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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 (β) (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°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 5th 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 1st, 3rd and 5th 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 χ^2_1 and χ^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 (β_v),
270 previously mated (β_m) or the focal male mated in a defensive role (β_{p1}) or an offensive role
271 (β_{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 (γ_v), previously mated (γ_m)
274 or the focal male mated in a defensive role (γ_{p1}) or an offensive role (γ_{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 (θ_i) and nonlinear selection that are described by eigenvalues (λ_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 γ_{m_1} and γ_{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 (λ) 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; ~ 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. β - 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. γ coefficients that describe the curvature of nonlinear selection on individual
330 traits); (a) stabilizing where γ coefficients are negative and individuals with intermediate
331 trait values have highest fitness, (b) disruptive where γ 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 γ 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 γ) selection on tooth length (TL), disruptive (positive γ)
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 γ 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 γ) selection which we visualized with \mathbf{m}_1 that had
353 the largest, albeit non-significant disruptive (positive γ) 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 γ) on wing length (Table 3C).
364 Canonical rotation of the γ 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 γ) 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 γ 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 (λ) 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

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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 1st (B), 3rd and 5th 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 (μ m)	58.23 \pm 0.12	0.61 \pm 0.08	0.07 \pm 0.02
Tooth length (μ 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

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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)		0.31 \pm 0.15	0.89 \pm 0.03	0.64 \pm 0.10
Tooth length (TL)	0.27 \pm 0.02		0.89 \pm 0.03	0.27 \pm 0.23
Tooth Number(TN)	0.84 \pm 0.01	0.01 \pm 0.03		0.70 \pm 0.11
Wing Length (WL)	0.45 \pm 0.02	0.44 \pm 0.02	0.23 \pm 0.02	

761

762 **Table 3.** The vector of standardized linear selection gradients (β) and the matrix of
 763 standardized nonlinear gradients (γ^\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).

	β	γ			
		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.

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775 **Table 4.** Linear (θ_i) and nonlinear (λ_i , the eigenvalue) selection gradients and the M matrix[†]
 776 of eigenvectors (m_i) from the canonical analysis of γ for (A) virgin mating success (B) non-
 777 virgin mating success (C) P1 experiment and (D) P2 experiment.

	θ_i	λ_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	-0.485	-0.059	0.869	-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	0.724	0.016	-0.687
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*	-0.824	0.0003	0.487	0.288
m_2	0.052	0.096*	-0.182	-0.444	0.197	-0.855
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).

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