

1 **Sexual selection and assortative mating: an experimental** 2 **test**

3 ALLAN DEBELLE¹, MICHAEL G. RITCHIE² AND RHONDA R. SNOOK³

4 ¹ *School of Life Sciences, University of Sussex, JMS Building, Brighton, BN1 9QG, UK*

5 ² *School of Biology, University of St Andrews, Dyers Brae House, St Andrews, Fife, KY16 9TH, UK*

6 ³ *Animal and Plant Sciences, University of Sheffield, Alfred Denny Building, Sheffield, S10 2TN, UK*

7 Correspondence:

8 A. Debelle

9 mailing address: JMS Building, University of Sussex, BN1 9QG Brighton, United Kingdom

10 e-mail address: allandebelle@gmail.com

11 phone number : +44 1273 877247

12 *Running title: Sexual selection and assortative mating*

13

14 **Abstract**

15 Mate choice and mate competition can both influence the evolution of sexual isolation
16 between populations. Assortative mating may arise if traits and preferences diverge in step,
17 and, alternatively, mate competition may counteract mating preferences and decrease
18 assortative mating. Here we examine potential assortative mating between populations of
19 *Drosophila pseudoobscura* that have experimentally evolved under either increased
20 ('polyandry') or decreased ('monogamy') sexual selection intensity for 100 generations.
21 These populations have evolved differences in numerous traits, including a male signal and
22 female preference traits. We use a 2 males: 1 female design, allowing both mate choice and
23 competition to influence mating outcomes, to test for assortative mating between our
24 populations. Mating latency shows subtle effects of male and female interactions, with
25 females from the monogamous populations appearing reluctant to mate with males from
26 the polyandrous populations. However, males from the polyandrous populations have a
27 significantly higher probability of mating regardless of the female's population. Our results
28 suggest that if populations differ in the intensity of sexual selection, effects on mate
29 competition may overcome mate choice.

30 *Keywords:* *Drosophila*; experimental evolution; mate competition; female preference; sexual
31 conflict; sexual isolation; speciation.

32

33 **Introduction**

34 Sexual selection is often thought to be an important force in the origin of sexual isolation
35 between populations, although this is subject to much debate (Mayr, 1963; Coyne & Orr,
36 2004; Rundle & Nosil, 2005; Sobel *et al.*, 2010; ITN Marie Curie Speciation, 2011). Intersexual
37 selection may facilitate sexual isolation because coevolution of mating signals and
38 associated preferences may lead to divergence between populations. This divergence would
39 then have the potential to generate assortative mating (i.e. a higher likelihood of mating
40 with an individual from the same population) if populations come into secondary contact
41 (Lande, 1981; Kirkpatrick, 1982; Price, 1998; Kirkpatrick & Ravigné, 2002; Uyeda *et al.*, 2009).
42 While divergence in preferences between populations is often matched by signal divergence
43 (Rodríguez *et al.*, 2013), strong preferences may theoretically decrease isolation if
44 preference genes introgress between species (Servedio & Bürger, 2014). Likewise, strong
45 sexual selection can influence mate competition, which may facilitate population
46 divergence, for example by reinforcing the action of mating preference on a given mating
47 signal. Strong mate competition may also constrain the expression of mating preferences by
48 reducing the opportunities to mate with preferred, but less competitive, mates (Wong &
49 Candolin, 2005; Hunt *et al.*, 2009). Thus, it is difficult to predict the overall influence of
50 sexual selection on sexual isolation.

51 Experimental sexual selection directly manipulates a species' mating system to observe, in
52 real time, the evolutionary consequences on sexual traits, mating patterns, and the
53 evolution of reproductive isolation (Holland & Rice, 1999; Martin & Hosken, 2003, 2004;
54 Wigby & Chapman, 2004, 2006; Crudgington *et al.*, 2005, 2010; Rundle & Chenoweth, 2005;
55 Snook *et al.*, 2005; Rundle *et al.*, 2006; Bacigalupe *et al.*, 2007, 2008). We have implemented

56 experimental sexual selection in *Drosophila pseudoobscura* by either enforcing monogamy
57 (1 male:1 female) or promoting polyandry (1 female:6 males) and found a variety of
58 evolutionary responses. For example, divergence between monogamous and polyandrous
59 populations in an important male courtship signal has occurred, with males from
60 polyandrous populations singing a faster courtship song compared to males from
61 monogamous populations (Snook *et al.*, 2005). There is also evidence for coevolution of
62 female preference for song; in playback experiments, females from the polyandrous
63 populations prefer polyandrous-like male song whereas females from monogamous
64 populations preferred monogamous-like song (Debelle *et al.*, 2014). Other traits that are
65 implicated in sexual selection, such as cuticular hydrocarbon profiles, have also diverged
66 between the sexual selection treatments (Hunt *et al.*, 2012).

67 Here we conduct what is referred to as a “choice” experiment in which mating trials involve
68 2 males: 1 female (Dougherty & Shuker, 2014) from replicate polyandrous and monogamous
69 populations to examine how the evolutionary history of these populations influences mating
70 patterns. This type of design was chosen as it usually results in a stronger expression of
71 mating preferences compared to no-choice designs (Dougherty & Shuker, 2014). Moreover,
72 such a design allows mating patterns to be influenced by both male-male and male-female
73 interactions, and is considered to be the most appropriate way to test for sexual isolation
74 between populations (Coyne *et al.*, 2005).

75 If female choice predominates mating interactions, we predict to observe a significant effect
76 of both male and female evolutionary history on mating patterns. These effects could
77 potentially result in assortative mating occurring within each replicate population of each
78 sexual selection treatment. However, we have previously found that there was little within-

79 treatment (i.e. between-replicate) variation in patterns of song-preference divergence
80 between the sexual selection treatments (Debelle et al. 2014), suggesting that sexual
81 selection treatment consistently influences the direction of signal-preference coevolution in
82 our populations (and other traits that may have diverged between treatments). We thus
83 predict that if female choice predominates mating interactions, then assortative mating by
84 treatment will occur (i.e. polyandrous females with polyandrous males and monogamous
85 females with monogamous males).

86 Alternatively, male-male competition could largely predominate mating interactions,
87 resulting in finding no effect of female evolutionary history on mating patterns. Males from
88 polyandrous populations present a higher courtship frequency (Crudgington *et al.*, 2010), a
89 trait that could be implicated in male-male competition (e.g. Shine et al. 2005; Kim and
90 Velando 2014). Additionally, male-male interactions are common between rival males of this
91 species placed in a choice design (e.g. chasing, courtship interruption, physical threats and
92 attacks; see Figure S1 in Appendix 1). We would therefore further predict that polyandrous
93 males, who continuously experience strong male-male competition, will win more matings
94 than monogamous males, regardless of female evolutionary history.

95 We test these alternative predictions by examining the mating patterns between the
96 experimental populations after 100 generations of experimental evolution. To standardise
97 female response against selection males, we also conduct the same experiment using
98 females from the ancestral population. Because these females do not discriminate between
99 male songs from the polyandrous and monogamous treatments (Debelle *et al.*, 2014), we
100 expect to observe random mating patterns. However, if male-male competition influences
101 mating outcome, then we expect ancestral females to show the same mating outcome

102 patterns as that of selection line females. We test for body size differences between our
103 populations and treatments, and include it as a covariate in our analyses, because body size
104 is frequently targeted by sexual selection and has a large influence on male mating success
105 (Blanckenhorn, 2000). In *Drosophila* species, larger males win more aggressive encounters
106 with other males (Partridge & Farquhar 1983; Partridge *et al.*, 1987a), deliver more courtship
107 (Partridge *et al.*, 1987a,b) and mate faster (Partridge & Farquhar, 1983). We discuss how
108 sexual selection influences mating outcome and the implications of these results for
109 population divergence and speciation.

110 **Material and Methods**

111 Sexual selection treatments

112 The selection lines are described in detail in Crudgington *et al.*, (2005). Briefly, an ancestral
113 wild-caught population of the naturally polyandrous species *Drosophila pseudoobscura* from
114 Tucson (Arizona, USA) was used to establish the selection lines. Four replicate populations
115 (replicate 1, 2, 3 and 4) of two different sexual selection treatments were established. Adult
116 sex-ratio in vials is manipulated by either confining one female with a single male
117 ('monogamy' treatment; **M**) or one female with 6 males ('elevated polyandry' treatment; **E**)
118 in vials. Henceforth, reference to E or M refers to the experimental sexual selection
119 treatment flies derive from. Effective population sizes are equalized between the treatments
120 (Snook *et al.*, 2009). At each generation, offspring are collected and pooled together for
121 each replicate population, and a random sample used to constitute the next generation in
122 the appropriate sex-ratios, thus proportionally reflecting the differential offspring
123 production across families. In total, 8 selection lines (M1, M2, M3, M4 and E1, E2, E3, E4) are

124 maintained, in standard food vials (2.5mm x 80mm) and with a generation time of 28 days.
125 The ancestral population (**A**) is also maintained, in bottles (57 mm x 132 mm) with an equal
126 sex-ratio of adult flies. All populations are kept at 22°C on a 12L:12D cycle, with standard
127 food media and added live yeast.

128 Experimental flies

129 To generate the experimental flies, 50 reproductively mature adults (25 males and 25
130 females) of each treatment (E and M) and replicate (1, 2, 3 and 4) were used as parents and
131 kept in mass-cultures, providing a common social context for parents of both sexual
132 selection treatments. The resulting larvae were raised in controlled density vials (100 first
133 instar larvae per food vial). Flies from these vials were collected and sexed on the day of
134 hatching using CO₂ anaesthetization. Virgin males and females were kept separate in
135 yeasted food vials with a maximum of 20 individuals per vial, and used in mating
136 experiments once they had reached sexual maturity (four to six days old; Snook & Markow,
137 2001). Experimental females from the ancestral population were also generated using the
138 same method.

139 To identify the population of origin of males, we clipped a small corner off the right lower
140 wing margin of half of the males, under CO₂ anaesthetization, two days before the
141 experiment. Wing clipping has no effect on male mating success in *D. pseudoobscura* (e.g.
142 Dodd, 1989) but, as a control, half the males from each treatment were clipped. The males
143 were then stored in vials of 12 individuals of the same population until the experiment.

144 Assortative mating design

145 We tested for assortative mating between the different populations by placing one female (E
146 or M) in a food vial with two males (one E and one M). Competing males always came from
147 the same replicate (e.g., one E1 and one M1 male, or one E3 and one M3 male). All the
148 female-male combinations between populations were tested: we crossed the 8 female
149 populations (E1-4; M1-4) with the 4 possible pairs of males (E1 and M1; E2 and M2; E3 and
150 M3; E4 and M4), for a total of 32 combinations. For each combination, the minimum sample
151 size was 40 females (N=1280 trials in total). Reproductively mature males were loaded first
152 into food vials, followed by reproductively mature females, and each vial was observed until
153 mating occurred, or for 20 minutes. If mating occurred, then the identity of the mating male
154 was recorded (E or M). If no mating occurred, then the trial was discarded (N=116 trials).
155 Both mating latency and mating outcome (i.e. the identity of the winning male: E or M) were
156 measured. Mating latency, defined here as the time between introducing the female into
157 the vial until the start of mating, is an important component of *Drosophila* male competitive
158 success and female preference (e.g. Bacigalupe *et al.*, 2007). Mating outcome was used to
159 predict the probability of an E or an M male winning with the different female populations.
160 The same design was used with females from the ancestral (A) population (one A female
161 with one E and one M male).

162 To examine a potential role of body size on mating patterns in our experiment, the length of
163 wing vein IV of each individual (male and female) was measured as an estimate of body size
164 (Crudginton *et al.*, 2005) and included in the statistical analyses. Wings were mounted in a
165 30% glycerol-70% ethanol medium, photographs taken using a Motic camera and Motic
166 Images Plus 2.0 software (Motic Asia, Hong Kong), and wing vein length measured with
167 ImageJ (v. 1.44e (Abramoff *et al.*, 2004). To control for potential temperature effects on

168 courtship behaviors (O'Dell, 2003), we measured temperature during trials using a Testo
169 735-1 thermometer (Testo Limited, United Kingdom), and subsequently used temperature
170 as a covariate in the analyses (mean temperature during the time of each trial). The
171 experiment was performed in 2-hour sessions, when the incubator lights came on, to mimic
172 the *D. pseudoobscura* activity pattern (Noor, 1998). The different crosses were randomly
173 assigned across the different days. The generations of the sexual selection treatments used
174 were: replicate 1= 102, 105 and 107; replicate 2= 101, 104 and 106; replicate 3= 100, 103
175 and 105; replicate 4= 98, 101 and 103. The generation of the ancestral population used was
176 124.

177 Predictions and statistical analyses

178 Our main objective was to distinguish between three alternative outcomes: assortative
179 mating could occur between replicate populations (i.e. a polyandrous male is more likely to
180 mate with a polyandrous female from its own replicate population), or between sexual
181 selection treatments (i.e. a polyandrous male is more likely to mate with a polyandrous
182 female regardless of their respective replicate population), or not occur at all (i.e. matings
183 could be mostly won by polyandrous males). We expect the non-coevolved ancestral
184 females to mate randomly, given that at least for song, they exhibit no distinct preference.
185 However this population is also subject to sexual selection, so predicting mating outcome is
186 more difficult than in the polyandrous and monogamous populations. Thus, results of
187 mating patterns for the females from the ancestral population were analysed separately.

188 Mating latency is used to measure female preference in *Drosophila*, with shorter latencies
189 usually implying a more preferred mate (see references in Bacigalupe *et al.*, 2007; Debelle *et*

190 *al.*, 2014). A simple prediction then would be that mating outcome patterns are reflected in
191 the mating latency patterns. However, this prediction is complicated by the potential action
192 of sexual conflict, that could lead to polyandrous (and/or bigger) females exhibiting more
193 resistance to mating, thereby increasing mating latency (Arnqvist & Rowe, 2005), and male-
194 male competition, that could also affect mating latency (Bretman *et al.*, 2009).

195 To test these predictions, we scored the winners of the mating encounters and measured
196 mating latency. For both mating outcome and latency, we also included 'type of cross' in the
197 model to test whether populations experiencing sexual conflict/sexual selection show
198 greater measures of sexual isolation (for review, see Gavrillets, 2014). The crosses involving a
199 male from the same population as the female (i.e. "coevolved"; e.g., an E1 female with an E1
200 and a M1 male or M1 female with an E1 and a M1 male) were considered as '*within*
201 *population*' crosses and all the other combinations were '*between populations*' crosses (e.g.,
202 an E1 female with an E2 and a M2 male). The category '*within population*' was further
203 divided into two subcategories, '*within E population*' when the E male and the E female were
204 from the same population (e.g., E1 female, E1 male, M1 male) and '*within M population*'
205 when the M male and the M female were from the same population (e.g., M2 female, M2
206 male, E2 male).

207 To examine any effect of male and female body size on male mating success, we first tested
208 for differences in absolute body size of males and females between the sexual selection
209 treatments. These were tested both within replicate (e.g., E1 males vs. M1 males, or E3
210 females vs. M3 females) and with all replicates combined (E males vs. M males, and E
211 females vs. M females), using Wilcoxon rank sum tests as size was not normally distributed.
212 P-values were adjusted using the Holm procedure for multiple comparisons (Holm, 2012).

213 Average body size differed significantly between the treatments, with both E males and
214 females being overall larger than their M counterparts, either taking all replicates into
215 account or across most replicates (Table 1). To disentangle the effect of body size on mating
216 patterns from the action of other traits that responded to sexual selection manipulation, we
217 ran statistical models analysing both mating outcome and latency either with absolute male
218 and female body size as covariates (presented within the text) or without (Appendix S1).

219 We analysed mating outcome (whether E or M males win) using a generalized linear mixed
220 model with a binomial distribution. We specifically investigated what variables influence the
221 probability of the two possible mating events (*'E male wins'* versus *'M male wins'*; *'E male*
222 *wins'* was used as the reference event). Female treatment, male replicate, E and M male
223 size, E and M male relative size difference, female size, the temperature and the type of
224 cross were included as fixed effects in the model. The interaction between female treatment
225 and male replicate was also tested. Male and female replicate were nested within their
226 respective sexual selection treatment. This analysis models the probability of an E male
227 winning. We ran the same model for A females, with the exception that 'female treatment',
228 'type of cross', and 'female replicate' were obviously not included as effects in the model.

229 To test the mating latency response, we first log-transformed mating latency and then
230 analysed it using a linear mixed model with a Gaussian distribution. Female treatment ('E'
231 was used as the reference level), winning male treatment ('E' was used as the reference
232 level), absolute body sizes of both males and of the female, temperature and type of cross
233 (*'between populations'* was used as the reference level) were included as fixed effects. In
234 addition to absolute male and female body sizes, the relative body size difference between
235 the E and the M male was also included in the model as a fixed effect (a factor with two

236 levels: 'E larger than M' or 'E smaller than M' than M; 'E smaller than M' was used as the
237 reference level). The interactions between winning male and female treatment (to test for
238 assortative mating within sexual selection treatment), and between type of cross and
239 winning male treatment (to test for a difference between the treatments in assortative
240 mating within population), were also tested. Male and female replicate were nested within
241 their respective sexual selection treatment, to account for variation among the replicated
242 populations (Garland & Rose, 2009). This analysis models the speed it takes males from the
243 different selection lines to mate with females of the different selection lines. We ran the
244 same model for A females, with the exception that 'female treatment' and 'type of cross'
245 could not be included as main effects and 'female replicate' could not be included as a
246 random effect in the model.

247 In all the mixed models described above, the significance of fixed effects was tested using
248 likelihood ratio tests. Normality and homoscedasticity of the residuals were checked
249 graphically. Model estimates were used in figures, adjusted for the effects of all the other
250 variables not included in the figure. All statistical analyses were performed in R (R
251 Development Core Team 2005). The *lme4* library was used for mixed-models (Bates &
252 Sarkar, 2007), and the *glht* function in the *multcomp* library was used for post-hoc analysis
253 of the mixed-model results (Hothorn *et al.*, 2008). Raw mating outcome and mating latency
254 data are also shown in Appendix S1 (see Fig. S2 and S3).

255 **Results**

256 There is no effect of the type of cross (that is, whether the female and the mating male are
257 from the same population or not) on either mating outcome or mating latency. Neither E nor

258 M males are faster to mate or more likely to mate when they are in the presence of a female
259 from their own population (Table 2). Instead, E males win significantly more matings with all
260 females and mate overall at least as quickly as M males.

261 In the case of mating outcome, E males win more matings than M males regardless of
262 female treatment (for E females: E males = 377, M males = 146, $\chi^2_1 = 98.83$, $P < 0.001$; for M
263 females : E males = 360, M males = 136, $\chi^2_1 = 101.16$, $P < 0.001$). The mixed-model approach
264 confirms this pattern, finding a much higher mating success of E males in comparison to M
265 males (i.e., E males have a mating probability greater than 0.5 regardless of their replicate
266 population; Fig. 1a; Table 2), and no significant effect of female treatment on the mating
267 outcome (Table 2). Neither the relative size difference between the males, nor male
268 absolute body sizes, have a significant effect on mating outcome (Table 2), meaning that the
269 higher mating probability of E males is not the result of their larger size. In contrast to males,
270 female size significantly influences the probability of an E male winning: E males are less
271 successful with larger females (Table 2; Fig. 2a). Running the model without male and female
272 body size shows the same pattern of treatment effect on mating outcome (see Table S1 of
273 Appendix S1).

274 For mating latency, there is a significant interaction between winning male treatment and
275 female treatment (Fig. 1b; Table 2). E females mate faster with E males when E males win,
276 and mate slower with M males when M males win. In contrast, M females mate as quickly
277 with M males as they do with E males. That is, when M males win, it takes them longer to
278 initiate copulation with E females than with M females. Male body size has a significant

279 effect on mating latency. The relative size difference between the E and the M male
280 influences mating latency, with mating latency being shorter when the E male is larger than
281 the M male (Fig. 2b ; Table 2). M male absolute size is negatively associated with mating
282 latency; that is, as M male size increases, males start mating with females earlier (Fig. 2c ;
283 Table 2). Overall, these results suggest that larger males, particularly M males, start mating
284 earlier than smaller males. In contrast, female size has no significant effect on mating
285 latency (Table 2). Running the model without male and female body size shows the same
286 direction of treatment effects on mating latency (see Table S1 of Appendix S1).

287 Mating trials with ancestral females show that E males also have a higher probability of
288 winning matings than M males (Fig. 3a; Table 3) and that M males take longer than E males
289 to achieve matings with ancestral females (Fig. 3b; Table 3). Ancestral female body size have
290 no effect on mating outcome or latency, likely because these females exhibit less variation in
291 body size than selection lines females (Levene's test: $F_1=39.57$, $P<0.001$). Running models
292 without body size shows the same pattern of treatment effects (see Table S2 of Appendix
293 S1).

294 **Discussion**

295 We used an experimental approach to understand how changes in sexual selection intensity
296 can influence assortative mating in a system in which we have quantified changes in traits
297 related to both intra- and inter- sexual selection. We find that assortative mating is not
298 observed, either between treatments, or within sexual selection treatments. Instead, males
299 from polyandrous populations, who evolved under mate competition, benefit from a much

300 higher mating success, winning about 4 times more often than M males, regardless of
301 female selection history.

302 What might cause these mating patterns? Predictions of assortative mating largely derive
303 from an expectation of greater male-female coevolution under strong sexual selection
304 (Lande, 1981; Kirkpatrick, 1982; Price, 1998; Kirkpatrick & Ravigné, 2002; Uyeda *et al.*, 2009).
305 There is evidence in our populations for coevolved song and female song preferences
306 (Debelle *et al.*, 2014) which may generate assortative mating. Song in this species is used as
307 a species-specific signal, suggesting it is important in determining mating success and in
308 reproductive isolation (Williams *et al.*, 2001). We have measured a variety of other male
309 traits in these populations that are thought to potentially influence pre-mating sexual
310 selection and found divergent responses between the treatments in some (cuticular
311 hydrocarbon profiles; courtship frequency; Hunt *et al.*, 2012; Crudgington *et al.*, 2010) but
312 not all (sex comb tooth number; Snook *et al.*, 2013) traits. The extent to which female
313 preferences has changed for non-song traits have not been measured.

314 However, because we find that E males equally win with all types of females, it seems
315 unlikely that male-female coevolution can explain our patterns of mating success. Yet, this
316 does not mean that coevolution between the sexes has not occurred. Patterns of mating
317 latency may provide some evidence of coevolution. Most interestingly, while E males mate
318 faster than M males with females from populations experiencing polyandry (E and A), this
319 difference is not seen with M females. When M males do win matings with M females, this is
320 achieved faster than when M males win matings with E females. Therefore M males do seem
321 to benefit from a relative mating advantage with M, and only M, females. This advantage
322 perhaps reflects M female mating preference for M male courtship song (Debelle *et al.*,

323 2014). However, E males mate as fast as M males with M females, implying that E males can
324 override this female preference.

325 Varying the intensity of sexual selection will also have targeted traits that evolve under
326 male-male competition. Mate competition can override female mating preference by
327 reducing the ability of females from detecting, evaluating and/or mating with preferred
328 mates (Wong & Candolin, 2005), for example by intensifying courtship (i.e. decreasing
329 courtship latency or increasing courtship rate) to maximise their mating success. Courtship
330 rate commonly increases in a competitive context, as shown in sticklebacks (Shine *et al.*,
331 2005), garter snakes (Kim & Velando, 2014) or fiddler crabs (Milner, 2012). Other
332 experimental evolution studies have found that males from monogamous populations
333 evolve reduced competitive mating success (Kawecki *et al.*, 2012). The fact that E males
334 initiate courtship faster and court more frequently than M males (Snook *et al.*, 2005;
335 Crudginton *et al.*, 2010) may then influence the ability of females to detect and evaluate
336 between males (Shaw & Lugo, 2001). Another trait potentially associated with competitive
337 mating success is body size (Blanckenhorn, 2000). We found that the relative size difference
338 between the E and the M male influenced mating latency, such that mating latency was
339 shorter when the E male was larger than the M male. Larger M males also experienced a
340 mating benefit; we found that as M male size increased, mating latency decreased.
341 Generally then larger body size, particularly of E males, may influence mating patterns. This
342 result has been shown in other *Drosophila* species where larger males mate faster due to
343 their increased locomotor activity (Partridge *et al.*, 1987b; Long & Rice, 2007).

344 While male body size was important in determining mating latency, neither absolute male
345 body size nor the relative difference in male body sizes influenced mating outcome. The role

346 of male body size in mediating mating success in *D. pseudoobscura* is unclear; in some
347 studies, larger males are more likely to be paired with females than smaller males (Partridge
348 *et al.*, 1987a) but in other studies this body size advantage was not observed (Markow, 1988;
349 Markow & Ricker, 1992). Instead of a male effect, we found that female body size had an
350 influence on what male won, with E males being more likely to win with smaller compared
351 to larger females. This suggests sexual conflict over mating decisions (Clutton-Brock &
352 Parker, 1995). Sexual conflict occurs in our polyandrous populations and is eliminated in our
353 monogamous populations (Crudgington *et al.*, 2005, 2010). Increased male mating
354 persistence can evolve under sexual conflict (Arnqvist & Rowe, 2005) and E males are more
355 persistent than M males (Crudgington *et al.*, 2010). Smaller, less resistant M females, may be
356 less able to resist such males. We did not observe an overall effect of female treatment on
357 mating latency or outcome, but the mating latency and size effects on mating success
358 described here suggest that subtle interactions influence the outcome of the mating trials.

359 Male-male competition and female preference are not mutually exclusive forms of selection.
360 For example, rapid, vigorous courtship may be selected for when mate competition is high,
361 but will also be indirectly targeted by female preferences. Females are likely to obtain
362 indirect benefits from mating with males who can out-compete other males. In this sense
363 separating sources of selection into intra- versus intersexual selection is simplistic. However,
364 the fact that we see polyandrous males succeeding in mating trials, despite some evidence
365 for coevolution between the sexes in the experiment, suggests that greater selection on
366 male competitive courtship ability in the polyandrous populations has overwhelmed any
367 selection likely to cause assortative mating between populations from the treatments (or
368 between replicate populations within the polyandry treatment). Parker & Partridge (1998)

369 suggested that if sexual conflict over mating outcome was strong, competitive males could
370 act as a force for gene flow and inhibit speciation (alternatively, if female choice
371 predominates, sexual conflict could increase speciation by assortative mating). Our results
372 are more compatible with the “males ahead” outcome of this model, with polyandrous
373 males, evolving under strong sexual selection, winning out in mating competitions with
374 males and females from different evolutionary histories. Overall, this suggests that sexual
375 selection has the potential to inhibit, as well as to increase, assortative mating and
376 speciation (Servedio, 2004).

377 **Acknowledgements**

378 We are grateful to undergraduate students Hugh Smith, Hanna Bennett, and David John, and
379 to lab members Helen Crudginton, Jessica Edwards, Sarah Fahle, Sarah Fellows and Nelly
380 Gidaszewski for their assistance with data collection, and to Alexandre Courtiol for
381 discussions about statistical analyses. This work was funded by the Marie Curie Initial
382 Training Network ‘Understanding the evolutionary origin of biological diversity’ (ITN-2008-
383 213780 SPECIATION), and by a US National Science Foundation grant (DEB 0093149) and
384 NERC grants (NE/B504065/1; NE/D003741/1) to RRS. We thank all the participants of this
385 network for fruitful comments and discussions about this project, and particularly Anneli
386 Hoikkala for her helpful insight throughout the project.

387 **References**

- 388 Abramoff, M.D., Magalhães, P.J. & Ram, S.J. 2004. Image processing with ImageJ.
389 *Biophotonics Int.* **11**: 36–42.
- 390 Arnqvist, G. & Rowe, L. 2005. *Sexual conflict*. Princeton University Press.
- 391 Bacigalupe, L.D., Crudgington, H.S., Hunter, F., Moore, A.J. & Snook, R.R. 2007. Sexual
392 conflict does not drive reproductive isolation in experimental populations of *Drosophila*
393 *pseudoobscura*. *J. Evol. Biol.* **20**: 1763–1771.
- 394 Bacigalupe, L.D., Crudgington, H.S., Slate, J., Moore, A.J. & Snook, R.R. 2008. Sexual selection
395 and interacting phenotypes in experimental evolution: a study of *Drosophila*
396 *pseudoobscura* mating behavior. *Evolution*. **62**: 1804–1812.
- 397 Bates, D. & Sarkar, D. 2007. *lme4: Linear mixed-effects models using S4 classes*.
- 398 Blanckenhorn, W.U. 2000. The evolution of body size: what keeps organisms small? *Q. Rev.*
399 *Biol.* **75**: 385–407.
- 400 Bretman, A., Fricke, C. & Chapman, T. 2009. Plastic responses of male *Drosophila*
401 *melanogaster* to the level of sperm competition increase male reproductive fitness.
402 *Proc. R. Soc. B Biol. Sci.* 1705–1711.
- 403 Clutton-Brock, T. & Parker, G. 1995. Sexual coercion in animal societies. *Anim. Behav.* **49**:
404 1345–1365.
- 405 Coyne, J.A., Elwyn, S. & Rolán-Alvarez, E. 2005. Impact of experimental design on *Drosophila*
406 sexual isolation studies: direct effects and comparison to field hybridization data.
407 *Evolution*. **59**: 2588–601.

- 408 Coyne, J.A. & Orr, H.A. 2004. *Speciation*. Sinauer Associates Sunderland, MA.
- 409 Crudgington, H., Fellows, S. & Snook, R.R. 2010. Increased opportunity for sexual conflict
410 promotes harmful males with elevated courtship frequencies. *J. Evol. Biol.* **23**: 440–446.
- 411 Crudgington, H.S., Beckerman, A.P., Brüstle, L., Green, K. & Snook, R.R. 2005. Experimental
412 removal and elevation of sexual selection: does sexual selection generate manipulative
413 males and resistant females? *Am. Nat.* **165**: S72–87.
- 414 Debelle, A., Ritchie, M. & Snook, R.R. 2014. Evolution of divergent female mating preference
415 in response to experimental sexual selection. *Evolution*. **68**: 2524–2533.
- 416 Dodd, D. 1989. Reproductive isolation as a consequence of adaptive divergence in
417 *Drosophila pseudoobscura*. *Evolution*. **43**: 1308–1311.
- 418 Dougherty, L.R. & Shuker, D.M. 2014. The effect of experimental design on the
419 measurement of mate choice: a meta-analysis. *Behav. Ecol.* **26**: 311–319.
- 420 Garland, T. & Rose, M.R. 2009. *Experimental evolution: concepts, methods, and applications*
421 *of selection experiments*. University of California Press.
- 422 Gavrillets, S. 2014. Is Sexual Conflict an “ Engine of Speciation ”? *Cold Spring Harb. Perspect.*
423 *Biol.* **6**: a017723.
- 424 Holland, B. & Rice, W.R. 1999. Experimental removal of sexual selection reverses intersexual
425 antagonistic coevolution and removes a reproductive load. *Proc. Natl. Acad. Sci. USA*
426 **96**: 5083–8.

- 427 Holm, S. 2012. Multiple confidence sets based on stagewise tests. *J. Am. Stat. Assoc.* **94**:
428 489–495.
- 429 Hothorn, T., Bretz, F. & Westfall, P. 2008. Simultaneous inference in general parametric
430 models. *Biometrical J.* **50**: 346–63.
- 431 Hunt, J., Breuker, C.J., Sadowski, J.A. & Moore, A.J. 2009. Male-male competition, female
432 mate choice and their interaction: determining total sexual selection. *J. Evol. Biol.* **22**:
433 13–26.
- 434 Hunt, J., Snook, R.R., Mitchell, C., Crudgington, H.S. & Moore, A.J. 2012. Sexual selection and
435 experimental evolution of chemical signals in *Drosophila pseudoobscura*. *J. Evol. Biol.*
436 **25**: 2232–41.
- 437 ITN Marie Curie Speciation. 2011. What do we need to know about speciation? *TREE* **27**: 27–
438 39.
- 439 Kawecki, T.J., Lenski, R.E., Ebert, D., Hollis, B., Olivieri, I. & Whitlock, M.C. 2012. Experimental
440 evolution. *TREE* **27**: 547–60.
- 441 Kim, S. & Velando, A. 2014. Stickleback males increase red coloration and courtship
442 behaviours in the presence of a competitive rival. *Ethology* **120**: 502–510.
- 443 Kirkpatrick, M. 1982. Sexual selection and the evolution of female choice. *Evolution.* **36**: 1–
444 12.
- 445 Kirkpatrick, M. & Ravigné, V. 2002. Speciation by natural and sexual selection: models and
446 experiments. *Am. Nat.* **159**: S22–S35.

447 Lande, R. 1981. Models of speciation by sexual selection on polygenic traits. *Proc. Natl. Acad.*
448 *Sci. USA.* **78**: 3721–3725.

449 Long, T. A. F. & Rice, W.R. 2007. Adult locomotory activity mediates intralocus sexual conflict
450 in a laboratory-adapted population of *Drosophila melanogaster*. *Proc. Biol. Sci.* **274**:
451 3105–12.

452 Markow, T. A. 1988. Reproductive behavior of *Drosophila melanogaster* and *D.*
453 *nigrospiracula* in the field and in the laboratory. *J. Comp. Psychol.* **102**: 169–173.

454 Markow, T. A. & Ricker, J.P. 1992. Male size, developmental stability, and mating success in
455 natural populations of three *Drosophila* species. *Heredity.* **69**: 122–7.

456 Martin, O. Y. & Hosken, D. 2004. Reproductive consequences of population divergence
457 through sexual conflict. *Curr. Biol.* **14**: 906–910.

458 Martin, O.Y. & Hosken, D.J. 2003. The evolution of reproductive isolation through sexual
459 conflict. *Nature* **423**: 979–982.

460 Mayr, E. 1963. *Animal species and evolution*. Harvard University Press.

461 Milner, R. 2012. Keeping up appearances: male fiddler crabs wave faster in a crowd. *Biol.*
462 *Lett.* **8**: 176–8.

463 Noor, M.A.F. 1998. Diurnal activity patterns of *Drosophila subobscura* and *D. pseudoobscura*
464 in sympatric populations. *Am. Midl. Nat.* **140**: 34–41.

- 465 O'Dell, K.M.C. 2003. The voyeurs' guide to *Drosophila melanogaster* courtship. *Behav.*
466 *Processes* **64**: 211–223.
- 467 Parker, G.A. & Partridge, L. 1998. Sexual conflict and speciation. *Philos. Trans. R. Soc.*
468 *London. Ser. B Biol. Sci.* **353**: 261–274.
- 469 Partridge, L., Ewing, A. & Chandler, A. 1987b. Male size and mating success in *Drosophila*
470 *melanogaster* : the roles of male and female behaviour. *Anim. Behav.* **35**: 555–562.
- 471 Partridge, L. & Farquhar, M. 1983. Lifetime mating success of male fruitflies (*Drosophila*
472 *melanogaster*) is related to their size. *Anim. Behav.* **31**: 871–877.
- 473 Partridge, L., Hoffmann, A. & Jones, J.S. 1987a. Male size and mating success in *Drosophila*
474 *melanogaster* and *D. pseudoobscura* under field conditions. *Anim. Behav.* **35**: 468–476.
- 475 Price, T. 1998. Sexual selection and natural selection in bird speciation. *Philos. Trans. R. Soc.*
476 *B Biol. Sci.* **353**: 251–260.
- 477 R Development Core Team. 2005. R: A language and environment for statistical computing.
- 478 Rodríguez, R.L., Boughman, J.W., Gray, D.A., Hebets, E.A., Höbel, G. & Symes, L.B. 2013.
479 Diversification under sexual selection: the relative roles of mate preference strength
480 and the degree of divergence in mate preferences. *Ecol. Lett.* **16**: 964–74.
- 481 Rundle, H., Chenoweth, S. & Blows, M. 2006. The roles of natural and sexual selection during
482 adaptation to a novel environment. *Evolution.* **60**: 2218–2225.

483 Rundle, H.D. & Chenoweth, S.F. 2005. Divergent selection and the evolution of signal traits
484 and mating preferences. *PLoS Biol.* **3**: 1988–1995.

485 Rundle, H.D. & Nosil, P. 2005. Ecological speciation. *Ecol. Lett.* **8**: 336–352.

486 Servedio, M.R. 2004. The what and why of research on reinforcement. *PLoS Biol.* **2**: 2032–
487 2035.

488 Servedio, M.R. & Bürger, R. 2014. The counterintuitive role of sexual selection in species
489 maintenance and speciation. *Proc. Natl. Acad. Sci. USA* **111**: 8113–8.

490 Shaw, K.L. & Lugo, E. 2001. Mating asymmetry and the direction of evolution in the Hawaiian
491 cricket genus *Laupala*. *Mol. Ecol.* **10**: 751–9.

492 Shine, R., Langkilde, T., Wall, M. & Mason, R. 2005. Alternative male mating tactics in garter
493 snakes, *Thamnophis sirtalis parietalis*. *Anim. Behav.* **70**: 387–396.

494 Snook, R., Robertson, A., Crudgington, H. & Ritchie, M. 2005. Experimental manipulation of
495 sexual selection and the evolution of courtship song in *Drosophila pseudoobscura*.
496 *Behav. Genet.* **35**: 245–255.

497 Snook, R.R., Brüstle, L. & Slate, J. 2009. A test and review of the role of effective population
498 size on experimental sexual selection patterns. *Evolution.* **63**: 1923–33.

499 Snook, R.R., Gidaszewski, N.A., Chapman, T. & Leigh, W. 2013. Sexual selection and the
500 evolution of secondary sexual traits : sex comb evolution in *Drosophila*. *J. Evol. Biol.* **26**:
501 912–918.

- 502 Snook, R.R. & Markow, T.A. 2001. Mating system evolution in sperm-heteromorphic
503 *Drosophila*. *J. Insect Physiol.* **47**: 957–964.
- 504 Sobel, J.M., Chen, G.F., Watt, L.R. & Schemske, D.W. 2010. The biology of speciation.
505 *Evolution.* **64**: 295–315.
- 506 Uyeda, J.C., Arnold, S.J., Hohenlohe, P. a & Mead, L.S. 2009. Drift promotes speciation by
507 sexual selection. *Evolution.* **63**: 583–94.
- 508 Wigby, S. & Chapman, T. 2004. Female resistance to male harm evolves in response to
509 manipulation of sexual conflict. *Evolution.* **58**: 1028–1037.
- 510 Wigby, S. & Chapman, T. 2006. No evidence that experimental manipulation of sexual
511 conflict drives premating reproductive isolation in *Drosophila melanogaster*. *J. Evol.*
512 *Biol.* **19**: 1033–1039.
- 513 Williams, M.A., Blouin, A.G. & Noor, M.A.F. 2001. Courtship songs of *Drosophila*
514 *pseudoobscura* and *D. persimilis*. II. Genetics of species differences. *Heredity.* **86**: 68–
515 77.
- 516 Wong, B.B.M. & Candolin, U. 2005. How is female mate choice affected by male
517 competition? *Biol. Rev. Camb. Philos. Soc.* **80**: 559–571.
- 518

519 **Tables**

520 **Table 1** Average body size values (in millimetres) between the sexual selection treatment, by
 521 sex and replicate. Standard deviation is given next to each average body size value. Wilcoxon
 522 rank sum tests were performed between E and M treatments for all replicates combined,
 523 and for each replicate, to compare body size differences between the sexual selection
 524 treatments. P is the p-value. The sample size is N = 1019. E = polyandry, M = monogamy, R =
 525 replicate.

	Males					Females				
	E		M		P	E		M		P
	Mean size	±SD	Mean size	±SD		Mean size	±SD	Mean size	±SD	
All	2.26	±0.071	2.22	±0.073	<0.001	2.46	±0.098	2.45	±0.082	0.0030
R1	2.22	±0.075	2.23	±0.072	0.17	2.43	±0.11	2.49	±0.072	<0.001
R2	2.29	±0.065	2.23	±0.078	<0.001	2.48	±0.13	2.45	±0.072	<0.001
R3	2.28	±0.062	2.23	±0.072	<0.001	2.48	±0.068	2.44	±0.078	<0.001
R4	2.24	±0.06	2.20	±0.065	<0.001	2.46	±0.069	2.43	±0.094	0.062

526

527 **Table 2** Output of the mixed-model for mating outcome and mating latency analyses for
528 selection line females, including model estimates and tests statistics. In the mating outcome
529 model, the response variable was the probability of an E male winning the mating. In the
530 mating latency model, the response variable was the mating latency of the winning male.
531 Winner treatment is the sexual selection treatment of the winning male (E or M), female
532 treatment is the sexual selection treatment of the female (E or M), type of cross
533 distinguishes between 'within E population', 'within M population' and 'between
534 populations' crosses, and E-M relative size difference is the relative size difference between
535 the males ('E larger' or 'E smaller'). The following elements are specified: the model
536 estimate(s) of each variable (β), the likelihood ratio statistic used to test the main effect of
537 each variable (LR) and the p-value of the likelihood ratio test (p). The sample size is N = 1019.
538 E = polyandry, M = monogamy.

Fixed effects	Factor level	MATING OUTCOME			MATING LATENCY		
		Parameters			Parameters		
		β	LR	P	β	LR	P
Winner treatment (WT)	M	-	-	-	0.30	16.2	0.0028
Female treatment (FT)	M	0.047	0.1	0.79	0.13	5.8	0.055
Type of cross (TC)	within E	0.049	0.9	0.64	0.13	5.6	0.23
	within M	-0.22					
E male body size	-	-0.26	0.0	0.85	-0.10	0.04	0.85
M male body size	-	0.74	0.3	0.80	-1.55	10.3	0.0013
E-M relative size difference	E > M	0.055	0.002	0.97	0.18	4.9	0.027
Female body size	-	2.33	7.5	0.0062	-0.44	1.8	0.17
Temperature	-	0.035	1.5	0.21	-0.0088	0.7	0.39
WT * FT	M winner and M female	-	-	-	-0.33	5.8	0.016
		-	-	-			

WT * TC	M winner and within E	-	-	-	0.075	1.6	0.44
	M winner and within M	-	-	-	0.25		
Global intercept		-8.56			9.48		
Random effects variance	female replicate	0.010			0.012		
	male replicate	0.064			0.00087		

539

540 **Table 3** Output of the mixed-model for mating outcome and mating latency analyses for ancestral females, including model estimates and tests
 541 statistics. In the mating outcome model, the response variable was the probability of an E male winning the mating with an ancestral female. In
 542 the mating latency model, the response variable was the mating latency of the winning male. Winner treatment is the sexual selection
 543 treatment of the winning male (E or M) and E-M relative size difference is the relative size difference between the males ('E larger' or 'E
 544 smaller'). The following elements are specified: the model estimate(s) of each variable (β), the likelihood ratio statistic used to test the main
 545 effect of each variable (LR) and the p-value of the likelihood ratio test (p). The sample size is N = 179. E = polyandry, M = monogamy.

		MATING OUTCOME			MATING LATENCY		
Fixed effects	Factor level	Parameters			Parameters		
		β	LR	P	β	LR	P
Winner treatment (WT)	M	-	-	-	0.39	6.1	0.014
E male body size	-	-4.32	0.7	0.42	-0.022	0.0001	0.99
M male body size	-	-1.69	0.1	0.72	-1.22	0.5	0.49
E-M relative size difference	E > M	-0.49	0.5	0.46	0.20	0.6	0.42
Female body size	-	3.38	0.7	0.39	0.83	0.3	0.58
Temperature	-	-0.15	0.1	0.73	0.24	2.6	0.11

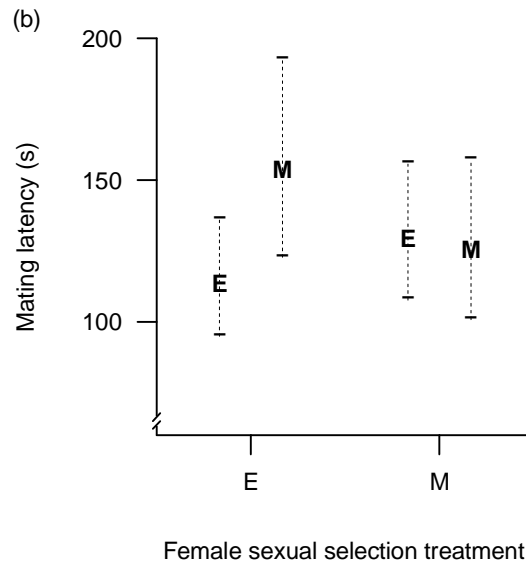
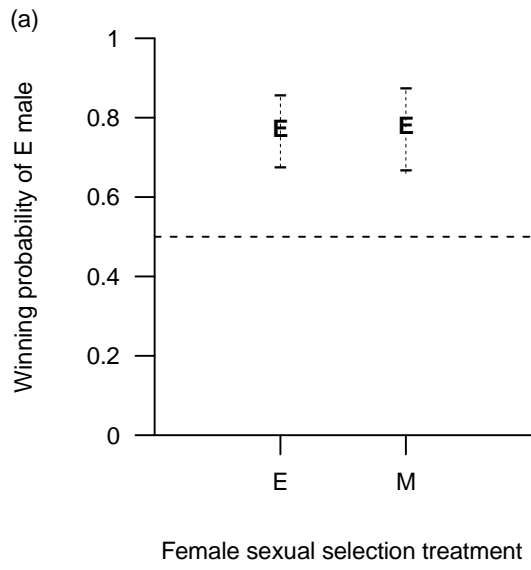
Global intercept		7.91	-0.62
Random effect variance	male replicate	0.43	<0.001

546

547 **Figures**

548 **Fig. 1** Mating outcome probability and mating latency of selection line females. (a) Mating
549 outcome (probability of an E male winning). The letters represent the fitted mating
550 probabilities estimated by the mixed-model of an E male winning depending on female
551 sexual selection treatment (labels of the x-axis). As these probabilities are superior to 0.5,
552 the figure shows that E males have overall a higher mating success than M males. (b) Mating
553 latency depending on male and female sexual selection treatment. The letters represent the
554 fitted mating latencies estimated by the mixed-model of a male winning depending on male
555 sexual selection treatment (the plotted values) and female sexual selection treatment (labels
556 of the x-axis). The figure shows that M males mate as fast as E males with M females. Post-
557 hoc tests adjusted for multiple comparisons show that mating latency significantly differs
558 between E and M males with E females, but not with M females (for E females: $z=-3.1$,
559 $p=0.0038$; for M females: $z=0.3$, $p=0.95$). In both (a) and (b), M is for monogamy, E is for
560 polyandry, and 95% confidence intervals around each predicted value are represented in
561 dotted lines. The model outputs are given in Table 2. E = polyandry, M = monogamy.

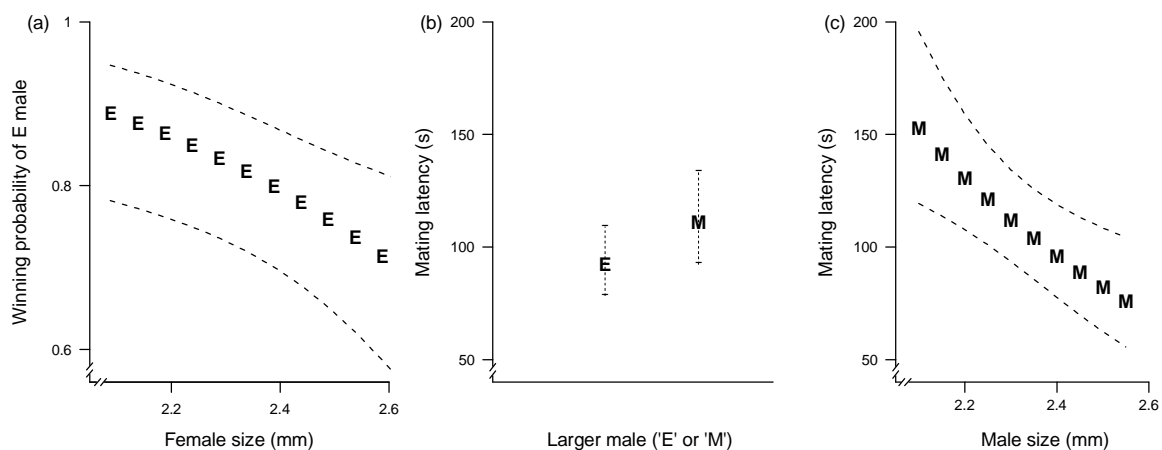
562



563

564 **Fig. 2** Body size effects on mating outcome probability and mating latency of selection line
 565 females. (a) Mating outcome depending on female body size. The letters represent the fitted
 566 mating probabilities estimated by the mixed-model of an E male winning depending on
 567 female body size. The figure shows that female size is negatively correlated with the
 568 probability of an E male winning. (b) Mating latency depending on the relative size
 569 difference between E and M males. The letters represent the fitted mating latencies
 570 estimated by the mixed-model depending on male relative size difference. The figure shows
 571 that mating latency is reduced when the E male is larger than the M male (representing 35%
 572 of the trials). (c) Mating latency depending on M male body size. The letters represent the
 573 fitted mating latencies estimated by the mixed-model depending on M male body size. The
 574 figure shows that mating latency is negatively correlated with M male body size. In all plots,
 575 M is for monogamous males and E is for polyandrous males, and 95% confidence intervals
 576 around predicted values are represented in dashed lines. The model outputs are given in
 577 Table 2. E = polyandry, M = monogamy.

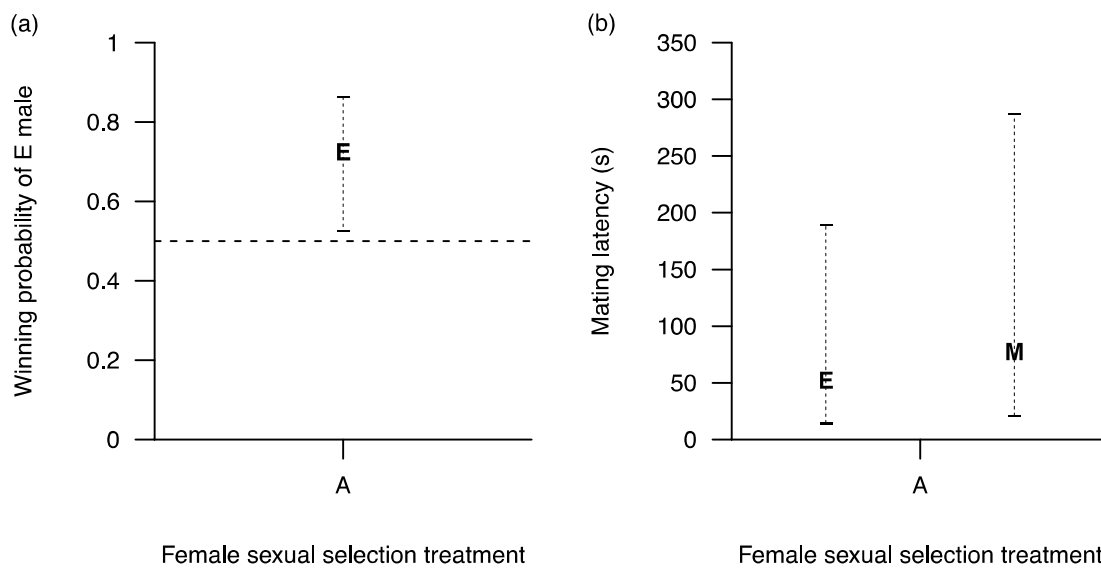
578



579

580

581 **Fig. 3** Mating outcome probability and mating latency of ancestral females. (a) Mating
 582 outcome (probability of an E male winning). The letter represents the fitted mating
 583 probability estimated by the mixed-model of an E male winning. As this probability is
 584 superior to 0.5, the figure shows that E males have a higher mating success than M males.
 585 (b) Mating latency depending on male sexual selection treatment. The letters represent the
 586 fitted mating latencies estimated by the mixed-model of a male winning depending on male
 587 sexual selection treatment (the plotted values). The figure shows that E males mate slightly
 588 faster than M males. In both (a) and (b), M is for monogamy, E is for polyandry, and 95%
 589 confidence intervals around each predicted value are represented in dotted lines. The model
 590 outputs are given in Table 3. A = ancestral, E = polyandry, M = monogamy.



591