.301 | -0.174 | -0.074 | -0.033 | 0.490 |
| \mathbf{m}_2 | 0.024 | 0.085 | -0.398 | -0.838 | -0.212 | 0.184 | -0.236 | 0.067 |
| \mathbf{m}_3 | 0.047 | 0.053 | -0.262 | -0.128 | -0.180 | -0.739 | 0.541 | 0.206 |
| \mathbf{m}_4 | -0.091* | 0.018 | -0.104 | -0.035 | 0.649 | -0.439 | -0.546 | 0.275 |
| \mathbf{m}_5 | -0.001 | -0.045 | -0.202 | 0.401 | -0.658 | -0.136 | -0.516 | 0.284 |
| \mathbf{m}_6 | -0.007 | -0.102 | -0.298 | 0.169 | 0.195 | 0.451 | 0.291 | 0.746 |

Randomization tests: *P < 0.05, **P < 0.01, ***P < 0.001

Table 3. Sequential model comparing the linear and nonlinear effects of sexual selection during different episodes of selection on body size, genital arch size, RW1, RW2, RW3 and RW4 in male *D. simulans*.

| | SS_R | SS_C | DF_1 | DF_2 | F | P |
|-------------------------------------|---------|---------|--------|--------|-------|--------------------------|
| Non-competitive mating vs P1 | | | | | | |
| Linear | 1004.83 | 992.58 | 6 | 793 | 1.63 | 0.136 |
| Quadratic | 975.90 | 958.93 | 6 | 781 | 2.30 | 0.032^A |
| Correlational | 939.891 | 924.356 | 15 | 751 | 0.841 | 0.631 |
| Non-competitive mating vs P2 | | | | | | |
| Linear | 878.97 | 868.34 | 6 | 858 | 2.22 | 0.109 |
| Quadratic | 861.28 | 850.15 | 6 | 846 | 1.84 | 0.087 |
| Correlational | 837.405 | 824.25 | 15 | 816 | 2.17 | 0.043^B |
| P1 vs P2 | | | | | | |
| Linear | 451.04 | 442.13 | 6 | 677 | 2.27 | 0.04^C |
| Quadratic | 427.59 | 422.91 | 6 | 665 | 1.23 | 0.289 |
| Correlational | 411.28 | 406.77 | 15 | 635 | 1.17 | 0.318 |

Univariate tests: **A** *WingxWing*: $F_{1,781}=4.718$, $P=0.03$. **B** *RW1xRW2*: $F_{1,816}=5.642$, $P=0.018$. **C** *RW4*: $F_{1,677}=3.174$, $P=0.05$.