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

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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.

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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 siring success) seems to the most universal

driver of genital evolution (e.g. Eberhard 1985, 2004; Hosken & Stockely 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).

52   Finally, direct evidence for sexual selection on genitals also comes from experimental

53   evolution studies where the strength of sexual selection is manipulated experimentally and

54   microevolutionary responses to this manipulation are assessed (insects: Cayetano et al.

55   2011; Hotzy and Arnqvist 2009; House et al. 2013; Hopwood et al. 2016; fish; Langerhans et

56   al. 2005; mammals; Simmons and Firman 2014). While these contrasting empirical

57   approaches provide compelling evidence that sexual selection acts on genitalia, surprisingly

58   few studies have quantified the strength and form of sexual selection across several

59   episodes of selection (but see Devigli et al. 2015; Dougherty and Shuker 2016; House et al.

60   2016). This is despite the fact that to fully understand genital evolution, identifying which

61   episodes of selection contribute most to the evolved phenotype is paramount (Hunt et al.

62   2009).

63   This importance is highlighted by the fact that different episodes of sexual selection

64   (i.e. pre- and postcopulatory) could act antagonistically (Hunt et al. 2009). Furthermore,

65   theory suggests that during sperm competition there may also be antagonistic selection on

66   males (Parker 1984). Selection is predicted to favour 'defensive' traits that protect sperm

67   from being usurped and 'offensive' traits that overcome these defences and it may be

68   difficult to maximise both functions (Parker 1984). Evidence from insects supports this

69   conjecture. In water striders, beetles, and earwigs different genital components improve

70   fertilization success during paternity defence (i.e. P1: the siring success of the first of two

71   males to mate with a female) (Wenninger and Averill 2006; Lieshout and Elgar 2011) and

72   offense (i.e. P2: the siring success of the second of two males to mate with a female)

73   (Arnqvist and Danielsson 1999; House and Simmons 2003). These studies suggest that males

74   may specialize in paternity defence or offense, consistent with the notion that selection on

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75 genital components can be antagonistic. However, the generality of this pattern is unclear

76 as too few studies have been undertaken.

77   Reflecting the general pattern (Eberhard 1985), the posterior lobes (i.e. secondary

78 genital grasping devices) of male *Drosophila* differ between closely related, sister species

79 (e.g. Coyne 1993; Jagadeeshan & Singh 2006). This is suggestive of a history of divergent

80 directional selection, an inference supported by QTL analysis of *D. mauritiana* and *D.*

81 *simulans* (Coyne 1993) and experimental evolution in *D. simulans* found that the male

82 posterior and ventral lobes evolved via sexual and natural selection (House et al. 2013).

83 During mating, the posterior lobes insert into the female's abdominal segments (VII and VIII)

84 and although they are not directly involved in sperm transfer, variation in the posterior

85 lobes is thought to be functionally significant (Price et al. 2001; Jagadeeshan & Singh 2006;

86 Polak and Rashed 2010; House et al. 2013; Grieshop and Polak 2010, 2014; Frazee and

87 Masly 2015; LeVasseur-Viens et al. 2015; Rice et al. 2019). A systematic analysis of *D.*

88 *melanogaster* and *D. simulans* posterior lobes during copulation confirmed this suggestion

89 by showing that male lobes were important in securing mounting and genital coupling

90 (Jagadeeshan and Singh 2006). Additionally, the experimental micro-ablation of genital

91 structures reduced male mating success in both *D. bipectinata* and *D. ananassae* (Polak and

92 Rashed 2010; Grieshop and Polak 2010, 2014), and similar results were found in *D. pachea*,

93 (Rhebergen et al. 2016). Posterior lobe alteration (i.e. surgical and genetic modification) was

94 used in *D. simulans* and *D. melanogaster* to test whether the lobe morphology influenced

95 mating and fertilization success (Frazee & Masly 2015; LeVasseur-Viens et al. 2015). In *D.*

96 *simulans*, the findings support that of Jagadeenshan & Singh (2006) altered lobes reduced

97 mating success but did not influence competitive fertilization success during sperm defence.

98 However, the influence of lobe alteration during sperm defence was not assessed. In a

99   similar study, the genetically modified lobe of male *D. melanogaster* significantly reduced

100   mating success and competitive fertilization success during sperm defence but had no

101   influence when males mate second (sperm offence) (Frazee & Masly 2015). Again we note

102   that *D. simulans* lobe form evolved due to experimental manipulation of sexual selection

103   strength (House et al. 2013), but currently it is not clear which elements of sexual selection

104   cause this evolution. However, post-copulatory sexual selection is the most likely

105   mechanism to have caused the microevolutionary shape changes of the posterior lobe that

106   we documented (House et al. 2013).

107   These studies suggest that sexual selection targets the lobes during mounting and

108   coupling (Polak and Rashed 2010; Grieshop and Polak 2010, 2014; LeVasseur-Viens et al.

109   2015; Jagadeeshan and Singh 2006; Frazee & Masly 2015) and may be specialized to

110   function in competitive fertilization. However, to date, the strength and form of selection

111   has not been directly estimated across multiple episodes of sexual selection for any one

112   species (i.e. across multiple reproductive events that potentially select on genital form) and

113   therefore previous studies are unable to test whether sperm competition antagonistically

114   selects on the lobe. It is also possible that cryptic female processes select on the genital

115   arch, but the form of selection that this may impose is unknown. Here we addressed this

116   knowledge gap by estimating the direction and strength of selection acting on the posterior

117   and ventral lobes of *D. simulans* across several episodes of selection, to determine the

118   relative importance of each in driving male genital evolution. We also test the theoretical

119   prediction (Parker 1984) that sperm offence (P2) and defence (P1) may act antagonistically

120   on genital form. Initially, we estimated multivariate linear and nonlinear sexual selection in

121   matings with virgin females in the absence of male-male competition. We then estimate

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

123   offense.

124

125   **Methods**
126   *Fly stocks*

127   Our wild-type population of *D. simulans* was originally collected from Tuncurry, Eastern

128   Australia and from this population, 20 isolines were maintained at the Centre for

129   Environmental Stress and Adaptation Research, La Trobe University, Australia. In our

130   laboratory, these isolines were mixed and maintained for at least 9 years prior to the start of

131   this study. Previously, we found that these lines are phenotypically and genetically variable

132   (e.g. Hosken et al. 2008; Wright et al. 2008; Okada et al. 2011; Sharma et al. 2011).

133   Furthermore, multivariate sexual selection and abundant genetic variation has been found

134   in the sex-combs of the wild-type flies that were measured at the same time as the

135   posterior and ventral lobes (Maraqa et al. 2017). The ebony flies – ebony are homozygous

136   for a recessive body colour marker – were derived from Tucson stock centre and maintained

137   for over 50 generations. The body-colour marker permits quick identification of paternity

138   when ebony females are mated to an ebony and wild-type male. All wild-type and *ebony*

139   population cages had an excess of 600 flies, overlapping generations and free mate choice.

140   Cages and experimental animals were maintained at 25˚C under a 12:12-h light-dark cycle

141   and maintained on an excess of *Drosophila* culture medium (Jazz Mix Drosophila Food,

142   Fisher Scientific; and Drosophila Quick Mix Medium, Blades Biological).

143

144   **Multivariate sexual selection**

145   *Experimental flies*

146   Excess experimental flies were collected by placing egg laying vials in wild-type cages (*n* = 4)

147   and *ebony* cages (*n* = 4) daily for 24 hrs over a 6-week period. These vials were incubated for

148   8 days until eclosions peaked and virgin flies were collected from these vials and separated

149   by sex. Virgin males (*n* = 40) were housed in large vials (> 30 vials that were randomly used

150   for the mating experiments) until 3 – 4 days of age to ensure they were sexually receptive.

151   Virgin females were aspirated directly into individual vials (> 700 tubes for each virgin

152   mating trial) containing *Drosophila* culture medium until 3 days of age when they were used

153   in mating trials.

154   All mating trials commenced when the flies are most sexually active at the beginning

155   of photophase (Manning 1967). Virgin males were aspirated into vials containing a single

156   female and observed for 2 hrs. No-choice mating assays were used for these trials, a

157   standard approach in sexual selection studies (e.g. Chenoweth and Blows 2005; Shakeleton

158   et al. 2005; Narraway et al. 2010) and identical outcomes are reported in choice and non-

159   choice assessments of *Drosophila* female mate-preference (e.g. Avent et al. 2008; Taylor et

160   al. 2008). It should also be noted that in our stock populations females are reluctant to

161   remate (Taylor et a. 2008) so initial matings are likely to be a source of considerable

162   selection. Males have a repertoire of courtship behaviours (i.e. wing flicking, wing

163   vibrations, leg rubbing and licking) and females indicate their mate choice by mating or

164   rejecting males based on these signals (Spieth, 1974; Hosken et al. 2019). We observed male

165   and female behaviour and recorded successful mating.

166

167   *Genital morphology and selection during non-competitive matings*

168   We initially tested whether variation in the posterior and ventral lobes of the genital arch

169   was associated with variation in offspring number when a male courted and/or mated a

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170   virgin female during a non-competitive mating. This is because previous work has suggested

171   genital form affects mating and genital coupling (Jagadeeshan and Singh 2006). To do this,

172   we recorded whether wild-type male *D. simulans* courted but was rejected (n = 154) or

173   courted and mated (n = 340) with a virgin, wild-type female. At the conclusion of the 2hr

174   observation period these males were separated from the female and frozen and stored at -

175   20˚C for morphometric measurement (n = 494). Pairs that neither courted or mated were

176   excluded from the study.

177

178   *Genital morphology and post-copulatory sexual selection*

179   Here we tested whether variation in the posterior and ventral lobes of the genital arch were

180   associated with competitive fertilization success when the focal male mated first (i.e.

181   defensive role, P1) or second (i.e. offensive role, P2). Mating trials were conducted as

182   described above except that ebony females mated to two virgin males; the focal male was

183   always the wild-type and his competitor was always ebony and they were used in one trial

184   only. During day 1 of mating trials, males that courted and mated were separated from the

185   female and stored for morphometric measurement. Once mated females are reluctant to

186   re-mate immediately (Taylor et al. 2008) and were transferred to a fresh food vial daily to

187   oviposit. On the 5th day, mated females and virgin males were aspirated into fresh food vials

188   and pairs were observed for mating as described previously. Females that did not mate

189   twice (together with their first mate) were excluded from the study (n ~ 600). Twice mated

190   females were transferred to fresh vials daily to oviposit (as before) until the 8th day when

191   they were aspirated into an Eppendorf vial and frozen at – 20˚C. Each female's vials (i.e. 8

192   vials) were incubated at 25˚C and checked daily for eclosion. Seven days after the first

193   emergence, the vials were inverted and stored in the freezer for processing. Subsequently

194   the number of offspring sired by the focal male (i.e. wild-type offspring) during defensive

195   (P1, *n* = 313) or offensive (P2, *n* = 378) mating were counted.

196

197   **Dissection and Morphometric Measurement**

198   *Body size measurement*

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

200   Digital images of the left and right wing were captured using a Leica M125 microscope with

201   a mounted camera that was linked to PC. The left and right wing vein (L3) was measured

202   using Image J and the average length of both was used in our analysis.

203

204   *Genital morphometric measurement*

205   The external genitalia were detached from the abdomen and soaked in 50:50 lactic acid and

206   glycerol for 60 min to soften the tissues prior to dissection. The genital arch is a paired

207   structure that is delicate and prone to tear and therefore the intact, left or right lobe was

208   mounted in Hoyers solution and a digital image was captured.

209   Geometric morphometric analysis was used to quantify the size and shape of the

210   posterior and ventral lobe of the genital arch. Previously, we identified 4 landmarks along

211   the outline that could be found consistently across all specimens and another 30 semi-

212   landmarks were placed around the outline (House et al. 2013). The repeatability of placing

213   landmarks along the genital lobe is high (House et al. 2013). Morphometric analysis was

214   conducted on the complete data set (i.e. non-competitive mating, paternity defence and

215   offense combined), so that centroid size and the relative warps (RW) were in the same

216   geometric space to allow comparison of the direction and form of selection in three

217   contexts. The landmarks and semi-landmarks were applied using TPSUTIL (version 1.46) and

218   TPSDig (version 2.14) programs (Rolf 2009). tpsRELW 1.46 (Rolf 2008) was used to extract

219   cartesian coordinates of the landmarks and normalized them for position, orientation and

220   scale (generalized least squares superimposition; Adams et al. 2009). tpsRELW 1.46 (Rolf

221   2008) was also used to estimate centroid size (which is the square root of the sum of

222   squared distances of the landmarks from the centroid; Cardini 2012) and for calculation of

223   relative warps (Adams et al. 2009) and visualize the shape of the genital arch as shape

224   deformations of thin plate splines. Although our shape analysis returned a total of 64 RW

225   scores, we only used the first four as they accounted for more than 70% of the shape

226   variation and subsequent RW scores explained progressively smaller amounts of variation

227   (from 5.15% to 0%) and the interpretation of subtle shape variation is difficult.

228

229   **Statistical Analysis**

230   *Multivariate Selection Analysis*

231   We used standard multivariate selection analysis to estimate linear and nonlinear sexual

232   selection on male body size (i.e. wing length, WL), genital size (CS) and shape (RW1, RW2,

233   RW3 & RW4) during a non-competitive mating with a virgin female or competitive

234   fertilization with a twice mated female during sperm defence (i.e. P1) or offense (i.e. P2).

235   Male fitness was assigned a continuous fitness score that was the total number of offspring

236   sired as we reasoned that offspring number was a common outcome across our three

237   episodes of selection and captures more of the variation in male fitness – from those males

238   that courted and mated or courted but failed to mate (non-competitive matings; i.e. 0 to

239   122 offspring), to those that mated but sired few or many offspring (competitive

240   fertilizations; i.e. 0 to 250 offspring). For each bout of selection, we transformed the

241   response variables to relative fitness by dividing individual scores by the mean fitness of the

242   population and standardized the male phenotypic traits to zero means and unit variances

243   (Lande and Arnold 1983). We then fitted separate, linear and polynomial regression models

244   for each of the three bouts of selection to estimate linear and nonlinear (i.e. quadratic and

245   correlational) selection gradients for male size and genital size and shape during non-

246   competitive (β_V and γ_V) and competitive (β_{P1}, β_{P2}, γ_{P1} and γ_{P2}) mating (Lande and Arnold 1983,

247   see Hunt et al. 2009 for details). All quadratic selection gradients were doubled as stabilizing

248   or disruptive selection is underestimated by a factor of 0.5 (Stinchcombe et al. 2008).

249   As our continuous fitness measures were not normally distributed, we assessed the

250   significance of our linear and nonlinear selection gradients using a re-sampling procedure

251   where the original measures were randomly shuffled to de-couple the individual fitness

252   score from the original male phenotype to obtain a null distribution for each gradient where

253   there is no relationship between trait and fitness (Mitchell-Olds and Shaw 1987). We then

254   tested the probability that the linear gradients of the pseudo-estimates (out of 9,999

255   permutations) was equal or less than the original estimated gradients for each episode of

256   selection. The same procedure was repeated for the full quadratic model (i.e. models

257   containing linear, quadratic and correlational terms).

258   As interpretation of individual γ-coefficients is difficult and may underestimate the

259   strength of nonlinear selection (Phillips and Arnold 1989; Blows and Brooks 2003), we

260   conducted canonical analyses to locate the major axes of selection using the Reynolds et al.

261   (2010) approach. The analysis generates a new matrix of vectors of linear selection

262   described by theta (θ) and nonlinear selection that are described by eigenvalues (λ) and

263   their corresponding eigenvectors (mi). The significance of the eigenvalues was tested using

264   the permutation procedure outlined in Reynolds et al. (2010) using the car function (Fox and

265   Weisberg 2011). To visualize the major axes of selection that were extracted from the

266   canonical rotations of θ_V, θ_{P1}, θ_{P2}, λ_V and λ_{P1}, we used thin-plate splines (Green and

267   Silverman 1994) using the Tps function in the fields package (Nychka et al. 2017) of R (R Core

268   Team 2018). The spline surfaces were fitted using the value of the smoothing parameter (λ)

269   that minimized the general cross-validation (GCV) score. In R, we plotted the perspective

270   and contour map of the surfaces. Finally, the difference in the linear, quadratic and

271   correlational selection gradients in non-competitive and competitive fertilization (i.e. P1 and

272   P2) were tested using a sequential model building approach (partial F-test) (Draper and John

273   1988; see Chenoweth and Blows 2005 for a detailed description of this procedure).

274

275   **Results**

276   *Variation in genital shape*

277   Our first four measures of genital shape (i.e. RW1, RW2, RW3 and RW4) explained more

278   than 72.5% of the variation and these were used in subsequent analyses. RW1 explained

279   32.70% of the total variance in genital shape with positive values corresponding with a

280   narrow space between the posterior process and ventral lobe and negative values with a

281   large space between the posterior process and ventral lobe that is down-ward facing (Figure

282   1). RW2 explained 18.83% of the variation in genital shape with positive values

283   corresponding with an elongated, long posterior process and negative values corresponding

284   with a thicker, wider posterior process (Figure 1). RW3 explained 12.68% of the variation in

285   genital shape with positive values corresponding with a posterior process with a laterally

286   elongated tip and negative values corresponding with a posterior process with a shortened

287   tip (Figure 1). Finally, RW4 explained 8.29% of the variation in genital shape with positive

288   values corresponding with a shallow, 'hook-like' posterior process and negative values

289   corresponding with a deep, 'hook-like' posterior process (Figure 1).

290

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 **m_1** and **m_6**, stabilizing selection along **m_4** and **m_5** and non-significant selection along vectors **m_2** and **m_3** (Table 2A). Visualization of the fitness surface against the significant axes of linear selection (**m_1** & **m_6**) show a region of highest fitness at positive values of m_1 and intermediate values of m_6 (Figure 2A & B). **m_1** is most heavily influenced by negative values of RW1 (down-ward facing ventral lobe) and RW2 (thicker, wider posterior process) and **m_6** is most heavily influenced by positive wing length (i.e. larger body size) (Figure 2A & B). Stabilizing selection on **m_4** and **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).

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314   Canonical analysis of the λ matrix of quadratic selection gradients revealed significant

315   positive, directional selection along vector $\mathbf{m_1}$, disruptive selection along $\mathbf{m_2}$ and non-

316   significant selection along vectors $\mathbf{m_3}$, $\mathbf{m_4}$, $\mathbf{m_5}$ and $\mathbf{m_6}$ (Table 2A). Visualization of the fitness

317   surface along the significant axes of linear selection ($\mathbf{m_1}$) and disruptive selection ($\mathbf{m_2}$)

318   showed a region of highest fitness at intermediate, positive values of $\mathbf{m_1}$ and extreme,

319   negative values of $\mathbf{m_2}$ (Figure 3A & B). $\mathbf{m_1}$ is most heavily influenced by negative values of CS

320   (small genital size) and RW1 (ventral lobe that is down-ward facing) and $\mathbf{m_2}$ is most heavily

321   influenced by positive values of WL (i.e. larger body size) and RW2 (elongated, long

322   posterior lobe) (Figure 3A & B).

323   When males mated in an offensive role (P2), linear selection on the genitalia was

324   significant and negative for RW1 and positive for RW3. Nonlinear selection was non-

325   significant for all other traits (Table 1C). Overall, canonical analysis of the λ matrix of

326   quadratic selection gradients revealed that selection was very weak during paternity

327   offense. Selection along all vectors was non-significant for all vectors, except for $\mathbf{m_4}$ (Table

328   2C) and visualization of the fitness surface against a non-significant, vector $\mathbf{m_1}$, shows a

329   region of highest fitness at negative values of $\mathbf{m_4}$ (Figure 3C & D). This vector is most heavily

330   influenced by positive values of RW1 (narrow space between the posterior and ventral lobe)

331   and negative values of RW3 (posterior lobe with a shortened tip) (Table 2C).

332

333   *The strength of selection across episodes*

334   Gradients of linear sexual selection differed significantly during sperm defence and offense

335   due to a marginal difference in selection on RW4, with positive selection during defence (P1)

336   and almost no selection during offense (P2) (Table 3). Quadratic selection differed

337   significantly in non-competitive mating and competitive fertilization during sperm defence,

338   with disruptive selection on body size (WL) when males mated virgin females and virtually

339   no selection during sperm defence (P1) (Table 3). Finally, correlational selection differed

340   significantly in non-competitive and competitive fertilization during paternity defence with

341   positive correlational selection between RW1 and RW2 in non-competitive mating and

342   negative correlational selection (albeit non-significant) between these same traits during

343   defence (P1). More striking than the changes in selection on individual traits - across

344   different episodes of selection, selection was weak across vectors during sperm defence and

345   offence (P1 and P2).

346

347   **Discussion**

348   Sexual selection often acts on traits across multiple episodes of selection to determine

349   sexual fitness (Hunt et al. 2009). Unfortunately, most genital studies (but see House et al.

350   2016; Devigili et al. 2015; Dougherty and Shuker 2016) have estimated linear and nonlinear

351   selection during just a single episode of selection and this may not provide a complete view

352   of net selection (Hunt et al. 2009). Under the standardized conditions in our laboratory, we

353   showed how successive episodes of sexual selection act on the size and shape of the male

354   posterior and ventral lobe and body size, although, if genital morphology is correlated to

355   another trait that influences mating and/or fertilization success, selection may be indirect

356   (Grafen 1988; Wade & Kalisze 1990; Krakauer et al. 2011). Furthermore, our estimates of

357   selection are limited to our rather simple paradigm and it is possible that variation in the

358   environment and social sexual environment could change our estimates of selection

359   strength and form. For example, previously, we found that aspects of the posterior and

360   ventral lobes changed in response to 47 generations of decreased or increased temperature

361   (i.e. 25°C or 27°C) and a lifetime of monogamy or polyandry (House et al. 2013).

362   Nonetheless, our findings are a useful complement to the experimental studies of *D.*

363   *melanogaster* (Frazee & Masly 2015) and *D. simulans* (LeVasseur-Viens et al. 2015) that

364   manipulated the posterior lobe to test the importance of this trait for mating and

365   fertilization success. In these studies, it could be argued that the significant influence of lobe

366   morphology was a by-product of the extreme reductions in the size/shape of the lobe

367   compared to the 'natural' lobe. However, in our naturally occurring lobe phenotypes, our

368   findings are remarkably consistent with Frazee & Masly (2015) and LeVasseur-Viens (2015)

369   and demonstrate how selection analyses and experimental manipulation can be effectively

370   used to isolate the effects of single traits and verify that they are the target of selection

371   (Grafen 1988; Wade & Kalisz 1990; Krakauer et al. 2011). Furthermore, our findings are

372   consistent with our experimental evolution work which documented micro-evolution of the

373   lobes in response to variation in sexual (and natural) selection (House et al. 2013). The lobe

374   shape that evolved through sexual selection in that work is remarkably similar to the

375   prominent posterior and downward facing ventral lobe favoured by pre-copulatory and

376   postcopulatory sexual selection during paternity defence that we document in the current

377   study.

378   Selection was strongest during non-competitive mating and suggests that mounting

379   and genital coupling is important for the evolution of the posterior and ventral lobes.

380   Perhaps this is not surprising as this component of the male genitalia does not enter the

381   female and previous work found these genital structures were important in securing

382   mounting and in genital coupling (Jagadeeshan and Singh 2006). A combination of linear and

383   stabilizing selection favoured a thicker, wider posterior process with a down-ward facing

384   ventral lobe that converges on the consensus genital size and shape. This finding is

385   consistent with work on *D. melanogaster* which reported variance in mating success that is

386 the strongest source of selection acting on males (Pichedda and Rice 2012). During post-

387 copulatory, competitive fertilization, when males mated in a defensive role (P1), linear and

388 disruptive sexual selection favoured a smaller genital arch with a similarly shaped ventral

389 lobe as during non-competitive mating but with a more elongated posterior lobe. Whereas,

390 when males mated in an offensive role (P2), selection favoured a lobe with a shortened tip

391 and a narrow space between the posterior and ventral lobe. However, despite these subtle

392 differences, we find that there is little difference in the strength and direction of linear and

393 non-linear selection on individual components of the genital lobes across the three episodes

394 of selection. Therefore, there is little evidence of antagonistic selection on the genital arch

395 when males compete, primarily because selection was weak during sperm defence and

396 offense. For example, the average linear selection during non-competitive mating is β =

397 |0.0246| compared to β = |0.0187| during sperm defence and β = |0.007| during offense.

398   Selection on the genital lobe during non-competitive mating is consistent with

399 previous studies that show that components of the external genitalia of *Drosophilia* are

400 important to establish genital coupling. For example, the asymmetrical epandrial lobe

401 bristles (*D. pachea*; Rhebergan et al. 2016) and genital spines (*D. bipectonata*; Polak &

402 Rashed 2010) are specialized to grasp the female during mating and stabilize coupling.

403 Correlational studies such as ours do not elucidate the functional mechanism(s) that are

404 driving selection (we only looked at outcome not process), but Jagadeeshan et al. (2006)

405 argued that variation in the morphology of the genital lobe is not driven by male-female

406 conflict as the female apparently lacks genital modifications that reduce the likelihood of

407 coupling, which contrasts to systems where morphological adaptations and counter-

408 adaptations have probably evolved via sexual conflict (e.g. Arnqvist and Rowe 2002;

409 Crudgington and Siva-Jothy 2000; Cayetano et al. 2011; Hotzy et al. 2012). Instead, it has

410 been argued that relatively large and broad posterior lobes of the *Drosophila* genitalia act as

411 hold-fasts that grasp the female oviscape so that copulation and sperm transfer is successful

412 (Jagadeeshan et al. 2006; House et al. 2013; LeVasseur-Viens et al. 2015). Alternatively, it is

413 equally possible that cryptic female choice (Eberhard 2009) imposes selection on the

414 posterior lobe when the lobes contact the oviscape and potentially stimulate females and

415 influence oviposition (Frazee & Masly 2015). As has been pointed out elsewhere, it is

416 extremely difficult, and perhaps even fruitless, to try and separate these processes (Pitnick

417 & Hosken 2010), but the main conclusion of our investigation, genital lobe shape impacts

418 male sexual fitness-components, remains unchanged regardless of the relative contribution

419 of these potential mechanistic explanations for the effects we document.

420   It has also been suggested that sperm competition generates antagonistic selection

421 on males to both protect paternity and overcome paternity assurance adaptations (Parker

422 1984). Here, we find no evidence of antagonistic selection on the posterior lobe during non-

423 competitive mating and sperm competition, which is consistent with work on *D.*

424 *melanogaster* (Frazee & Masly 2015). Instead, we found that selection during sperm

425 defence favoured a similar posterior lobe shape to that favoured during non-competitive

426 mating (i.e. thicker, wider lobe), albeit selection was weaker during defence. Interestingly, in

427 *D. melanogaster*, a prominent hook-like lobe morphology was also associated with greater

428 reproductive success during sperm defence, suggesting that sexual selection tends to favour

429 more exaggerated lobe phenotypes (Frazee &; Masly 2015). In our study, selection during

430 sperm offense was weakest and overall we find limited evidence that lobe morphology

431 influences this element of sperm competition. This is consistent with previous studies that

432 also found little evidence that the lobes influence sperm offense in *D. simulans* (LeVasseur-

433 Viens et al. 2015) or *D. melanogaster* (Frazee & Masly 2015). Nonetheless, our study shows

434   considerable variation in fertilization success (i.e. the least and most fit males have a

435   difference of 200 offspring), consistent with the fact that male traits other than genital

436   form, like behaviour (i.e. Hosken et al. 2008), morphology (i.e. body size; Taylor et al. 2008a)

437   and physiology (i.e. Hosken et al. 2008; Taylor et al. 2008b) also affect male sexual fitness.

438   In other arthropod systems, evidence for antagonistic sexual selection on genital

439   traits is weak even when post-copulatory selection on genitals has been documented. For

440   instance, in the oriental beetle, *Anomala orientalis* (Wenninger and Averill 2006) and

441   earwig, *Euborellia brunneri* (van Lieshout 2011; van Lieshout & Elgar 2011), the aedeagus is

442   under selection during sperm defence, but selection on genitals during sperm offense was

443   not detected (Wenninger and Averill 2006; van Lieshout 2011; van Lieshout & Elgar 2011).

444   In the water strider, *Gerris lateralis* and dung beetle, *Onthophagus taurus* the aedeagus

445   have pairs of sclerites that function in either sperm defense or offense, and superficially, it

446   appears that selection on the sclerites may be antagonistic. However, in *O. taurus*, this

447   hypothesis is not supported as the genetic correlations between sclerites suggest that the

448   size of the sclerites is optimized so that males may be successful in both roles (House et al.

449   2005). Conclusive evidence for antagonistic selection on genital structures ideally requires

450   empirical studies that combine estimates of selection on genitalia across more than one

451   episode (Parker 1984; Hunt et al. 2009). Therefore, whilst there is no evidence of

452   antagonistic selection on the posterior lobe in some *Drosophila* species, general conclusions

453   cannot be made until more evidence is gathered.

454   In a separate study in *D. simulans*, we found that the morphology of the genital lobes

455   evolved in response to experimentally manipulated sexual and natural selection (House et

456   al. 2013). In this study, there were similarities in the lobe shape favoured by sexual selection

457   (i.e. non-competitive mating and sperm defence) and the shape that evolved under elevated

458   sexual (i.e. polyandry) and natural selection (House et al. 2013). In both studies, a thicker

459   wider posterior lobe with a down-ward facing ventral lobe evolved were favoured. Thus,

460   selection that we documented here is similar with aspects of the evolutionary divergence

461   that we documented previously (House et al. 2013).

462   Finally, although we detect linear selection on some aspects of the genital arch,

463   overall, selection was not especially strong (median, β = |0.009|) compared with estimates

464   of selection on non-genital, morphological traits (median, β = |0.16|) across species

465   (Kingsolver et al. 2001). Weak linear selection on the posterior and ventral lobe is likely to

466   limit the response to selection. This was unexpected because an experimental evolution

467   study documented significant and rapid microevolution of the lobe (House et al. 2013) as

468   expected if selection acted on it and there was (appropriate) genetic variation in the

469   posterior lobe. More broadly a finding of weak selection on the lobe seems somewhat

470   paradoxical as *Drosophila* species are morphologically similar but have strikingly different

471   genitalia across species (e.g. Coyne 1993; Eberhard 1985; Arnqvist 1998; Simmons et al.

472   2009) - which seems to imply relatively strong selection on genitals – although we cannot

473   know whether this divergence is due to strong selection in the past, and this appears to be a

474   general pattern for genitals (Hosken et al. 2019). Perhaps the genital arch is relatively free

475   from constraining genetic correlations, for which there is evidence as the lobe is less

476   sensitive than other traits to genetic regulators of size (Dreyer and Shingleton 2011; Dreyer

477   and Shingleton 2019; Shingleton et al. 2008). Additionally, perhaps selection and the G

478   matrix of the posterior lobe are aligned such that evolution is facilitated (Blows et al. 2004)

479   despite of weak selection. This would be consistent with findings that sexually selected

480   traits in animals tend to evolve faster than life history traits and morphological traits even

481   though selection on them does not appear to be stronger (Pitchers et al. 2014). In short, we

482 believe that examination of how the G matrix is aligned with the major axes of selection on

483 the posterior lobe is required to reliably conclude that the lobe will or will not evolve in our

484 population and this will be the subject of a future study.

485

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697

699   **FIGURE LEGENDS**

700

701   **Figure 1.** Frequency distribution of the four relative warp (RW) scores characterizing the

702   variation in male genital shape (A, B, C and D). For each RW, we provide thin-plate spline

703   visualizations (inset) that characterize a positive and negative score.

704

705   **Figure 2.** Thin-plate spline visualizations of the two major axes of linear (m_1 and m_6) and

706   nonlinear (m_4 and m_5) selection on the fitness surface for males during non-competitive

707   mating. The three-dimensional surfaces on the left (A & C) show a perspective-view while

708   the contour plots on the right (B and D) show the same surface from above. In each contour

709   plot, white colouration represents regions of highest fitness, whereas red colouration

710   represents regions of lowest fitness. Individual data points are provided as black circles on

711   the surface.

712

713   **Figure 3.** Thin-plate spline visualizations of the two major axes of linear and disruptive

714   selection during paternity defence (m_1 and m_2) and two axes of linear selection (m_1 and m_4)

715   during paternity offense – only selection along m_4 is significant. The three-dimensional

716   surfaces on the left (A & C) show a perspective-view while the contour plots on the right (B

717   and D) show the same surface from above. In each contour plot, white colouration

718   represents regions of highest fitness, whereas red colouration represents regions of lowest

719   fitness. Individual data points are provided as black circles on the surface.

720

721

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

732   **Table 2:** Linear (θ_i) and nonlinear (λ_i) selection gradients and the M matrix of eigenvectors

733   from the canonical analysis of Y for (A) non-competitive, virgin mating success (B) paternity

734   defence (i.e. P1) and (C) paternity offense (i.e. P2) in male *D. simulans*. The sign of λ_i

735   describes the form of quadratic selection acting along each eigenvector, with a positive λ_i

736   indicating disruptive selection and a negative λ_i indicating stabilizing selection.

737   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								
m_1	**0.071***	0.296	0.118	-0.377	-0.534	-0.731	0.114	-0.109
m_2	-0.120	0.083	-0.019	0.441	0.029	-0.065	0.781	-0.435
m_3	-0.040	-0.030	-0.251	-0.559	-0.120	0.364	0.565	0.398
m_4	0.059	**-0.166****	-0.024	0.496	-0.770	0.247	-0.059	0.309
m_5	0.084	**-0.205***	0.047	0.310	0.327	-0.472	0.184	0.734
m_6	**0.075*****	-1.120	0.959	-0.094	-0.0001	0.213	0.139	0.080
B. Canonical analysis of P1								
m_1	**0.143***	0.279	-0.156	**-0.884**	**-0.304**	0.094	0.106	0.284
m_2	-0.089	**0.198***	**0.603**	-0.010	-0.089	**0.605**	-0.480	0.179
m_3	0.029	0.084	0.621	-0.179	0.202	-0.694	-0.037	0.242
m_4	0.057	0.003	-0.371	0.290	-0.267	-0.229	-0.463	0.662
m_5	0.028	-0.021	0.164	0.261	0.017	0.245	0.719	0.572
m_6	-0.091	-0.231	0.249	0.180	-0.887	-0.178	0.157	-0.249
C. Canonical analysis of P2								
m_1	0.023	0.250	0.795	-0.301	-0.174	-0.074	-0.033	0.490
m_2	0.024	0.085	-0.398	-0.838	-0.212	0.184	-0.236	0.067
m_3	0.047	0.053	-0.262	-0.128	-0.180	-0.739	0.541	0.206
m_4	**-0.091***	0.018	-0.104	-0.035	**0.649**	-0.439	**-0.546**	0.275
m_5	-0.001	-0.045	-0.202	0.401	-0.658	-0.136	-0.516	0.284
m_6	-0.007	-0.102	-0.298	0.169	0.195	0.451	0.291	0.746

738   Randomization tests: *P < 0.05, **P < 0.01, ***P < 0.001
739

740 **Table 3.** Sequential model comparing the linear and nonlinear effects of sexual selection

741 during different episodes of selection on body size, genital arch size, RW1, RW2, RW3 and

742 RW4 in male *D. simulan*s.

	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

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

744 *RW4:* $F_{1,677}=3.174$, P=0.05.

745