

1 **Published in J Evol Biol**

2

3 **Constrained evolution of the sex comb in *Drosophila simulans***

4 **Maraqqa et al.**

5 **Abstract**

6 Male fitness is dependent on sexual traits that influence mate acquisition (pre-copulatory  
7 sexual selection) and paternity (post-copulatory sexual selection), and while many studies  
8 have documented the form of selection in one or the other of these arenas, fewer have  
9 done it for both. Nonetheless, it appears that the dominant form of sexual selection is  
10 directional, although theoretically, populations should converge on peaks in the fitness  
11 surface, where selection is stabilizing. Many factors, however, can prevent populations from  
12 reaching adaptive peaks. Genetic constraints can be important if they prevent the  
13 development of highest fitness phenotypes, as can the direction of selection if it reverses  
14 across episodes of selection. In this study, we examine the evidence that these processes  
15 influence the evolution of the multivariate sex comb morphology of male *Drosophila*  
16 *simulans*. To do this, we conduct a quantitative genetic study together with a multivariate  
17 selection analysis to infer how the genetic architecture and selection interact. We find  
18 abundant genetic variance and covariance in elements of the sex comb. However, there was  
19 little evidence for directional selection in either arena. Significant nonlinear selection was  
20 detected prior to copulation when males were mated to non-virgin females, and post-  
21 copulation during sperm offence (again with males mated to non-virgins). Thus contrary to  
22 our predictions, the evolution of the *D. simulans* sex comb is limited neither by genetic  
23 constraints nor by antagonistic selection between pre- and post-copulatory arenas, but  
24 nonlinear selection on the multivariate phenotype may prevent sex combs from evolving to  
25 reach some fitness maximising optima.

26 **Keywords: *Drosophila*, sex combs, genetic constraints, selection gradients, pre-copulatory**  
27 **selection & post-copulatory selection.**

## 28 1. INTRODUCTION

29 Male sexually selected traits typically evolve rapidly (Andersson 1994; Arnqvist 1998)  
30 through both pre- and post-copulatory sexual selection (Partridge & Halliday 1984). Pre-  
31 copulatory mechanisms of sexual selection include male-male competition and female mate  
32 choice and post-copulatory mechanisms of sexual selection include sperm competition and  
33 cryptic female choice (Parker 1970; Eberhard 1985; Andersson & Simmons 2006; Hunt et al.  
34 2009). Given the complexity of the mechanisms of sexual selection, an understanding of the  
35 form and strength of selection that pre- and post-copulatory sexual selection impose is  
36 required to gain an understanding of the extravagance of the traits that they produce (Hunt  
37 et al. 2009).

38 In the last decade an increasing number of studies have used multivariate statistical  
39 techniques to describe the form and strength of selection on sexually selected traits  
40 (reviewed in; Hunt et al. 2009; Kingsolver & Diamond 2011), and it is striking that directional  
41 selection is the dominant form of selection that has been documented (Hunt et al. 2009;  
42 Kingsolver & Diamond 2011). This is intriguing as, theoretically, populations should evolve  
43 towards areas of high fitness on fitness landscapes (Philips & Arnold 1989; Kingsolver &  
44 Diamond 2011) and as populations move closer to these regions, selection should become  
45 stabilising with moves in any direction acting to lower fitness (Chenoweth et al. 2012). There  
46 are a number of mechanisms that may explain why populations never reach peaks on a  
47 fitness landscape, but one explanation is the presence of trade-offs that could arise from  
48 either the genetic covariance structure among traits under selection, or from antagonism of  
49 selection on the multivariate phenotype across episodes of selection (e.g., pre and post-  
50 copulatory episodes).

51 Genetic constraints may arise due to associations among traits (i.e. the genetic  
52 covariance structure) so selection on one will indirectly select on others (Cheverud 1984;  
53 Phillips & Arnold 1989; Blows & Brooks 2003; Moore et al. 2004; Bentson et al. 2006; Hunt  
54 et al. 2007a; Pitcher et al. 2014). If the genetic covariance or correlation ( $r_G$ ) between traits  
55 is negative with respect to each traits' (directional) effect on fitness (e.g.  $r_G < 0$  between two  
56 positively selected traits) this should limit selection towards an adaptive peak (Fear & Price  
57 1998; Blows & Hoffmann 2005). Evidence consistent with bivariate genetic constraints have  
58 been found in a cricket (*Gryllus lineaticeps*; Wagner et al. 2012), dung beetle (*Onthophagus*  
59 *taurus*; House & Simmons 2005) and a cockroach (*Nauphoeta cinerea*; Moore et al. 2004).  
60 More recently, however, a focus on bivariate correlations to infer constraints has been  
61 criticized, as the data from long term studies suggest that populations do not evolve as  
62 predicted from bivariate genetic architecture alone (reviewed in Walsh & Blows 2009).  
63 Instead a multivariate approach that combines the genetic variance-covariance (**G**) matrix  
64 (i.e. the genetic variance across a suite of traits and the genetic covariances among them)  
65 with the vectors of linear selection gradients ( $\beta$ ) (i.e. estimation of linear selection across  
66 suites of traits) has been advocated to assess the potential for genetic constraints (Walsh  
67 and Blows 2009; Walling et al. 2014).

68 If trade-offs can occur between traits, they can also occur across discrete episodes of  
69 selection if trait values that increase fitness in one selective bout decrease it in another  
70 (Kingsolver & Diamond 2011; Hunt et al. 2009; Andersson & Simmons 2006). For instance, if  
71 selection on a trait is positive during mate acquisition and negative during sperm  
72 competition this can result in no net selection on traits (Hunt et al. 2009). However, the  
73 empirical evidence for these sorts of trade-offs is mixed. For example, pre- and post-

74 copulatory selection appear to be reinforcing in the guppy (*Poecilia reticulata*; Evans et al.  
75 2003), cricket (*Achete domesticus*; Head et al. 2006), fly (*Drosophila simulans*; Hosken et al.  
76 2008) and stalk-eyed fly (*Teleopsis dalmanni*; Rogers et al. 2008). In contrast, episodes of  
77 pre- and post-copulatory selection are antagonistic in the water strider (*Gerris lacustris*;  
78 Danielsson 2001), dung beetles (*Onthophagus* species; Simmons & Emlen 2006), fire fly  
79 (*Phontinus greeni*; Demary & Lewis, 2007), gulf pipefish (*Syngnathus scovelli*; Rose et al.  
80 2013) and the flour beetle (*Gnatocerus cornatus*; Okada et al. 2014). So at least sometimes,  
81 the trait values that would be of highest fitness in one selective episode may not be highest  
82 in another selective bout and therefore evolution is constrained by antagonistic selection.

83         Many male *Drosophila* have a secondary sexual trait on their forelegs, the sex  
84 comb(s) (Kopp & True 2002). These are used to grasp the female's abdomen and genitalia  
85 prior and during copulation. The design of the sex combs is highly variable across closely  
86 related species, with comb and tooth number being especially variable (Markow et al.  
87 1996). Field and laboratory studies provide evidence that these interspecific patterns of  
88 phenotypic variation are partly due to sexual selection. For instance, during pre-copulatory  
89 sexual selection there is positive (directional) selection on comb size and comb symmetry in  
90 *D. bipectinata* (wild population; Polak et al. 2004), while positive selection on tooth number  
91 has been reported in *D. melanogaster* (experimental lines; Promislow et al. 1998). There is  
92 also post-copulatory selection on sex comb traits in *D. bipectinata*, with positive selection  
93 on comb size (artificial lines; Polak & Simmons 2009) and non-linear (disruptive) selection  
94 against intermediate tooth number in *D. melanogaster* (wild populations; Robinson et al.  
95 2012). However, a number of other studies have found less evidence for selection. For  
96 instance no relationship between sex comb tooth number and mating success was found in

97 either *D. melanogaster* (wild populations; Markow et al. 1996; experimental lines; Snook et  
98 al. 2013) or *D. pseudoobscura* (experimental lines; Snook et al. 2013). This poses a paradox  
99 because while *Drosophila* sex combs have characteristics expected of a sexually selected  
100 trait (e.g. rapid divergence among lineages) the evidence that these characters are under  
101 strong sexual selection is inconsistent. One resolution may be that sex comb traits are the  
102 target of selection that has not been measured and/or selection on sex combs across pre-  
103 and post-copulatory selection is antagonistic.

104 In this study we investigate the hypothesis that the evolution of the paired *D. simulans*  
105 sex comb is constrained by genetic constraints and/or antagonistic selection across episodes  
106 of sexual selection. Sexual selection has been intensely studied in *D. simulans* for a number  
107 of traits (for example, Hosken et al. 2008; Taylor et al. 2008; Ingleby et al. 2014), and  
108 previous research suggests that sex comb tooth number is under negative directional  
109 selection through pre-copulatory mating success (Markow et al. 1996). However, tooth  
110 number represents just one component of the multivariate comb phenotype and little is  
111 known about if (and how) selection differs depending on whether it occurs pre- versus post-  
112 copulation. It is also unknown whether pre-copulatory selection is itself contingent on  
113 whether females have previously mated. Nonetheless, prior work has shown the single sex  
114 comb on the fore-tarsus of this species is functionally important, being used to grasp the  
115 female abdomen and genitalia and spread her wings prior to and during copulation (Sharma  
116 et al 2011). We therefore expect that overall comb morphology will be subject to directional  
117 selection. To start, we used a half-sib breeding design to estimate the genetic variance for  
118 and covariances among components of the sex comb (and body size). Next we quantified  
119 the form and strength of sexual selection across four episodes of sexual selection; pre-

120 copulatory selection when females were virgin or mated and post-copulatory sexual  
121 selection during sperm competition, when the focal male was first to mate (i.e. P1, sperm  
122 defence) or second to mate (i.e. P2, sperm offence).

123

## 124 **2. METHODS**

### 125 **1. Fly stocks**

126 Our laboratory wild-type populations of *Drosophila simulans* were derived from 20 isolines  
127 (supplied by Centre for Environmental Stress and Adaptation Research, La Trobe University,  
128 Australia) that originally came from individuals that were caught in Tincurry, Eastern  
129 Australia, in March 2004. In the laboratory these isolines were mixed and maintained for at  
130 least 7 years prior to the start of this study and have been found to be genetically and  
131 phenotypically variable for all traits that have been assayed (Hosken et al. 2008; Wright et  
132 al. 2009; Sharma et al. 2011; Okada et al. 2011). In addition to the wild type population,  
133 laboratory populations of ebony flies, which carry a homozygous recessive phenotypic  
134 marker, were derived from a strain obtained from the Tucson stock centre and maintained  
135 as above for over 50 generations. The grey-black cuticle of ebony flies allows the easy  
136 discrimination between progeny of ebony females sired by ebony versus wild-type males  
137 (Ashburner et al. 2005). All population cages (wild-type and ebony) had an excess of 600  
138 flies with overlapping generations and free mate choice. All stock and experimental  
139 offspring were maintained at 25°C under a 12:12 H light: dark cycle and maintained on  
140 *Drosophila* culture medium (Jazz Mix *Drosophila* Food, Fisher Scientific and *Drosophila* Quick  
141 Mix Medium, Blades Biological) with an excess of food. This reduces the risk of

142 environmental influences affecting mating and remating probabilities because of stress  
143 response (Zera et al 2001).

## 144 **2. Breeding design**

### 145 (a) Parental generation

146 For our experimental breeding design, wild-type flies were initially collected from  
147 population cages. Egg laying vials were placed in the cages of two wild-type populations  
148 daily and left for 24 hours. These vials were incubated until peak eclosion (ca. 8-9 days after  
149 egg laying). Offspring that eclosed overnight were killed and virgins were collected ca. 7hrs  
150 later (Sharma et al. 2010). Virgin males were maintained in standard culture vials, with ca.  
151 80 males per vial. Virgin females were aspirated into ca. 800 individual vials containing  
152 culture medium. These virgin females and males were the parents for our design and were 3  
153 days old before breeding commenced to ensure full sexual receptivity (Manning 1967).

### 154 (b) Breeding and rearing

155 A conventional half-sibling breeding design was used (Lynch & Walsh 1998), where 130 sires  
156 were each mated with 5 dams. Details of the mating regime are as follows; a sire was  
157 housed with a randomly selected, virgin female for 24 hrs to maximize the probability that  
158 the pair would mate. The following day the male was aspirated from the vial and transferred  
159 to a new vial that contained a virgin female for 24 hrs. The process was repeated three more  
160 times until the sire had been housed with a total of 5 dams. The mated dams were housed  
161 singly in oviposition vials and transferred daily to new oviposition vials for a total of 4 days.  
162 The oviposition vials were stored at 25°C for 12 days under a 12/12h light: dark cycle until  
163 the offspring began to emerge. Six days after the first eclosion, the offspring were collected,



164 labelled and frozen at  $-20^{\circ}\text{C}$  for subsequent dissection, measurement and quantitative  
165 genetic analysis (see below).

166

## 167 **2. Multivariate sexual selection**

### 168 (a) Experimental design

169 For experimental mating assays, a sample of ebony and wild-type flies (not the same as  
170 those that were used for the breeding design) were collected as virgins from population  
171 cages using the protocols described above (see above, '*Parental generation*'). Virgin females  
172 and males were used for mating trials when the females were 3 days old and males were 3 -  
173 4 days old, to ensure full sexual receptivity (Manning 1967). Mating trials began at the  
174 beginning of the photophase of the light: dark cycle as this is when the flies are most  
175 reproductively active (Sakai & Ishida 2001). In all trials, each male was aspirated into a  
176 female housing vial, and continuously observed for 2 hours during which courtship (i.e. wing  
177 flicking, wing vibration, leg rubbing and licking) and mating were recorded (Spieth 1974).

### 178 (b) Sex comb morphology and pre-copulatory sexual selection

179 In the first part of the study we investigated whether variation in sex comb morphology  
180 predicts mating success with virgin females (Virgin Trial) or with mated females (Non-Virgin  
181 Trial). To do this, we used no-choice mating assays that are a standard method to assess  
182 overall male attractiveness (for example, Hedge & Krishna 1997; Koref-Santibanez 2001;  
183 Gowaty et al. 2002; Yeniseti & Hedge 2003; Shackleton et al. 2005) and the results of assays  
184 with single and multi-males are the same (Taylor et al. 2008). During Virgin Trials, males that  
185 courted but were rejected ( $n = 154$ ) or courted and mated ( $n = 340$ , total  $n = 494$ ) were

186 separated from the females and frozen at -20°C for morphometric measurement. During  
187 Non-Virgin Trials, we used a new set of flies that were derived from the same stock  
188 population. The females were once mated but detailed observation of their mating  
189 behaviour was not recorded. All females were 7 days old, having mated 4 days before their  
190 second exposure to virgin males. The mating procedure in this trial was identical to that  
191 described above (Virgin Trial). All males that courted but were rejected (n =329) or courted  
192 and mated (n =154, total n = 483) were frozen at -20°C for morphometric measurement.

### 193 (c) Sex comb morphology and post-copulatory sexual selection

194 In the second part of the selection study we investigated whether variation in sex comb  
195 morphology predicts fertilization success. Ebony females were sequentially mated with a  
196 focal, wild type male followed by an ebony male (paternity defence – P1) or an ebony male  
197 followed by a focal, wild type male (paternity offence – P2). Males mated once only and in a  
198 single role – defensive or offensive. During the observation period, if copulation occurred,  
199 the male was removed from the chamber, aspirated into an Eppendorf and stored at -20°C  
200 for dissection and measurement. Following the first mating, females were transferred daily  
201 into fresh food vials to oviposit for 4 days before their second exposure to virgin males. The  
202 second mating procedure for mated females was identical to that described above. Ebony  
203 females that did not mate with the second mating partner during the 2 hour assay were  
204 excluded from the dataset, along with their first mate (n ~ 600 – *D. simulans* are reluctant to  
205 mate, particularly with mutant strains). Following their second mating, twice mated females  
206 were once again transferred daily into fresh food vials to oviposit for 4 days. On the 5<sup>th</sup> day  
207 the female was aspirated into an Eppendorf and stored at -20°C. Vials that had contained  
208 the mated females were stored at 25°C and monitored daily until offspring emerged. Seven

209 days after the first emergence, the vials were inverted and stored in the freezer and the  
210 ebony and wild type offspring from each of the female's 8 vials was subsequently counted  
211 to determine the number of offspring that were sired by the focal (i.e. wild type) male  
212 during defensive (P1, n = 308) or offensive mating (P2, n = 355).

213

#### 214 4. Dissection and Morphometric Measurement

215 The left and right fore-legs and wings of focal, wild type males or sons from our breeding  
216 design were carefully pulled free from the body of each male and then mounted on glass  
217 slides in a droplet of Hoyer's Medium. Digital images for wings (X30) and sex combs (X100)  
218 were captured using a Leica dissecting microscope (M125) connected to a Leica camera  
219 (DFC295). Wing length and sex comb components were measured using Image J v1.46r (RSB  
220 National institute of Mental Health, USA) (Figure 1).

221 We used wing length (WL) as an index of body size (Markow & Ricker 1992; Gilchrist  
222 & Partridge 1999; Sharma et al. 2011) and both left and right wings of each male were  
223 measured and an average value was calculated. Three components of sex comb morphology  
224 were measured; the comb length (CL), tooth length (TL), measured as the average length of  
225 the 1<sup>st</sup>, 3<sup>rd</sup> and 5<sup>th</sup> teeth), and comb tooth number (TN) (Figure 1). All sex comb  
226 characteristics, including CL, TL, and TN were estimated as the average of the  
227 measurements on the left and right body sides. The precision of the measurements were  
228 assessed by blindly measuring all traits twice on a sub-sample of wings and sex combs (N =  
229 20). Two measures of the same trait were tightly correlated (TL:  $r^2 = 0.919$ ,  $P < 0.05$ ; CL:  $r^2 =$   
230  $0.982$ ,  $P < 0.001$ ; TN:  $r^2 = 1.00$ ,  $P < 0.001$ ; WL:  $r^2 = 0.992$ ,  $P < 0.001$ ).

231

## 232 5. Statistical Analysis

### 233 (a) Genetic Analyses

234 Data were analysed using animal models fitted with restricted maximum likelihood in  
235 ASReml (version 3.0; VSN International Ltd) with assumed Gaussian errors (see Wilson et al.  
236 2010). First we tested for additive genetic variance using univariate models fitted to each of  
237 the sex comb component traits (comb length CL, tooth length TL, and tooth number TN) and  
238 size (wing length WL). Each model contained the mean as a fixed effect and random effects  
239 of additive genetic merit and a “maternal identity” effect. The latter was included to protect  
240 against upward bias from maternal (or other common environment) effects shared by full-  
241 sibs. For each trait we compared this to a reduced model with the additive effect dropped  
242 using a likelihood ratio test and assuming that twice the difference in log-likelihoods is  
243 distributed as a 50:50 mix of  $\chi^2_1$  and  $\chi^2_0$  (subsequently denoted  $\chi^2_{0,1}$ ). Having detected  
244 significant genetic variance in all traits (see results), we formulated a multivariate animal  
245 model which was used to estimate the additive variance-covariance matrix (**G**) and derived  
246 parameters. To facilitate convergence in the multivariate model, traits were scaled to unit  
247 variance by dividing by their (observed) standard deviations. Heritability ( $h^2$ ) was estimated  
248 for each trait as  $V_A/V_P$  where  $V_A$  is the additive genetic variance and  $V_P$ , the phenotypic  
249 variance, determined as the sum of  $V_A$ ,  $V_M$  (maternal variance) and  $V_R$  (residual variance).  
250 We similarly estimated the magnitude of the maternal effect as  $m^2$ , where  $m^2 = V_M/V_P$ .  
251 Genetic correlations ( $r_G$ ) were determined for each pair of traits (1,2) as  $r_{G(1,2)} =$   
252  $COV_{A(1,2)}/(V_{A1} * V_{A2})^{0.5}$  where  $COV_A$  is the estimated additive genetic covariance. For  
253 comparison we also estimated the corresponding phenotypic correlations  $r_P$ .

254 (b) Multivariate Selection Analysis

255 To determine whether male phenotypic traits (CL, TL, TN and WL) influenced fitness during  
256 pre-copulatory or post-copulatory selection we used a standard multivariate selection  
257 analysis approach. In pre-copulatory bouts of selections, a male was assigned a score of 1 if  
258 the male courted and mated and a 0 if the male courted only. In these mating success trials,  
259 the female was always presented with a wild type male to increase the likelihood that a  
260 male would attempt to court and mate. As a consequence, we would have been unable to  
261 determine the number of offspring that were sired by the focal male when mating a  
262 previously mated female without extensive genotyping work, hence the binary fitness  
263 measure. In post-copulatory, fertilization success trials, male fitness was assigned a  
264 continuous value - the number of offspring that were sired by the focal male which ranged  
265 from 0 – 200. The mating and fertilization success response variables were transformed to  
266 relative fitness by dividing individual scores by the mean for each data set. The male  
267 phenotypic traits were standardized to zero means and unit variances as suggested by  
268 Lande & Arnold (1983). We then fitted a separate linear multiple regression for each of the  
269 4 bouts of selection to estimate linear selection gradients when females were virgins ( $\beta_v$ ),  
270 previously mated ( $\beta_m$ ) or the focal male mated in a defensive role ( $\beta_{p1}$ ) or an offensive role  
271 ( $\beta_{p2}$ ) (Lande & Arnold 1983). Next we applied a quadratic regression model including all  
272 linear, quadratic and cross-product (i.e. correlational) terms to estimate the matrix of  
273 nonlinear selection gradients for males when females were virgin ( $\gamma_v$ ), previously mated ( $\gamma_m$ )  
274 or the focal male mated in a defensive role ( $\gamma_{p1}$ ) or an offensive role ( $\gamma_{p2}$ ). Quadratic  
275 regression coefficients were doubled to yield the standardised non-linear selection  
276 gradients (see Stinchcombe et al. 2008). As our binary and continuous fitness measures did

277 not conform to a normal distribution, we used a re-sampling procedure to assess the  
278 significance of our linear and nonlinear selection gradients. Our fitness scores were  
279 randomly shuffled across individual phenotypes 10000 times to generate a null distribution  
280 of pseudo-selection gradients expected in the absence of a causal phenotype-fitness  
281 relationship (Mitchell-Olds & Shaw 1987). The probability that the gradient pseudo-estimate  
282 was equal to or less than the original estimated gradient (out of 9,999 permutations) was  
283 then tested. We conducted separate randomization analyses for the multiple regression  
284 models for directional selection (i.e. model containing only linear terms) and for the full  
285 quadratic model (i.e. model containing linear, quadratic and correlational terms).

286 To establish the extent of nonlinear selection acting on male phenotypic traits we  
287 conducted a canonical analysis using the approach suggested by Reynolds et al. (2010). The  
288 analysis generates a new matrix that consists of vectors of linear selection described by  
289 theta ( $\theta_i$ ) and nonlinear selection that are described by eigenvalues ( $\lambda_i$ ) and their  
290 corresponding eigenvectors ( $\mathbf{m}_i$ ). Tests of the significance of the eigenvalues were  
291 conducted using the permutation procedure outlined in Reynolds et al. (2012). We used  
292 thin-plate splines (Green & Silverman 1994) to visualize the major axes of the fitness  
293 surfaces extracted from the canonical rotation of  $\gamma_{m_1}$  and  $\gamma_{p2}$ . Tps functions in the fields  
294 package of R (version 2.13.0; available via <http://www.r-project.org>) were used to fit spline  
295 surfaces using the value of the smoothing parameter ( $\lambda$ ) that minimized the generalized  
296 cross-validation (GCV) score. We then plotted surfaces in R using both the perspective and  
297 contour map views. Finally, to test whether the linear, quadratic and correlational selection  
298 gradients differed when females had previously mated compared to when males mated in

299 the offensive role we used a sequential model building approach (partial F-test) (Draper &  
300 John 1988; see Chenoweth & Blows, 2005 for a detailed description of this procedure).

301

## 302 **Results.**

### 303 *Genetic architecture*

304 Comparison of full and reduced univariate models indicated significant additive genetic  
305 variance for comb length (CL:  $\chi^2_{0,1} = 25.0$ ,  $P < 0.001$ ), tooth length (TL:  $\chi^2_{0,1} = 5.48$ ,  $P = 0.010$ ),  
306 tooth number (TN:  $\chi^2_{0,1} = 40.2$ ,  $P < 0.001$ ) and wing length (WL:  $\chi^2_{0,1} = 4.78$ ,  $P = 0.014$ ).

307 Estimates of maternal variance were non-zero in all cases except for TN where  $V_M$  was  
308 bound at zero (full results not shown), so we formulated the multivariate model with a 4x4  
309 **G** matrix but a 3x3 maternal effect covariance matrix (i.e. no maternal effect on TN). Under  
310 this multivariate model,  $h^2$  estimates for sex comb components ranged from moderate to  
311 high (Table 1). The heritability of wing length (which is a proxy for body size) was similar to  
312 previously published heritability of body size for *Drosophila* ( $h^2 \sim 0.4$ ; Robertson 1957;  $\sim 0.5$ ;  
313 Coyne & Beecham 1987). All genetic correlations between sex comb component traits were  
314 positive and nominally significant (based on  $|r_G| > 2SEs$ ; Table 2). Genetic correlations  
315 between wing length and all sex comb components were also positive although not  
316 significantly for WL and TL. While noting that estimated standard errors are approximate  
317 and so not necessarily robust for formal inference, the model was a significantly better fit to  
318 the data than a reduced version in which all off-diagonal (ie  $COV_A$ ) terms in the **G** matrix  
319 were constrained to zero ( $\chi^2_6 = 112$ ,  $P < 0.001$ ). Thus it is clear that **G** contains significant

320 additive genetic covariance among the traits, and estimates are uniformly positive across all  
321 trait pairs.

322

### 323 *Sexual selection on sex combs*

324 Rather surprisingly, given the evidence from a previous study in *D. simulans* (Markow et al.  
325 1996) we found no evidence of significant directional selection (i.e.  $\beta$  - linear selection that  
326 increases/decreases the trait mean) acting on any component of the sex comb in any of the  
327 four selective contexts (Table 3). However, we found evidence for non-linear selection,  
328 which acted differently in each context. There are three different forms of nonlinear  
329 selection (i.e.  $\gamma$  coefficients that describe the curvature of nonlinear selection on individual  
330 traits); (a) stabilizing where  $\gamma$  coefficients are negative and individuals with intermediate  
331 trait values have highest fitness, (b) disruptive where  $\gamma$  coefficients are positive and  
332 individuals with extreme low or high trait values have highest fitness and (c) correlational  
333 selection where pairs of traits are jointly acted upon (Hunt et al. 2009). We find evidence for  
334 all three forms of nonlinear selection.

335

### 336 *Pre-copulatory Sexual Selection*

337 Nonlinear selection was weak and non-significant when males courted virgin females with  
338 the exception of significant positive correlational selection between tooth number (TN) and  
339 wing length (WL) (Table 3A). Canonical rotation of the  $\gamma$  matrix of nonlinear selection  
340 gradients produced one positive and three negative eigenvalues, which describe the  
341 curvature of selection on the major axes of selection, rather than on individual traits (Table  
342 4A – i.e. positive eigenvalue is indicative of disruptive selection along  $\mathbf{m}_1$  and negative



343 eigenvalue is indicative of stabilizing selection along  $\mathbf{m}_2 - \mathbf{m}_4$ ). However, selection on the  
344 eigenvectors ( $\mathbf{m}_1 - \mathbf{m}_4$ ) was non-significant (Table 4A).

345 Nonlinear selection was stronger when males courted non-virgin females. There was  
346 significant stabilising (negative  $\gamma$ ) selection on tooth length (TL), disruptive (positive  $\gamma$ )  
347 selection on the tooth number (TN) as well as positive correlational selection between tooth  
348 length (TL) and wing length (WL) (Table 3B). Canonical rotation of the  $\gamma$  matrix of nonlinear  
349 selection gradients produced a combination of disruptive selection along the  $\mathbf{m}_1$  and  $\mathbf{m}_2$  axis  
350 and stabilizing selection along the  $\mathbf{m}_3$  and  $\mathbf{m}_4$  axis however, there was only significant  
351 selection along eigenvector  $\mathbf{m}_4$  (Table 4B). This axis of significant selection for the non-virgin  
352 mating phase shows stabilizing (negative  $\gamma$ ) selection which we visualized with  $\mathbf{m}_1$  that had  
353 the largest, albeit non-significant disruptive (positive  $\gamma$ ) eigenvalue. These represent parts of  
354 the fitness surface that curve downward and upward respectively to create a saddle like  
355 fitness surface in the  $\mathbf{m}_1 - \mathbf{m}_4$  plot (Figure 2A). Along the  $\mathbf{m}_4$  axis, highest fitness occurred  
356 along a ridge which corresponds with intermediate values and was heavily influenced by  
357 tooth length (TL) and wing length (WL) (i.e. in each row of M table 4, the magnitude of the  
358 values indicates the contribution of individual traits to an eigenvector). A contour-view  
359 visualization of the same fitness surface, with an overlay of the data points shows that many  
360 of the males are spread along the ridge on the  $\mathbf{m}_4$  axis (Figure 2B).

### 361 *Post-copulatory Sexual Selection*

362 Nonlinear selection was weak and non-significant when males mated in a defensive role  
363 with the exception of, significant disruptive selection (positive  $\gamma$ ) on wing length (Table 3C).  
364 Canonical rotation of the  $\gamma$  matrix of quadratic selection gradients produced a combination

365 of disruptive selection along the  $\mathbf{m}_1$  and  $\mathbf{m}_2$  axis and stabilizing selection along the  $\mathbf{m}_3$  and  
366  $\mathbf{m}_4$  axis, however selection along these vectors ( $\mathbf{m}_1 - \mathbf{m}_4$ ) was non-significant (Table 4C).

367 Nonlinear selection was stronger during competitive mating when males mated in  
368 the offensive role (P2). There was disruptive (positive  $\gamma$ ) selection on comb length (CL) and  
369 negative correlational selection between comb length (CL) and tooth number (TN) and comb  
370 length (CL) and wing length (WL) (Table 3, D). Canonical rotation of the  $\gamma$  matrix of quadratic  
371 selection gradients produced a combination of disruptive selection along the  $\mathbf{m}_1$  and  $\mathbf{m}_2$  axis  
372 and stabilizing selection along the  $\mathbf{m}_3$  and  $\mathbf{m}_4$  axis but selection along these vectors was only  
373 significant for  $\mathbf{m}_1$  and  $\mathbf{m}_2$ . These axes of significant selection for the competitive, offensive  
374 mating phase (P2) showed disruptive selection along the  $\mathbf{m}_1$  and  $\mathbf{m}_2$  axes which curved the  
375 fitness upwards to create an inverted fitness surface in the  $\mathbf{m}_1$ - $\mathbf{m}_2$  plot (Figure 3, A). Along  
376 the ridge of highest fitness (i.e. intermediate values of  $\mathbf{m}_1$  and positive values of  $\mathbf{m}_2$ ), high  
377 paternity was correlated with a long sex comb, few but long comb teeth and large body size.  
378 However, a contour-view visualization of the same fitness surface, with an overlay of the  
379 data points shows that few males occupy this region on the landscape (Figure 3B).

380

### 381 *The strength and form of linear and nonlinear selection across episodes*

382 To test for possible differences in selection on the sex comb and body size (i.e. WL) during  
383 bouts of significant pre- and post-copulatory sexual selection, we compared the strength of  
384 linear, quadratic and correlational selection across selective bouts. The strength of linear  
385 ( $F_{4,826} = 1.192, P = 0.313$ ), quadratic ( $F_{4,818} = 1.576, P = 0.179$ ) and correlational selection  
386 ( $F_{6,806} = 0.469, P = 0.759$ ) did not differ significantly between these bouts of selection.

387

## 388 **Discussion**

389 We find that there is substantial genetic variation in the male sex comb trait components  
390 which are positively genetically correlated to each other and with body size. However, there  
391 was no evidence of directional selection on the sex comb across any bout of sexual  
392 selection. Thus, contrary to our predictions, it is the absence of directional selection that is  
393 the primary limitation to the evolution of the *D. simulans* sex comb rather than genetic  
394 constraints arising from among-trait covariance and/or antagonistic linear selection across  
395 episodes of selection. While evidence for linear selection was conspicuous by its absence,  
396 we did find complex patterns of significant nonlinear selection. In particular, we found  
397 disruptive selection acting on male sex combs during post-copulatory selection when  
398 females are already mated.

### 399 *Genetic (co)variance among components of the sex comb*

400 A breadth of studies find that sexually selected traits harbour abundant genetic variation  
401 (reviewed in Roff & Mousseau 1987; Houle 1992; Pomiankowski & Moller 1995; Walsh &  
402 Blows 2009). Our average  $h^2$  estimate for sex comb components ( $h^2 = 0.46$ ) is high and  
403 comparable with other  $h^2$  estimates for morphological traits (Roff & Mousseau 1987; Houle  
404 1992; Pomiankowski & Moller 1995). The maintenance of genetic variation in sexually  
405 selected traits is an evolutionary puzzle and a number of models have been developed to  
406 explain the phenomena (Taylor & Williams 1992; Mousseau & Roff 1987; Pomiankowski &  
407 Moller 1995; Rowe & Houle 1996). Here, it appears that the lack of significant directional  
408 selection coupled with stabilizing and disruptive selection, (which may promote genetic

409 variation), has maintained genetic variance in the sex comb. We also found positive genetic  
410 correlations between component traits of the comb and body size which should result in  
411 positively correlated indirect selection responses (see below).

#### 412 *Linear selection on the sex comb across selective episodes*

413 A previous study of *D. simulans* found that directional selection during pre-copulatory  
414 sexual selection favoured fewer teeth in the comb. Whereas we find no evidence that  
415 directional selection acts on sex comb components during any bout of selection. More  
416 generally, the evidence that selection acts on components of the sex comb of *Drosophila*  
417 species is mixed. In part, this may be a result of experimental design – typically, estimates of  
418 selection on the sex comb are univariate (Polak et al. 2004; Markow et al. 1996; Promislow  
419 et al. 1998; Polak and Simmons 2009; Snook et al. 2013) even though this may  
420 underestimate the strength of selection (Blows & Brooks 2003). For instance, if nonlinear  
421 selection was acting, it could result in linear selection gradients being estimated that simply  
422 cross two points of a nonlinear selection gradient (Hunt et al. 2009). The results of this  
423 study, and from a field study of *D. melanogaster*, where sexual selection on the sex comb  
424 was disruptive (Robinson et al. 2012), suggest that this may be an oversight as nonlinear  
425 selection was the dominant form of selection.

426         Given that directional selection on male sex comb components was absent, it is clear  
427 that trade-offs (between component traits and/or pre- versus post- selective episodes) are  
428 neither present, nor required to explain evolutionary stasis. Among previous studies of  
429 sexual selection on male traits, pre-copulatory selection for elaborate male traits is often  
430 reinforced by post-copulatory fertility benefits (Rogers et al. 2008), sons with high  
431 fertilization success (Hosken et al. 2008) and/or high quality sons (Head et al. 2006). In *D.*

432 *simulans*, pre-copulatory selection acting on the sex comb is weak so it seems unlikely that  
433 females exercise mate choice on the basis of male sex comb morphology. Furthermore,  
434 during post-copulatory sexual selection more than one sex comb phenotype is correlated  
435 with fertilization success during competitive mating (see below) and therefore it seems  
436 unlikely that sex combs provide a clear signal of sire or offspring reproductive quality.

#### 437 *Nonlinear selection on the sex comb across selective episodes*

438 When females were already mated, pre-copulatory sexual selection favours males with  
439 intermediate tooth length (TL) and wing sizes which resulted from a blend of stabilizing and  
440 correlational selection on these traits. A similar pattern of stabilizing selection has been  
441 found in *D. melanogaster* following successful (artificial) linear selection for high or low  
442 tooth number (Ahuja & Singh 2008). After ten generations of relaxed selection, tooth  
443 number regressed back to intermediate, control numbers, demonstrating the action of net  
444 stabilizing selection on this component of the comb (Ahuja & Singh 2008). Interestingly,  
445 males within the low tooth number lines were less likely to successfully mate if they had  
446 very few teeth compared to those that had more sex comb teeth. However, among the  
447 control and high tooth number lines, the effect of tooth number on mating success was  
448 nonsignificant (Ahuja & Singh 2008).

449 Here, non-linear post-copulatory selection on the sex combs was stronger and  
450 disruptive when measured as sperm offence. As the significant eigenvalues ( $\lambda$ ) are positive,  
451 it suggests that the fitness surface is concave and best described as a bowl (Figure 3A; Hunt  
452 et al. 2009) and along the height of the bowl fertilization success is approximately  
453 equivalent (Figure 3B). Two other studies have shown that particular morphologies of  
454 *Drosophila* sex combs enhance competitive fertilization success. In *D. bipectinata*, artificial

455 selection was used to develop lines with relatively short or long combs and relatively long  
456 combs were found to confer an advantage during sperm offense (Polak and Simmons 2009).  
457 In contrast, in a field study of *D. melanogaster*, sexual selection on the sex comb was  
458 disruptive (Robinson et al. 2012) as we report here.

459         The patterns of selection that we found may be explained if extreme combinations  
460 of sex comb components are most effective at grasping the female and aligning her genitalia  
461 during insemination, with intermediate combinations being less effective. Similar patterns  
462 of disruptive selection have been found in naturally selected traits (Brodie 1992; Smith  
463 1993; Bolnick 2004) and sexually selected traits (Blows et al. 2003) and in three of these  
464 studies, competition for limiting resources appears to generate this pattern of selection  
465 (Smith 1993; Blows et al. 2003; Bolnick 2004). For instance, the African finch exhibit small or  
466 large bill size and feed exclusively on soft or hard-seeded sedge respectively (Smith 1993). In  
467 the three-spine stickleback, intraspecific competition selects for extreme trophic  
468 morphology (i.e. large or small gill raker length) (Bolnick 2004) and female choice selects for  
469 rare male phenotypes in guppies (Blows et al. 2003). The wider implication of this pattern of  
470 selection is subject to debate but theoretically can force niche expansion (Roughgarden  
471 1972), sexual dimorphism (Slatkin 1984; Bolnick & Doebeli 2003) and speciation (Doebeli  
472 1996; Dieckmann & Doebeli 1999).

### 473 *Nonlinear selection and genetic correlations between sex comb components*

474 In this study, all genetic correlations between body size and the sex comb components were  
475 positive so that genotypes predisposing to larger size, also result in longer combs with both  
476 more numerous and longer teeth. However, highest post-copulatory, paternity offense (i.e.  
477 P2) was correlated with a long sex comb but few comb teeth (i.e. intermediate  $m_1$  and

478 positive  $m_2$ ) yet the positive genetic covariance between these sex comb traits means that  
479 few male genotypes occupy this region of the landscape. This may reflect an underlying  
480 mechanistic constraint as sex combs are positively allometric (Sharma et al. 2011) so the  
481 scaling of sex comb trait components with body size largely prevents this combination.  
482 Evidence from other species, suggests that the cause of genetic covariance may originate  
483 from developmental or functional constraints that place limits on trait combinations. For  
484 example, negative genetic covariance between the call rate and chirp duration of a cricket  
485 (Wagner et al. 2012) and ejaculate size and sperm quality in a cockroach (Moore et al. 2004)  
486 may reflect the energetics of calling (Wagner et al. 2012) and sperm production (Moore et  
487 al. 2004). Whereas, the negative covariance among colour pattern components in a guppy  
488 (Brooks and Endler 2001) and the correlated evolution of beak morphology and vocal  
489 repertoire of Darwin's finches (Podos 2001) may be due to physical constraints. For  
490 instance, in the guppy, spots occupied by one colour may preclude another (Brooks and  
491 Endler 2001) and, in finches, beaks that become adapted for increased bite force are less  
492 able to perform rapid movements that are required for certain songs (Podos 2001).

#### 493 *Opportunity for sexual selection across selective episodes*

494 *D. simulans* belong to a clade in which female re-mating is infrequent and females can be  
495 more choosy after mating as they can use stored sperm to continue to produce offspring  
496 (Taylor et al. 2007, 2008a,b). More broadly in *Drosophila sp*, it is striking that secondary  
497 sexual traits, like the sex comb, are only present in clades where females rarely re-mate.  
498 Theoretically, this should increase the variance in male mating success and thus the  
499 opportunity for selection on male secondary sexual characters (Emlen & Oring 1977;  
500 Markow 2002; Collet et al. 2012). Our results are partially consistent with this expectation,

501 with no evidence of selection on male sex combs during any bout of selection when females  
502 are virgin whereas we detect some nonlinear selection on sex combs when females are  
503 mated. For example, during pre-copulatory selection, virgin females rejected ~ 30% of male  
504 courtship displays and this increased to ~ 60% when females were mated which was  
505 matched by the detection of selection on the sex comb, albeit weak selection. Similarly,  
506 post-copulatory sexual selection on sex combs was only significant for sperm offense - so  
507 selection on the sex combs is acting through males' ability to displace a mated female's  
508 stored sperm.

### 509 *Conclusions*

510 Given the lack of directional selection acting on the sex comb, formal analysis of the extent  
511 to which the covariance structure in **G** constrains a selection response is not particularly  
512 informative. Studies that have implemented this approach show that genetic covariances  
513 constrain evolution sometimes (Hine et al. 2004; Von Homrigh et al. 2007; Hunt et al. 2007;  
514 Hall et al. 2010; Ingleby et al. 2014) but not always (Ingleby et al. 2014; Welch et al. 2014;  
515 Walling et al. 2014). Here, selection is non-linear, comprising a combination of stabilizing  
516 and disruptive processes as described by the fitness surfaces. These forms of selection,  
517 coupled with the lack of net linear selection may contribute to the maintenance of genetic  
518 variation and prevent male sex combs from evolving to a single optimal phenotype.  
519 Furthermore, data from other systems suggest that disruptive selection, as we find here,  
520 may be important for divergent evolution and speciation (Schluter 2000).

521

### 522 **Acknowledgments**



523 **References**

- 524 Ahuja, A. & Singh, R. S. 2008. Variation and evolution of male sex combs in *Drosophila*:  
525 Nature of selection response and theories of genetic variation for sexual traits. *Genetics*  
526 **179**: 503-509.
- 527 Andersson, M. 1994. *Sexual selection*. Princeton University Press, New Jersey.
- 528 Andersson, M.B. & Simmons, L.W. 2006. Sexual selection and mate choice. *Trend in Ecol.*  
529 *Evol.* **21**: 296-302.
- 530 Ashburner, M., Golic, K.G. & Hawley, R.S. 2005. *Drosophila: a Laboratory Handbook*. Cold  
531 Spring Harbour Press, Cold Spring Harbour, New York.
- 532 Bentsen, C.L., Hunt, J., Jennions, M.D. & Brooks, R. 2006. Complex multivariate sexual  
533 selection on male acoustic signalling in a wild population of *Telogyllus commodus*. *Am.*  
534 *Nat.* **167**: 102-116.
- 535 Blows, M.W. & Brooks, R. 2003. Measuring nonlinear selection. *Am. Nat.* **162**: 815-820.
- 536 Blows, M.W., Brooks, R. & Kraft, P.G. 2003. Exploring complex fitness surfaces: Multiple  
537 ornamentation and polymorphism in male guppies. *Evolution* **57**: 1622-1630.
- 538 Blows, M.W. & Hoffmann, A.A. 2005. A reassessment of genetic limits to evolutionary  
539 change. *Ecology* **86**: 1371-1384.
- 540 Bolnick, D.I. & Doebeli, M. 2003. Sexual dimorphism and adaptive speciation: Two sides of  
541 the same ecological coin. *Evolution* **57**: 2433-2449.
- 542 Bolnick, D.I. 2004. Can intraspecific competition drive disruptive selection? An experimental  
543 test in natural populations of sticklebacks. *Evolution* **58**: 608-618.
- 544 Brodie, E.D. III 1992. Correlational selection for colour pattern and antipredator behaviour in  
545 the garter snake *Thamnophis ordinoides*. *Evolution* **46**: 1284-1298.
- 546 Brooks, R. & Endler, J.A. 2001. Direct and indirect sexual selection and quantitative genetics  
547 of male traits in guppies (*Poecilia reticulata*). *Evolution* **55**: 1002-1015.

- 548 Chenoweth, S.F. & Blows, M.W. 2005. Contrasting mutual selection on homologous signal  
549 traits in *Drosophila serrata*. *Am. Nat.* **165**: 281-289.
- 550 Chenoweth, S.F., Hunt, J. & Rundle, H. 2012. Analyzing and comparing the geometry of  
551 individual fitness surfaces. In *The Adaptive Landscape in Evolutionary Biology* (E.  
552 Svensson & R. Calsbeek, ed.), pp. 126-320. Oxford University Press, Oxford.
- 553 Cheverud, J.M. 1984. Quantitative genetics and developmental constraints on evolution by  
554 selection. *J. Theor. Biol.* **110**: 155-171.
- 555 Collet, J., Richardson, D.S., Worley, K. & Pizzari, T. 2012. Sexual selection and the differential  
556 effect of polyandry. *PNAS* **109**: 8641-8645.
- 557 Coyne, J.A. & Beecham, E. 1987. Heritability of two morphological characters within and  
558 among natural populations of *Drosophila melanogaster*. *Genetics* **117**: 727-737.
- 559 Danielsson, I. 2001. Antagonistic pre- and post-copulatory sexual selection on male body  
560 size in a water strider (*Gerris lacustris*). *Proc. R. Soc. Lond. B.* **268**: 77-81.
- 561 Demary, K.C. & Lewis, S.M. 2007. Male courtship and paternity success in *Photinus greeni*  
562 fireflies. *Evolution* **61**: 431-439.
- 563 Dieckmann, U. & Doebeli, M. 1999. On the origin of species by sympatric speciation. *Nature*  
564 **400**: 354-357.
- 565 Doebeli, M. 1996. A quantitative genetic competition model for sympatric speciation. *J. Evol.*  
566 *Biol.* **9**: 893-909.
- 567 Draper, N.R. & John, J.A. 1988. Response-surface designs for quantitative and qualitative  
568 variables. *Technometrics* **30**: 423-428.
- 569 Eberhard, W.G. 1985. *Sexual selection and Animal Genitalia*. Harvard University Press,  
570 Cambridge MA.
- 571 Emlen, S.T. & Oring, L.W. 1977. Ecology, sexual selection and the evolution of mating  
572 systems. *Science* **197**: 215-223.
- 573 Evans, J.P., Zane, L., Francescato, S. & Pilastro, A. 2003. Directional postcopulatory sexual  
574 selection revealed by artificial insemination. *Nature* **421**: 360-363.

- 575 Fear, K.K. & Price, T. 1998. The adaptive surface in ecology. *Oikos* **82**: 440-448.
- 576 Gilchrist, A.S. & Partridge, L. 1999. A comparison of the genetic basis of wing size divergence  
577 in three parallel body size clines of *Drosophila melanogaster*. *Genetics* **153**: 1775-1787.
- 578 Gowaty, P.A., Steinichen, R., Anderson, W.W. 2002. Mutual interest between the sexes and  
579 reproductive success in *Drosophila pseudoobscura*. *Evolution* **56**: 2537-2540.
- 580 Green, P. J. & Silverman, B. W. 1994. *Nonparametric regression and generalized linear*  
581 *models*. Glasgow: Chapman & Hall, Glasgow.
- 582 Hall, M.D., Lailvaux, S.P., Blows, M.W. & Brooks, R.C. 2010. Sexual conflict and the  
583 maintenance of multivariate genetic variation. *Evolution* **64**: 1697-1703.
- 584 Head, M.L., Hunt, J. & Brooks, R. 2006. Genetic association between male attractiveness and  
585 female differential allocation. *Ecol. Evol.* **2**: 341-344.
- 586 Hedge, S.N. & Krishna, M.S. 1997. Size-assortative mating in *Drosophila malerkotliana*. *Anim.*  
587 *Behav.* **54**: 419-426.
- 588 Hine, E., Chenoweth, S.F. & Blows, M.W. 2004. Multivariate quantitative genetics and the  
589 lek paradox: genetic variance in male sexually selected traits of *Drosophila serrata*  
590 under field conditions. *Evolution* **58**: 2754-2762.
- 591 Hosken, D.J., Taylor, M.L., Hoyle, K., Higgins, S. & Wedell, N. 2008. Attractive males have  
592 greater success in sperm competition. *Current Biol.* **18**: R553-R554.
- 593 Houle, D. 1992. Comparing evolvability and variability of quantitative traits. *Genetics* **130**:  
594 195-204.
- 595 House, C.M. & Simmons, L.W. 2005. The evolution of male genitalia: Patterns of genetic  
596 variation and covariation in the genital sclerites of the dung beetle *Onthophagus taurus*.  
597 *J. Evol. Biol.* **18**: 1281-1292.

- 598 Hunt, J., Blows, M.W., Zajitschek, F., Jennions, M.D. & Brooks, R. 2007b. Reconciling strong  
599 stabilizing selection with the maintenance of genetic variation in a natural population of  
600 black field crickets (*Teleogryllus commodus*). *Genetics* **177**: 875-880.
- 601 Hunt, J., Wolf, J.B. & Moore, A. J. 2007a. The biology of multivariate evolution. *J. Evol. Biol.*  
602 **20**: 1-8.
- 603 Hunt, J., Breuker, C.J., Sadowski, J.A. & Moore, A.J. 2009. Male-male competition, female  
604 mate choice and their interaction: determining total sexual selection. *J. Evol. Biol.* **22**:  
605 13-26.
- 606 Ingleby, F.C., Hosken, D.J., Flowers, K., Hawkes, M.F., Lane, S.M., Rapkin, J., House, C.M.,  
607 Sharma, M.D. & Hunt, J. 2014. Environmental heterogeneity, multivariate sexual  
608 selection and genetic constraints on cuticular hydrocarbons in *Drosophila simulans*. *J.*  
609 *Evol. Biol.* **27**: 700-713.
- 610 Kingsolver, J.G. & Diamond, S.E. 2011. Phenotypic selection in natural populations: What  
611 limits directional selection? *Am. Nat.* **177**:346-357.
- 612 Kopp, A. & True, J.R. 2002. Evolution of male sexual characters in the oriental *Drosophila*  
613 *melanogaster* species group. *Evol. Dev.* **4**: 278-291.
- 614 Koref-Santibanez, S. 2001. Effects of age and experience on mating activity in the sibling  
615 species *Drosophila pavani* and *Drosophila gaucha*. *Behav. Genet.* **31**: 287-297.
- 616 Lande, R. & Arnold, S. J. 1983. The measurement of selection on correlated characters.  
617 *Evolution* **37**: 1210-1226.
- 618 Lynch, M. & Walsh, B. 1998. Genetics and analysis of quantitative traits. Sinauer Associates,  
619 Sunderland, Massachusetts.
- 620 Manning, A. 1967. The control of sexual receptivity in female *Drosophila*. *Anim. Behav.* **15**:  
621 239-250.
- 622 Markow, T.A. & Ricker, J.P. 1992. Male size, developmental stability, and mating success in  
623 natural populations of three *Drosophila* species. *Heredity* **69**: 122-127.

- 624 Markow, T.A., Bustoz, D. & Pitnick, S. 1996. Sexual selection and a secondary sexual  
625 character in two *Drosophila* species. *Anim. Behav.* **52**: 759-766
- 626 Markow, T.A. 2002. Female remating, operational sex ratio, and the arena of sexual  
627 selection in *Drosophila* species. *Evolution* **59**: 1725-1734.
- 628 Mitchell-Olds, T. & Shaw, R. G. 1987. Regression analysis of natural selection: statistical  
629 inference and biological interpretation. *Evolution* **41**: 1149-1161.
- 630 Moore, P.J., Harris, E., Montrose, T., Levin, D. & Moore, A.J. 2004. Constraints on evolution  
631 and postcopulatory sexual selection: Trade-offs among ejaculate characteristics.  
632 *Evolution* **58**: 1773-1780.
- 633 Mousseau, T.A. & Roff, D.A. 1987. Natural selection and the heritability of fitness  
634 components. *Heredity* **59**: 181-197.
- 635 Okada, K., Blount, J.D., Sharma, M.D., Snook, R.R. & Hosken, D.J. 2011. Male attractiveness,  
636 fertility and susceptibility to oxidative stress are influenced by inbreeding in *Drosophila*  
637 *simulans*. *J. Evol. Biol.* **24**: 363-371.
- 638 Okada, K., Katsuki, M., Sharma, M., House, C.M. & Hosken, D.J. 2014. Sexual conflict over  
639 mating in *Gnatoceus cornutus*? Females prefer lovers no fighters. *Proc. R. Soc. B* **281**:  
640 20140281.
- 641 Parker, G.A. 1970. Sperm competition and its evolutionary consequences in the insects. *Biol.*  
642 *Rev.* **45**: 525-567.
- 643 Partridge, L. & Halliday, T. 1984. Mating patterns and mate choice. In *Behavioural Ecology:*  
644 *an evolutionary approach* (J. R. Krebs & N. B. Davies, ed.), pp. 222-250. Blackwell  
645 Scientific, Oxford.
- 646 Phillips, P.C. & Arnold, S.J. 1989. Visualizing multivariate selection. *Evolution* **43**: 1209-1222.
- 647 Pitcher, W., Wolf, J.B., Tregenza, T., Hunt, J. & Dworkin, I. 2014. Evolutionary rates for  
648 multivariate traits: the role of selection and genetic variation. *Phil. Trans. R. Soc. B* **369**:  
649 20130252.

650 Podos, J. 2001. Correlated evolution of morphology and vocal signal structure in Darwin's  
651 finches. *Nature* **409**: 185-188.

652 Polak, M., Starmer, W.T. & Wolf, L.L. 2004. Sexual selection for size and symmetry in a  
653 diversifying secondary sexual character in *Drosophila bipectinata* Duda (Diptera:  
654 Drosophilidae). *Evolution* **58**: 597-607.

655 Polak, M. & Simmons, L.W. 2009. Secondary sexual trait size reveals competitive fertilization  
656 success in *Drosophila bipectinata* Duda. *Behav. Ecol.* **20**: 753-760.

657 Pomiankowski, A. & Moller, A.P. 1995. A resolution of the lek paradox. *Proc. R. Soc. B* **260**:  
658 21-29.

659 Promislow, D.E.L., Smith, E.A. & Pearse, L. 1998. Adult fitness consequences of sexual  
660 selection in *Drosophila melanogaster*. *PNAS* **95**: 10687-10692.

661 Reynolds, R.J, Childers, D.K., Pajewski, N.M. 2010. The distribution and hypothesis testing of  
662 eigenvalues from the canonical analysis of the gamma matrix of quadratic and  
663 correlation selection gradients. *Evolution* **64**: 1076-1085.

664 Robertson, F.W. 1957. Studies in quantitative inheritance XI. Genetic and environmental  
665 correlation between body size and egg production in *Drosophila melanogaster*. *J.*  
666 *Genet.* **55**: 428-443.

667 Robinson, S.P., Kennington, W.J. & Simmons, L.W. 2012. Assortative mating for relatedness  
668 in a large naturally occurring population of *Drosophila melanogaster*. *J. Evol. Biol.* **25**:  
669 716-725.

670 Roff, D.A. & Mousseau, T.A. 1987. Quantitative genetics and fitness: lessons from  
671 *Drosophila*. *Heredity* **58**: 103-118.

672 Rogers, D.W., Denniff, M., Chapman, T., Fowler, K. & Pomiankowski, A. 2008. Male sexual  
673 ornament size is positively associated with reproductive morphology and enhanced  
674 fertility in the stalk-eyed fly *Teleopsis*. *BMC Evolutionary Biology* **8**: 236  
675 doi:10.1186/1471-2148-8-236.

- 676 Rose, E., Paczolt, K.A. & Jones, A.G. 2013. The contribution of premating and postmating  
677 selection episodes to total selection in sex-role-reversed gulf pipefish. *Am. Nat.* **182**:  
678 410-420.
- 679 Roughgarden, J. 1972. Evolution of niche width. *Am. Nat.* **106**: 683-718
- 680 Rowe, L. & Houle, D. 1996. The lek paradox and the capture of genetic variance by condition  
681 dependent traits. *Proc. R. Soc. Lond. B* **263**: 1415-1421.
- 682 Sakai, T. & Ishida, N. 2001. Circadian rhythms of female mating activity governed by clock  
683 genes in *Drosophila*. *PNAS*. **98**: 9221-9225.
- 684 Schluter, D. 2000. The ecology of adaptive radiation. Oxford University Press, Oxford
- 685 Shackleton, M.A., Jennions, M.D., Hunt, J. 2005. Fighting success and attractiveness as  
686 predictors of male mating success in the black field cricket, *Teleogryllus commodus*: the  
687 effectiveness of no-choice tests. *Behav. Ecol. Sociobiol.* **58**: 1-8.
- 688 Sharma, M.D., Tregenza, T. & Hosken, D.J. 2010. Female mate preferences in *Drosophila*  
689 *simulans*: evolution and costs. *J. Evol. Biol.* **23**: 1672-1679.
- 690 Sharma, M.D., Tregenza, T. & Hosken, D.J. 2011. Sex combs, allometry, and asymmetry in  
691 *Drosophila*. *Biol. J. Linn. Soc.* **103**: 913-934.
- 692 Simmons, L.W. & Emlen, D.J. 2006. Evolutionary trade-off between weapons and testes.  
693 *PNAS* **103**: 16346-16351.
- 694 Slatkin, M. 1984. Ecological causes of sexual dimorphism. *Evolution* **38**: 622-630.
- 695 Smith, T.B. 1993. Disruptive selection and the genetic basis of bill size polymorphism in the  
696 African finch *Pyrenestes*. *Nature* **363**: 618-620.
- 697 Snook, R.R., Gidaszewski, N.A., Chapman, T. & Simmons, L.W. 2013. Sexual selection and the  
698 evolution of secondary sexual traits: sex comb evolution in *Drosophila*. *J. Evol. Biol.* **26**:  
699 912-918.
- 700 Spieth, H.T. 1974. Courtship behaviour in *Drosophila*. *Annu. Rev. Entomol.* **19**: 385-405.

701 Stinchcombe, J.R., Agrawal, A.F., Hohenlohe, P.A., Arnold, S.J. & Blows, M.W. 2008.  
702 Estimating non-linear selection gradients using quadratic regression coefficients: double  
703 or nothing? *Evolution* **62**: 2435-2440.

704 Taylor, M.L., Wedell, N. & Hosken, D.J. 2007. The heritability of attractiveness. *Current Biol.*  
705 **17**: R959-R960.

706 Taylor, M.L, Wedell, N. & Hosken, D. J. 2008a. Sexual selection and female fitness in  
707 *Drosophila simulans*. *Behav. Ecol. Sociobiol.* **62**: 721-728.

708 Taylor, M.L., Wigmore, C., Hodgson, D.J., Wedell, N. & Hosken, D.J. 2008b. Multiple mating  
709 increases female fitness in *Drosophila simulans*. *Anim. Behav.* **76**: 963-970.

710 Taylor, P.D. & Williams, G.C. 1982. The lek paradox is not resolved. *Theor. Popul. Biol.* **22**:  
711 392-409.

712 Von Homrigh, A., Higgie, M., McGuigan, K. & Blows, M.W. 2007. The depletion of genetic  
713 variance by sexual selection. *Current Biol.* **17**: 528-532.

714 Wagner, W.E., Beckers, O.M., Tolle, A.E. & Basolo, A. 2012. Tradeoffs limit the evolution of  
715 male traits that are attractive to females. *Proc. R. Soc. B* **279**: 2899-2906.

716 Walling, C.A., Morrissey, M.B., Foerster, K., Clutton-Brock, T.H., Pemberton, J.M. & Kruuk,  
717 L.E. 2014. A multivariate analysis of genetic constraints to life history evolution in a wild  
718 population of red deer. *Genetics* **198**: 1735-1749.

719 Walsh, B. & Blows, M.W. 2009. Abundant genetic variation + strong selection = Multivariate  
720 genetic constraints: A geometric view of adaptation. *Annu. Rev. Ecol. Evol. Syst.* **40**: 41-  
721 59.

722 Welch, A.M., Smith, M.J. & Gerhardt, H.C. 2014. A multivariate analysis of genetic variation  
723 in the advertisement call of the gray treefrog, *Hyla versicolor*. *Evolution* **68**: 1629-1639.



- 724 Wilson, A.J., Réale, D., Clements, M.N., Morrissey, M.M., Postma, E., Walling, C.A., Kruuk,  
725 L.E.B. & Nussey, D.H. 2010. An ecologist's guide to the animal model. *J. Anim. Ecol.* **79**:  
726 13-26.
- 727 Wright, L.I., Tregenza, T. & Hosken, D.J. 2009. Inbreeding, inbreeding depression and  
728 extinction. *Conserv. Genet.* **9**: 833-843.
- 729 Yeniseti, S.C. & Hedge, S.C. 2003. Size-related mating and reproductive success in a  
730 drosophilid: *Phorticella striata*. *Zool. Stud.* **42**: 203-210.
- 731 Zera, A.J. & Harshman, L.G. 2001. The physiology of life history trade-offs in animals. *Annu.*  
732 *Rev. Ecol. Syst.* **32**: 95-126.
- 733
- 734

735 **FIGURE LEGENDS**

736 **Figure 1:** Morphological measures of male *Drosophila simulans* (i) wing and (ii) sex comb. The length  
737 of the wing was measured as the distance between points A and B. Three components of the sex  
738 comb were measured; comb length (CL; A), tooth length (TL) which was measured as the average  
739 length of the 1<sup>st</sup> (B), 3<sup>rd</sup> and 5<sup>th</sup> tooth and comb tooth number (TN).

740

741 **Figure 2.** Thin-plate spline visualizations (A; perspective view and B; contour view) of the two major  
742 axes of nonlinear selection ( $\mathbf{m}_1$  and  $\mathbf{m}_4$ ) on the fitness surface when males courted non-virgin  
743 females. In the contour view, yellow to white colouration represents regions of highest fitness,  
744 whereas red colouration represents regions of lowest fitness. Individual data points are provided as  
745 black circles on the surface.

746

747 **Figure 3.** Thin-plate spline visualizations (A; perspective view and B; contour view) of the two major  
748 axes of nonlinear selection ( $\mathbf{m}_1$  and  $\mathbf{m}_2$ ) on the fitness surface when males mated in the offensive  
749 role (P2). In the contour view, yellow to white colouration represents regions of highest fitness,  
750 whereas red colouration represents regions of lowest fitness. Individual data points are provided as  
751 black circles on the surface.

752

753 **Table 1.** Phenotypic means and estimates of heritability ( $h^2$ ) and maternal effect ( $m^2$ ) for  
 754 male body size and sex comb components, (N sires = 110, N offspring = 1449). Estimates are  
 755 from the multivariate animal model (see text for details).

Trait (unit)	Mean	$h^2$ (SE)	$m^2$ (SE)
Comb length ( $\mu$ m)	58.23 $\pm$ 0.12	0.61 $\pm$ 0.08	0.07 $\pm$ 0.02
Tooth length ( $\mu$ m)	39.23 $\pm$ 0.05	0.26 $\pm$ 0.11	0.27 $\pm$ 0.05
Tooth Number	9.90 $\pm$ 0.02	0.53 $\pm$ 0.06	NA
Wing Length (mm)	1154.08 $\pm$ 1.12	0.45 $\pm$ 0.06	0.40 $\pm$ 0.06

756

757 **Table 2.** Additive genetic correlations above the diagonal and phenotypic correlations below  
 758 the diagonal for sex comb components; comb length (CL), tooth length (TL), tooth number  
 759 (TN) and wing length (WL). Significant genetic ( $|r_G| > 2SEs$ ) and phenotypic correlations are  
 760 in bold (after bonferroni correction).

	CL	TL	TN	WL
Comb length (CL)		<b>0.31 <math>\pm</math> 0.15</b>	<b>0.89 <math>\pm</math> 0.03</b>	<b>0.64 <math>\pm</math> 0.10</b>
Tooth length (TL)	<b>0.27 <math>\pm</math> 0.02</b>		<b>0.89 <math>\pm</math> 0.03</b>	0.27 $\pm$ 0.23
Tooth Number(TN)	<b>0.84 <math>\pm</math> 0.01</b>	0.01 $\pm$ 0.03		<b>0.70 <math>\pm</math> 0.11</b>
Wing Length (WL)	<b>0.45 <math>\pm</math> 0.02</b>	<b>0.44 <math>\pm</math> 0.02</b>	<b>0.23 <math>\pm</math> 0.02</b>	

761

762 **Table 3.** The vector of standardized linear selection gradients ( $\beta$ ) and the matrix of  
 763 standardized nonlinear gradients ( $\gamma^\dagger$ ) for sex comb morphological traits in male *D. simulans*  
 764 during pre-copulatory sexual selection when a male courted and/or mated a (A) virgin  
 765 female or (B) non-virgin female and during post-copulatory selection in a competitive role  
 766 when a male mated in a (C) defensive role (i.e. P1) or (D) offensive role (i.e. P2).

	$\beta$	$\gamma$			
		CL	TL	TN	WL
A. Standardized selection gradients when a male courted and/or mated a virgin female					
CL	0.020	0.264			
TL	0.019	-0.051	-0.012		
TN	-0.029	-0.161	-0.041	-0.012	
WL	-0.004	-0.108	0.027	0.196*	-0.004
B. Standardized selection gradients when a male courted and/or mated a non-virgin female					
CL	-.172	0.104			
TL	-.051	.031	-0.300*		
TN	.010	-.249	-.022	0.422*	
WL	.121	.069	.228**	-.017	0.270
C. Standardized selection gradients when a male mated in a defensive role (P1)					
CL	-0.108	-0.030			
TL	-0.010	0.099	-0.106		
TN	0.127	-0.118	0.041	0.228	
WL	-0.119	-0.018	-0.073	-0.121	0.234*
D. Standardized selection gradients when a male mated in an offensive role (P2)					
CL	-0.055	0.806**			
TL	0.038	0.025	-0.154		
TN	0.094	-0.509*	-0.026	0.25	
WL	-0.047	-0.282*	0.119	0.157	0.13

767  
 768 CL, comb length; TL, tooth length; TN, tooth number; WL, wing length. Randomization tests: \* $P < 0.05$ , \*\* $P <$   
 769  $0.01$ , \*\*\* $P < 0.001$

770 † Nonlinear selection gradients include quadratic ( $z_{ii}^2$ ) gradients on the diagonal and  
 771 correlational ( $z_i z_j$ ) gradients below the diagonal.

772  
 773  
 774

775 **Table 4.** Linear ( $\theta_i$ ) and nonlinear ( $\lambda_i$ , the eigenvalue) selection gradients and the M matrix<sup>†</sup>  
 776 of eigenvectors ( $m_i$ ) from the canonical analysis of  $\gamma$  for (A) virgin mating success (B) non-  
 777 virgin mating success (C) P1 experiment and (D) P2 experiment.

	$\theta_i$	$\lambda_i$	M			
			CL	TL	TN	WL
A. Canonical analysis of virgin mating success						
$m_1$	0.029	0.286	0.696	0.428	-0.575	-0.038
$m_2$	0.015	-0.010	0.629	-0.148	0.623	0.441
$m_3$	-0.020	-0.139	-0.021	-0.629	-0.530	0.568
$m_4$	-0.011	-0.249	0.345	-0.632	-0.007	-0.693
B. Canonical analysis of non-virgin mating success						
$m_1$	0.086	0.565	<b>-0.485</b>	-0.059	<b>0.869</b>	-0.074
$m_2$	0.076	0.004	-0.674	-0.379	-0.440	-0.456
$m_3$	-0.132	-0.099	0.555	-0.573	0.223	-0.560
$m_4$	-0.128	-0.515**	0.047	<b>0.724</b>	0.016	<b>-0.687</b>
C. Canonical analysis of P1						
$m_1$	0.184	0.374	-0.145	0.133	0.715	-0.671
$m_2$	0.049	0.173	-0.489	-0.258	0.542	0.633
$m_3$	-0.070	-0.022	-0.614	-0.575	-0.379	-0.385
$m_4$	-0.023	-0.200	0.602	-0.764	0.227	-0.041
D. Canonical analysis of P2						
$m_1$	0.078	1.204*	<b>-0.824</b>	0.0003	<b>0.487</b>	0.288
$m_2$	0.052	0.096*	-0.182	<b>-0.444</b>	0.197	<b>-0.855</b>
$m_3$	0.047	-0.052	0.527	0.008	0.846	0.078
$m_4$	-0.068	-0.216	0.095	-0.896	-0.090	0.424

778 Randomization tests: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$

779 † Values in bold, contributed most to that eigenvector ( $m_i$ ).

780

781

782

783

784

785

786